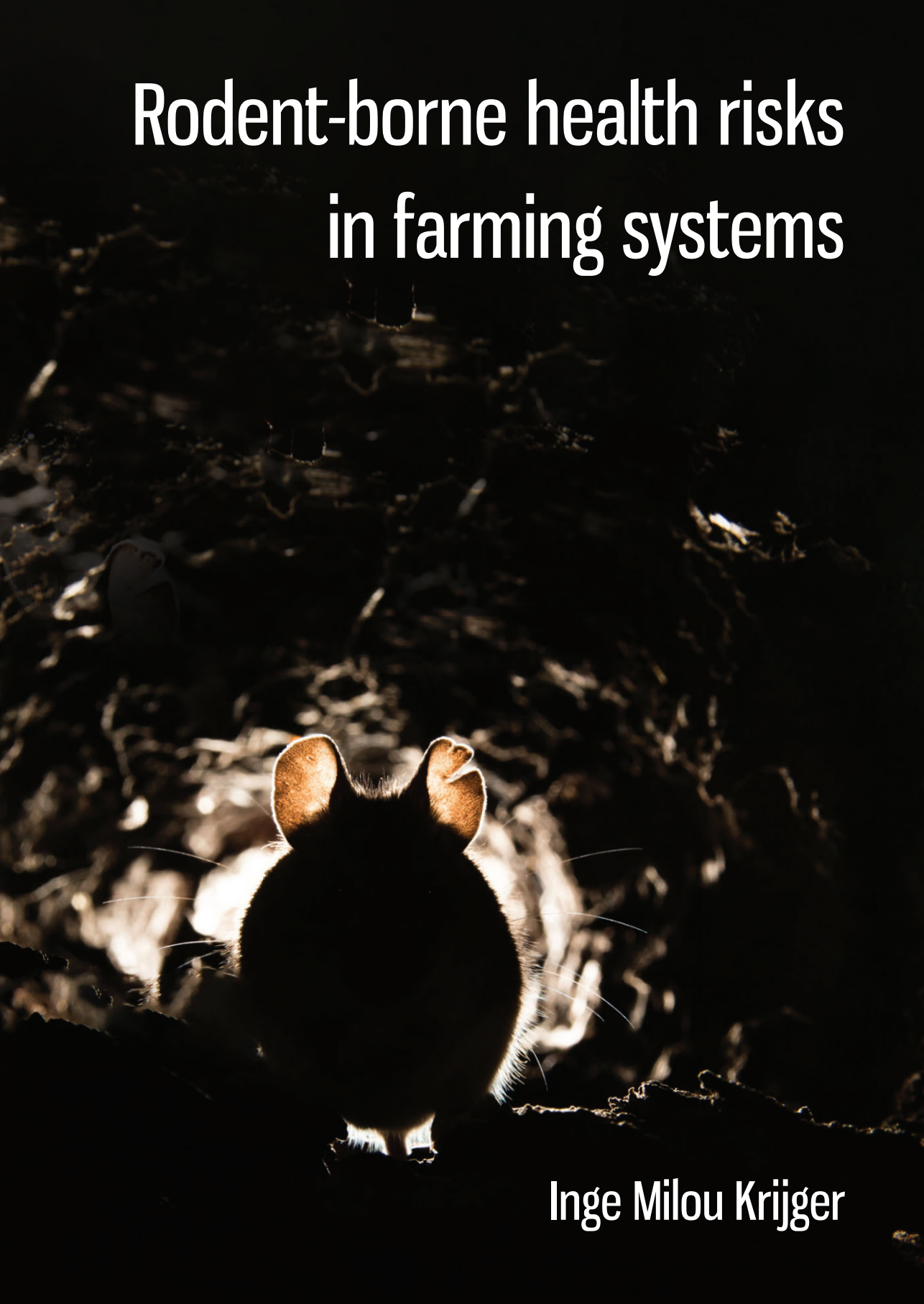
Rodent-borne health risks in farming systems



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Rodent-borne health risks in farming systems

Inge Milou Krijger

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Contents

Chapter 1.	General introduction	9
Chapter 2.	Wild rodents and insectivores as carriers of pathogenic *Leptospira* and *Toxoplasma gondii* in the Netherlands	29
Chapter 3.	Evidence of *Toxoplasma gondii* in rodents from Bangladesh	43
Chapter 4.	Prevalence of *Leptospira* infection in rodents from Bangladesh	53
Chapter 5.	*Clostridium difficile* in wild rodents and insectivores in the Netherlands	77
Chapter 6.	Efficacy of rodent management and monitoring methods on post-harvest losses by rodents in Bangladesh	87
Chapter 7.	The need to implement the landscape of fear within rodent pest management strategies	109
Chapter 8.	General discussion	123
	References	143
	Summary	173
	Dankwoord/Acknowledgements	181
	Curriculum Vitae	187
	List of publications	191
	Training and Education statement	196

Chapter 1

General introduction

Rodents

Rodents represent the largest order of mammals (covering over 40% of all mammals) and (this order) consists of ample 2000 species. Rodents are from the order *Rodentia* which is derived from the Latin *rodere,* meaning 'to gnaw'. Rodents are characterised by their continuously growing upper and lower pairs of incisors, which they use for gnawing. Most rodents are seed eaters, but there are also insectivorous and omnivorous rodent species. Many rodent species have impressive individual adaptability and behavioural flexibility, which enables them to adapt to multiple environments and enable them to go everywhere on earth humans go. Rodents encompass an awesome variety of traits such as their small size, their accurate and sensitive senses, their nutritional opportunism, and their athleticism. Furthermore, most species are active at night.

A small portion (<10%) of all rodent species can be referred to as pest-species (Singleton, G. R., Brown, Jacob, & Aplin, 2007). Commensal rodents such as brown rats, black rats, and house mice are usually found in association with humans. These rodents are capable of using their generalist body plan to feed and breed under any circumstances and they use their sophisticated behavioural patterns to avoid attempts to rodent management or eradication by humans. Although the terminology 'commensal' indirectly expresses a relation between two kinds of organisms in which one obtains food or other benefits from the other without damaging the host, commensal rodents should better be referred to as klepto-parasitic (Macdonald, D., Fenn, & Gelling, 1994).

From a human perspective, rodents have always been connected with disease. This association is mainly due to the 'Black Death', a large and deadly pandemic in the human history caused by the bubonic plague. The arrival of the bubonic plague in Europe around 1350 led to the death of nearly a third of the human population (Battersby, 2015; Keeling & Gilligan, 2000; Slack, 1989). For years and years rats were assumed to be the primary vector for spreading the plague. However, presently we know that not rats, but primarily ectoparasites such as human fleas and body lice were the major plague vectors during these epidemics in the past (Bramanti, Dean, Walløe, & Stenseth, 2019; Dean *et al.*, 2018). Although the bubonic plague is commonly thought of as a disease of predominantly historical importance, there are increasing reports of occurrence (D'Ortenzio *et al.*, 2018; Keeling & Gilligan, 2000; Lowell *et al.*, 2009; Melman *et al.*, 2018; Stenseth *et al.*, 2008). Thus also in modern times, rodent presence can form a direct threat for public health (Meerburg, B. G., Singleton, & Kijlstra, 2009). Besides the bubonic plague, there are numerous more pathogens that can be transferred from rodents to humans. Infectious pathogens

that can be transferred between animals (usually vertebrates) and humans are called zoonoses.

Zoonoses

Public health and global economies are negatively influenced by emerging infectious diseases (EIDs) (Binder, Levitt, Sacks, & Hughes, 1999; Lederberg, Hamburg, & Smolinski, 2003; Morens, Folkers, & Fauci, 2004). A disease is classified as emerging infectious disease when the incidence in humans has increased over the past two decades, or portend to upsurge in the nearby future. Worldwide, 15.8% of all deaths are due to infectious diseases (Wang *et al.*, 2016). Furthermore, infectious diseases account for 43.7% of deaths in low-resource countries (Wang *et al.*, 2016). A study from 2008 looked at which factors drive disease outbreaks and analyzed these factors to understand global temporal and spatial patterns of emerging infectious diseases (Jones, K. E. *et al.*, 2008). Of all emerging infectious diseases, 60.3% is zoonotic (Jones, K. E. *et al.*, 2008). Globally, there are over 200 zoonotic diseases recognized as a threat for both human and animal health. Zoonotic diseases are assessed to be accountable for 2.5 billion cases of human disease and 2.7 million human deaths per year worldwide (Gebreyes *et al.*, 2014). Besides, (emerging) zoonoses are responsible for some of the most dangerous and harmful epidemics (Nabarro & Wannous, 2014; Salyer, Silver, Simone, & Barton Behravesh, 2017; WHO, 2019).

The majority of the emerging zoonotic diseases (70.8%) originates from wildlife (Jones, K. E. *et al.*, 2008). More specifically, the majority of this wildlife group from which zoonoses can emerge, consists of terrestrial mammal species. As the order *Rodentia* contains the highest species richness (over 2,050 species) within the terrestrial mammals, it is thus also linked to the largest diversity of zoonoses (Han, Barbara A., Kramer, & Drake, 2016). It is known that the risk of zoonotic disease increases with species diversity (Hawlena *et al.*, 2018).

Continents with a relative high risk of an emerging infectious disease incident caused by zoonotic pathogens from wildlife are Asia, Europe, and Africa (Figure 1.1) (Allen *et al.*, 2016; Jones, K. E. *et al.*, 2008). Continents with a high relative risk of an emerging infectious disease incident caused by zoonotic pathogens from non-wildlife are Europe and Asia (Jones, K. E. *et al.*, 2008).

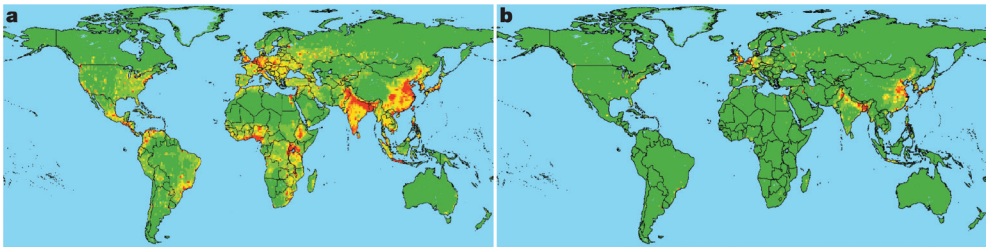

Figure 1.1 Global distribution of relative risk for emerging disease events caused by **a)** zoonotic pathogens from wildlife, and **b)** zoonotic pathogens from non-wildlife mapped on a linear scale from green (lower values) to red (higher values). Adapted from "Global trends in emerging infectious diseases", Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., & Daszak, P. (2008). Nature, 451(7181), 990-993.

Rodents and zoonoses

There are over 80 rodent-borne zoonoses known (Bordes, Frédéric, Blasdell, & Morand, 2015; Gratz, 1994; Han, Barbara A. *et al.*, 2016; Han, Barbara A, Schmidt, Bowden, & Drake, 2015; Luis *et al.*, 2013; Meerburg, B. G. *et al.*, 2009; Mills & Childs, 1998). Rodents can contaminate produce with their droppings, urine, and saliva, which could possibly harbour zoonotic pathogens (Belmain et al., 2015; Hussain and Iqbal, 2002; Meerburg et al., 2009). In order to mitigate and prevent zoonotic disease risks to humans, it is essential to understand the transmission route and life cycle of the pathogen. Therefore, it is of the essence to focus on that point in the food production chain where the chance of both indirect and direct contact between rodents and humans is highest. Therefore we decided to focus in this thesis on the rodent – as host of zoonotic pathogens – in and around farming systems.

Each pathogen has its own, specific lifecycle and requirements to its environment (Beard, Garafalo, & Gage, 2015; Hansen *et al.*, 2016; Meerburg, B. G. & Kijlstra, 2009; Rood, Goris, Pijnacker, Bakker, & Hartskeerl, 2017; Young *et al.*, 2017). Although the disease transmission involves more than one cause, global climate change could be a significant driver. Climate can influence infectious disease by influencing pathogen survival and transmission, host defenses, the life cycle of vectors, and habitats. There are numerous vector borne diseases that are sensitive to rainfall events. For example the Ross River virus, which is a mosquito-borne disease found throughout Australia. Other zoonotic pathogens that are sensitive to weather and climate changes are leptospirosis and Rift Valley fever, which were present in multiple epidemics worldwide over the last decade (Epstein, 2000; King, 2004). Pathogen vectors or hosts are susceptible for climate change as well

(King, 2004). Although climatic changes do not have a direct effect on evolution, climate changes could lead to new ecological opportunities by changing vegetation (Renaud *et al.*, 2005). There are numerous potential infection possibilities of rodents with zoonotic micro-parasites e.g. viruses, bacteria, and protozoans. There are multiple factors that interact in the occurrence/existence and transfer of rodent-borne zoonoses, see the simplified framework (Figure 1.2). This model consists of several components, with rodents as common denominator.

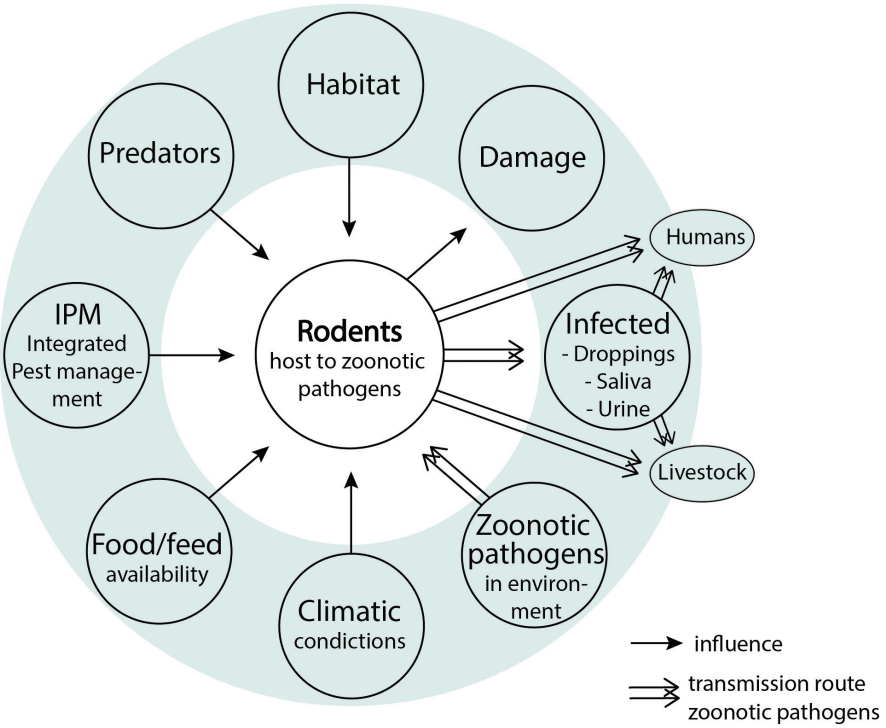

Figure 1.2. Framework showing aspects of rodent presence and density on potential transmission routes of rodent-borne zoonoses to humans or livestock.

Factors playing a role in the transmission of rodent borne zoonoses are host habitat, predator influence (e.g. the level of fear), IPM, availability of food, and climatic conditions.

In this thesis the focus is on rodent borne zoonoses, with high risks for humans. It was decided to study zoonotic pathogens in rodents that are able to survive and thrive in different climates. Therefore two continents were selected to conduct

research on rodents: Asia and Europe. Asia has a warm, humid climate with two main seasons; dry and wet, and is predisposed for (infectious-) disease emergence (Coker, Hunter, Rudge, Liverani, & Hanvoravongchai, 2011; Morand, Jittapalapong, Suputtamongkol, Abdullah, & Huan, 2014). Bangladesh was selected as study case for Asia, as it has a high population density which could result in many human-rodent interactions.

Little research has been conducted on current rodent-borne zoonoses in regions of Asia, which raises the need to determine pathogen prevalence to gain insight in the current situation. There is also impaired knowledge on the biology and habitat specialisations and distribution of many rodent species in Asia (Blasdell *et al.*, 2015), which is essential for species specific pest-rodent management.

Europe is considered to be a global zoonotic host hotspot, mainly due to the diversity in rodent and insectivore species that are hosts to pathogens. Europe's weather patterns follow four different seasons: spring, summer, autumn, and winter. The Netherlands was selected as country in Europe, also because there is impaired knowledge on zoonotic pathogens in rodents and insectivores in the Netherlands, on which will be elaborated later on in this chapter. As long as direct or indirect contact between reservoir (rodent) and host (human) exists, it is essential to monitor infection prevalence of the reservoir, in order to target control of the reservoir host at times and places where the risk is highest (Begon, 2003).

To our knowledge there is no unequivocal list of global or continental emerging zoonotic pathogens. Therefore two rodent-borne zoonotic pathogens were chosen to study in both selected countries: *Toxoplasma gondii* and *Leptospira* spp. To indicate the significance of these pathogens: *T. gondii* is listed 2nd in the top 5 pathogens resulting in death from food-borne illness (CDC), and also listed 2nd based on DALYs for foodborne pathogens in Europe (Bouwknegt *et al.*, 2018). For the Netherlands, *T. gondii* is 2nd on the list of 86 prioritized emerging zoonoses (Havelaar *et al.*, 2010). Due to a paucity of research, the *Toxoplasma gondii* infection status of rodents in most Asian countries is poorly known (Herbreteau *et al*., 2012), resulting in an information gap. By acquiring information on the infection status of rodents in and around food-production sites, the risks for humans could be identified and communicated in order to prevent infections.

Leptospirosis is known as a disease of epidemic potential that has a significant health impact in many parts of the world. In 1999 it was estimated that yearly 500,000 cases of leptospirosis occur globally (WHO, 1999). Research conducted in 2008 into the worldwide incidence concluded that "Leptospirosis is a re-emerging

zoonosis of global importance" with South-East Asia being one of the most significant centres of the disease (Pappas *et al*., 2008). Information on leptospirosis and its consequences is extremely limited in many regions in Asia, amongst which Bangladesh (Singleton, 2003b). This information gap results in lack of precautions taken when handling rats or when preparing and consuming potential contaminated food (Singleton, 2003b). Asia is an endemic area for leptospirosis, and leptospirosis affects rural communities in Asian countries negatively (Bahaman and Ibrahim, 1988; LaRocque *et al*., 2005; Light *et al*., 1971; Van *et al*., 1998; Victoriano *et al*., 2009). In Thailand for example, the cases of human leptospirosis markedly increased over 1995-2000, with in 2000 leptospirosis being associated with 320 deaths reported among rice farmers (Singleton, 2003b). In the Netherlands leptospirosis occurs at an average yearly incidence of 1 case per 400,000 people with a case fatality rate of 6.5% (Rood *et al.*, 2017). For Europe, a three-fold rise in the number of leptospirosis cases is expected compared to 2014 (Suk, Vaughan, Cook, & Semenza, 2019).

For the Netherlands, *Leptospira interrogans* is 10nd on the list of 86 prioritized emerging zoonoses (Havelaar *et al.*, 2010). Emergence of leptospirosis infections in the Netherlands is closely linked to the environment (leptospirosis shows seasonal dynamics as the bacteria thrives in humid and wet circumstances)(Rood *et al.*, 2017). Human and mammal exposure to *Leptospira* spp. might be intensified by heavy rainfall and flooding, and the amount of flood events in Europe is increasing and is predicted to continue to increase in coming years (Suk *et al.*, 2019) . Another motivation to choose for *Leptospira* spp., is that this pathogen uses rodents as main reservoir host. Rodents are thought to be the most important host for a variety of *Leptospira* serovars.

For the Netherlands, a third potentially zoonotic pathogen was researched: the opportunistic anaerobic bacteria *Clostridium difficile*. In Europe *C. difficile* is in the top six of healthcare-associated infections, with an estimated yearly burden of 175.000 cases, leading to an estimated number of 7.000 deaths yearly (Cassini *et al.*, 2016). These numbers highlight the need for increased efforts for prevention and control of this pathogen. However, there is little research on *C. difficile* in rodents whilst rodents are potential carriers and transmitters of this zoonotic bacterium. With rodents living close by humans and leaving their droppings everywhere they come, it is important to gain insight on the risks of their presence in order to take appropriate measures.

So three pathogens were selected to research: *Toxoplasma gondii, Leptospira* spp.*,* and *Clostridium difficile.* For each pathogen now the lifecycle and risks for human health will be described.

Toxoplasma gondii

Felids are the definitive hosts for the protozoan parasite *Toxoplasma gondii*. All felid species are able to excrete *T. gondii* oocysts in their faeces. Presence of excreted oocysts in the environment enables *T. gondii* to be taken up by numerous warm-blooded animal species, which therewith act as the parasite its intermediate hosts (Dubey and Beattie, 1988). Humans for example, can become infected by ingesting food or beverages contaminated with oocysts, by (accidentally) ingesting oocysts from the environment, or by ingesting tissue cysts present in undercooked meat (Figure 3) (Dubey and Beattie, 1988). Other ways of infection occur via organ transplants from an infected person (Schaffner, 2001; Shulman and Appleman, 1991), or prenatally via the mother.

Toxoplasmosis is usually asymptomatic in healthy people, only a minor part of the immunocompetent human hosts infected with *T. gondii* will develop symptoms, such as fever, malaise, lymphadenopathy, pulmonary dysfunctions, or ocular problems (Hakes and Armstrong, 1983; Mechain *et al*., 2000; Montoya and Liesenfeld, 2004). Reactivation of a latent toxoplasmosis infection however, is one of the major killers of immunocompromised people in sub-Saharan countries. Examples of immunocompromised persons can be transplant recipients, HIV-infected people, or cancer patients receiving anticancer therapies (immunosuppressive drugs, chemotherapy) (Bachmeyer *et al*., 2006; Israelski and Remington, 1993; Khabaz

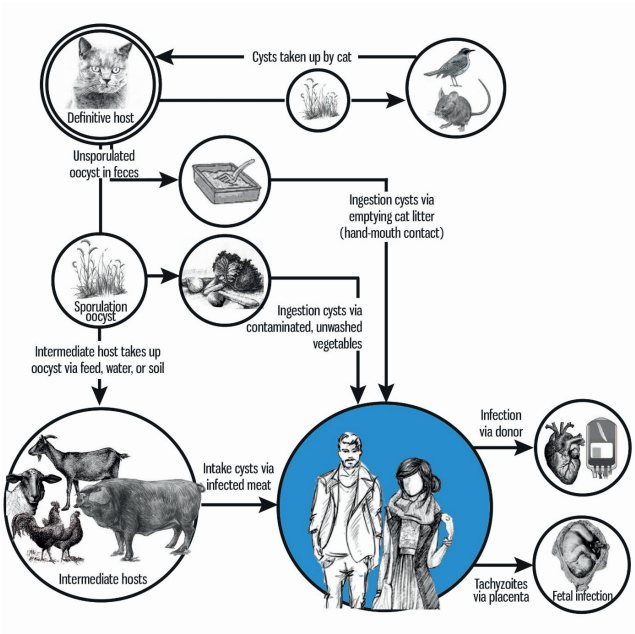

Figure 1.3 Life cycle of *Toxoplasma gondii* (figure created by author).

et al., 2011; Luma *et al*., 2013; Muluye *et al*., 2013). Another major risk lies with congenital infections. Infection of pregnant women can lead to many different foetal manifestations such as abortion, still-birth, and problems with the central nervous system (CNS). Furthermore, children that are born asymptomatically can develop neurological or ocular problems later in their life (Guerina *et al*., 1994; McAuley *et al*., 1994).

Research from Dubey *et al*. (1995) showed that rodents are able to carry infectious *T. gondii*. By being a prey to cats, rodents contribute to completion of the parasite life cycle. As cats are often held as rodent management method, *T. gondii* transmission is expected to occur (Brown & Khamphoukeo, 2010; Elton, 1953). Besides passing on the parasite to the cat, it is also possible that rodents transmit *T. gondii* directly to humans, as rodents are a common food source in Asia (Fiedler, 1990; Suwannarong & Chapman, 2014; Khiem & Van Chien, 2003). With rodents being a food source, and the fact that both cats and rodents are present in food storages, serious risks of *T. gondii* transmission need to be taken into account. Rodent *T. gondii* infection could reach 73%, depending on rodent species, topographical region, and season (Tenter *et al*., 2000). Considering the perilous effects of primary infection during pregnancy, ingestion of *T. gondii* via consuming infected meat need to be taken into account as a risk factor.

Leptospira spp.

Another zoonotic disease occurring globally is Leptospirosis. Leptospirosis is caused by the *Leptospira* spirochaete bacteria, which is classified into over 200 serovars (Hartskeerl & Terpstra, 1996). Leptospira spp. are widespread and able to affect humans from urban as well as rural environments, in both temperate and tropical climates (Vinetz, 2001). Several Leptospira serovars show host preferences. Rats for example serve mostly as reservoirs of the Icterohaemorragiae serogroup whereas the ballum serogroup is mostly found in house mice (*Mus musculus*) (Bharti *et al*., 2003; Levett, 2001; Thiermann, 1981). However, almost each rodent species can carry and excrete leptospires (Faine, 1994). *Leptospira* serovars usually do not cause disease in reservoir hosts, but do cause disease in the dead-end host, which in this case is the human.

Humans can acquire infection by contact with infected animals, animal tissue, animal excretions, or by contact with contaminated water (Figure 4) (Waitkins, 1987). Leptospirosis is an infectious disease which causes feverish illness in humans, and when severe it can result in Weil's disease (Faine, 1994). Weil's disease is characterized by jaundice, acute renal failure and bleeding. Another emerging disease type caused by *Leptospirosa* is leptospirosis-associated pulmonary

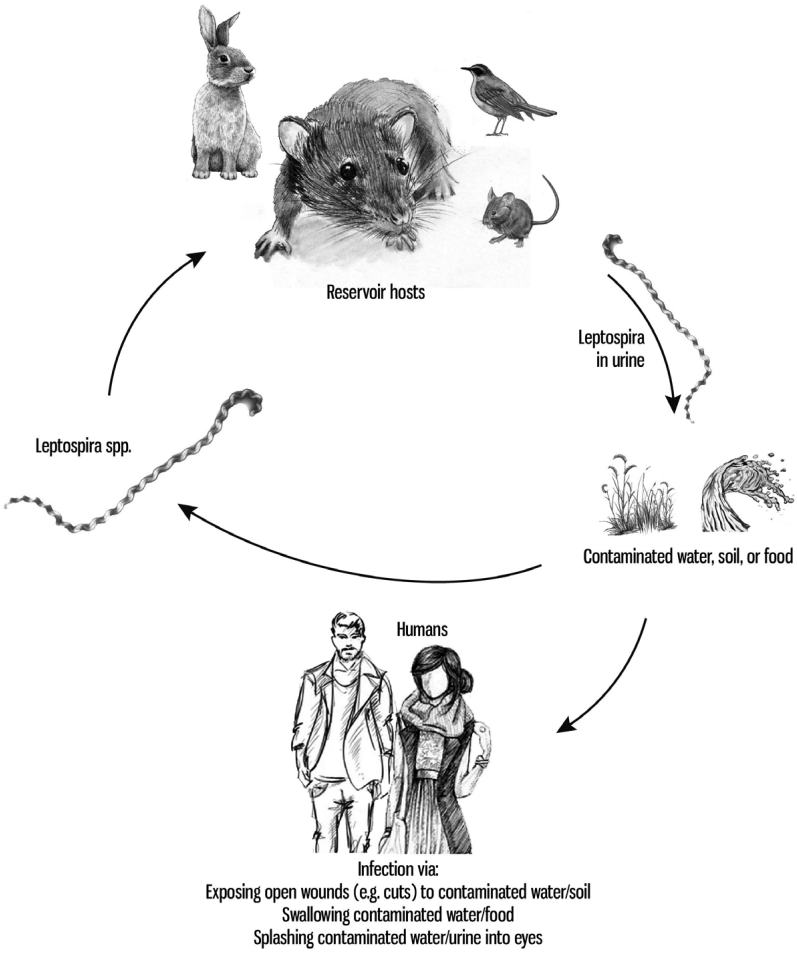

Figure 1.4 How humans can contract leptospirosis. (Figure created by author).

haemorrhage syndrome (fatality rate >50%)(McBride *et al*., 2005). With the symptoms being flu-like, the disease is often mistaken for a significant proportion of several diagnoses and neglected until serious damage occurs, such as kidney damage, meningitis, liver failures, or even respiration problems (Goeijenbier *et al.*, 2013; Laras *et al.*, 2002).

Granaries offer a rich source of food for rodents, as well as suitable circumstances for the survival of leptospires. As several leptospirosis reservoir hosts (e.g. rodents and cats) live in the same locations, granaries harbour a potential epidemiological niche for Leptospira transmission to humans (Natarajaseenivasan *et al*., 2002).

Clostridium difficile

C. difficile is a globally distributed enteropathogen for both humans and animals (Freeman *et al*. 2010) with over 800 ribotypes known. This gram-positive bacteria can be found in the intestinal tract of many animal species, but also in water, soil, and on meat (Al Saif & Brazier 1996; Songer *et al*. 2009; de Boer *et al*. 2011; Fawley, 2018). *C. difficile* infection (CDI) is one of the most frequently observed sources of mucosal injury and inflammation in hospital patients, leading to diarrhoea or inflammation of the colon (Kelly & LaMont ,1998). However, it is also described in patients who did not visit an hospital (Chernak *et al*., 2005). CDI is an emerging disease, both in human patients and in animals used for food (Keessen *et al*. 2011; Balsells *et al*. 2018; Crobach *et al*. 2018; Rodriguez Diaz *et al*. 2018). The bacterium *C. difficile* not only causes disease in humans, it is also able to cause enteric disease in several animal species, such as horses, piglets, calves, and other domestic animals (Båverud, 2002; Rupnik, 2007; Rupnik *et al*. 2009; Kecerova *et al*. 2019). This finding suggests that animals and humans may share a common source (Rupnik, 2007), and it has been shown that there is substantial overlap of *C. difficil*e strains present in humans and animals (Keessen *et al*. 2011; Rodriguez Diaz *et al*. 2018). This overlap of *C. difficile* types could indicate zoonotic spread amongst animals and humans. With wild rodents being present around humans and their living, working, and food production environments, it is important to gain knowledge of the zoonotic pathogens present in rodents in order to assess human health risks and when and how to apply rodent management (Meerburg *et al*. 2009; Meerburg, 2010; Himsworth *et al*. 2014).

Rodent damage

Besides transmitting pathogens, rodents are also known to cause losses to stored human food and for causing damage to insulation and wiring due to their gnawing behaviour (Belmain, Steven R, Htwe, Kamal, & Singleton, 2015; Hussain & Iqbal, 2002; Meerburg, B. G. *et al.*, 2009). In 2017 about 820 million people were undernourished globally (FAO). Southern Asia has the highest undernourishment rate with an estimated number of over 275 million people suffering from hunger (FAO, IFAD, UNICEF, WFP, & WHO, 2018). Although Asia has the largest share of rice production, pre- and post-harvest losses are part of the underlying problems causing undernourishment as rodents are able to cause a loss of approximately 11 kg food per person per year in Asia (Singleton, Grant R, 2003). Asia is the continent which would avail most (54323 million tons versus 1885 million tons in Europe) from proper rodent management in order to minimize harvest losses (Meerburg, B. G., Singleton, & Leirs, 2009). For Asia, it is known that significant losses of stored rice occur mostly because current rice storage systems in Asia are not rodent-proof. By

sustainably reducing pre- and post-harvest losses by rodents, nearly 280 million undernourished people could meet their daily energy requirements (Meerburg, B. G. *et al.*, 2009).

There are only few reports on post-harvest losses of cereals due to rodents available. Nowadays in the modern Europe, the extent of crop damage is not the reason for starvation (Jacob & Tkadlec, 2010). However, agricultural production and/or forestry in Europe can suffer significant losses due to rodents, especially during rodent outbreaks. Rodent outbreaks in agricultural areas can endanger the survival of individual farming businesses (Jacob & Tkadlec, 2010). Reports on produce losses due to rodents in Europe are mainly on pre-harvest losses (e.g. grasslands, clover, sugar beets, maize, alfalfa, winter cereals, fruit trees, potatoes) due to vole species. Those pre-harvest losses can be up to 80%. In Germany damage to agriculture by rodents is caused by two vole species: *Arvicola terrestris* and *Microtus arvalis.* These voles are known to be able to cause significant economic damage to the German pomiculture by gnawing the root system of trees, with an estimated national damage value of between € 3.5 and >35 million per year (Walther, Fülling, Malevez, & Pelz, 2008). Repeated and extensive damage by common voles make this rodent species the most severe rodent pest in European agriculture (Jacob & Tkadlec, 2010).

In the literature no information on studies on post-harvest losses in Europe are present. This could be due to the fact that storage facilities for food products are further developed compared to third world countries in Asia. However, a storage facility made of robust material such as steel does not guarantee rodent absence. When screening the literature for contamination of stored produce with rodent droppings in Europe, a study from the Czech republic revealed heavy contamination of grain stored in steel silos (up to 26 droppings/m^2 grain surface) (Fraňková, Stejskal, Rödl, & Aulický, 2016) (Stejskal & Aulický, 2014). Furthermore, plant seeds (e.g. barley and wheat) are mainly stored in materials which can be easily gnawed on by rodents (paper, cardboard, plastic bags)(Fraňková *et al.*, 2016). These findings suggest that also in Europe, post-harvest losses due to rodents might be larger than we think. Although this research gap exists, Bangladesh was selected as in this country the population density is higher than in European countries, and due to the fact the undernourishment in Bangladesh is high.

Rodent management

Besides food loss, current storage methods also lead to damage and contamination of food by rodents, and to potential disease transmission via contamination of the food by rodent droppings, urine, and saliva (Meerburg, B. G. *et al.*, 2009). Inferior or absence of rodent management could lead to an increase of rodents living and foraging nearby households, which upsurges both undernourishment and the probability of zoonotic disease transmission. Therefore it is necessary to assess what rodent management is needed and how to apply this.

With rodent pest species around, the need for management arises in order to reduce the risk of disease transmission from rodent to human. An often used way of control, is by placing rodenticides in and around buildings. However, the risk of rodents developing resistance to rodenticides (with resistance being defined as the loss of effectiveness of rodenticides on rodents) and the possibility of poisoning non-target species is substantial (Figure 1.5).

The use of rodenticides is one of the last options in rodent control, which urges the need to research what ecologic rodent pest management methods are available (Singleton *et al*., 2007). Research conducted in 2014 in The Netherlands revealed that a considerable part of the rodent population has developed resistance to anticoagulant rodenticides, respectively 56% in rat tails (n=61), and 25% in rat droppings (n=169) (Meerburg et al., 2014). Resistance to first generation anticoagulant rodenticides is also reported for several rodent species in other continents, from 1966 onwards (Deoras, 1966, 1967; Fernando et al., 1967).

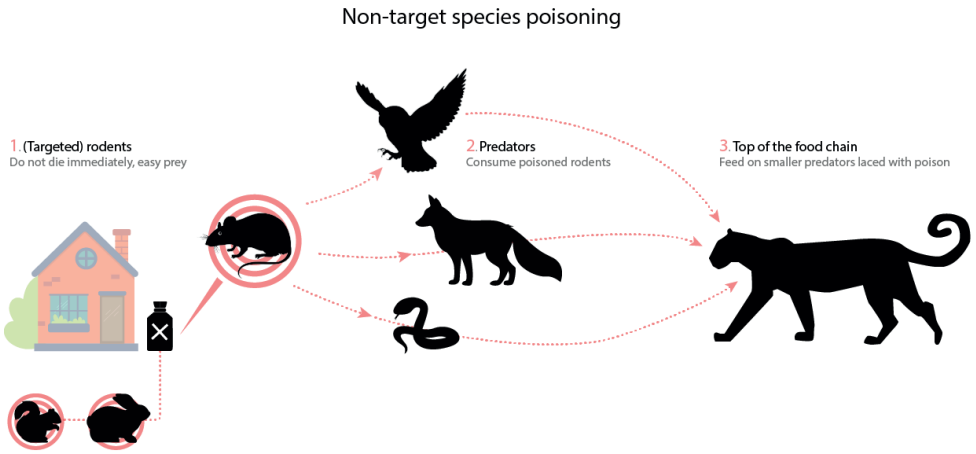

Figure 1.5 Rodent poison routes and unintentional effects on other places in the food chain. (Figure created by author)

It is crucial to research rodent ecology to understand their patterns of behaviour and feeding for effective ecologic rodent pest management (John, 2014). A recent on-farm study conducted in Bangladesh showed that over a period of 90 days, farmers without rodent management on average lost 2.5% of their stored rice stocks, but when applying rodent management they reduced the loss to 0.5% (Belmain *et al*., 2015). Thus, it is stated that proper rodent management leads to decrease of stored-produce loss. This on-farm research used snap-traps and showed that coordinated snap-trapping can be effective and therewith could be an opportunity to reduce rodenticide use (Belmain *et al*., 2015). The demand for rodent control strategies lies either with less reliance on chemicals or more specific targeting of pest species. So, the need to use ecologically-based pest management rises, which is based on integrated pest management methods (Singleton *et al*., 1999).

Integrated Pest Management (IPM) is the integration of several management methods to provide more effective management of a pest species together than each management method used on its own. IPM is based two elements; (I) prevention and (II) control. Forms of prevention are removing pest species shelter, food, or water. An important aspect of IPM is the aim to use methods which are the least interruptive to the ecological systems (Smith & van den Bosch, 1967).

Singleton (1997) reviewed the progress of IPM on rodents in Asia (field rat, *Rattus argentiventer*) and Australia (mouse, *Mus musculus).* In this review it was found that IPM of rodent pests lags seriously behind IPM of insect pests. It is suggested that translation of IPM programmes for insect pests to rodent pest methodologies could increase the IPM effects of rodent pests. In both of the by Singleton (1997) reviewed pest cases, the IPM methodology was based on detailed rodent population ecology studies, but lacked management training, research on rodent pests, and revealed a lack of extension of programmes on rodent management.

An example of strategies applied to insect pests are *push-pull strategies* (Cook *et al*., 2006), which bring together several elements of different pest management strategies. This can be based on the ecology of the pest, and the sense organs of the pest species. The aim of the *push*-component in the *push-pull* method is to make the resource unattractive or unsuitable for the pest. *Pull* components are used to divert pests from the protected resource (Cook *et al*., 2006).

The rationale of this research is to improve IPM for rodent pests, in order to increase the effectivity of the method. Although not used yet, this push-pull methodology might also prove to be highly effective in managing rodent pests. For example, as

push component, the resource (rice) could be made impervious to the rodents. Another opportunity for rodent control strategies could be to focus on the *Landscape of Fear* of the rodents, as fear is known to influence ecological processes. The *Landscape of Fear* reflects levels of fear of predation a prey species perceives on different locations within its home range (Laundré *et al*., 2010). The *Landscape of Fear* was used as a model to visualise how fear could alter area usage of prey as it tries to reduce the risk of predation (Altendorf *et al*., 2001; Laundré *et al*., 2001). In practice, the landscape of fear (LOF) is a mapping of habitat use as a result of perceived fear, which shows where bait or traps are most likely to be encountered and used by rodents.

When combining the perceived risk of predation with rodent behavioural responses, spatial use patterns of individuals could be explained (Laundré *et al*., 2010). This creates an opportunity, as this strategy could be very effective to concentrate rodent management on those areas knowing where rodents perceive the least levels of predation/risk. However, it is unknown what role quitting-harvest rates or giving-up densities (GUDs) could play in rodent management. To our knowledge very few papers have directly used GUDs in relation to pest management strategies.

Outsmarting the rodent

Rodents are clever and reproduce fast (for example: 2 brown rats can create a 15.000 population within one year) (Davis, H. N., Gray, & Dewsbury, 1977). Therefore, the need for pest management is high, and it is necessarily to act before the population is too large to manage. Recently, Reinhold et al. (Reinhold, Sanguinetti-Scheck, Hartmann, & Brecht, 2019) demonstrated that rats can play hide-and-seek with a human. Recordings in the medial prefrontal cortex detected neurons that were sensitive to the game structure. In the “seek” condition, rats were trained to search a hidden human and retained looking for until they found them. In the “hide” condition, the rats learned to hide and wait until being found. It was found that the rats vocalized when seeking and finding and were silent when hiding. The research of Reinhold et al. (Reinhold *et al.*, 2019) shows that rodents are not only clever, but also able to learn easily. This underpins the fact that it can be difficult to manage a rodent pest, especially when there is an outbreak.

On the other hand, Xu et al (2019) studied the memory of rats brains and demonstrated that they were able to predict where rats would go. Certain neurons in the rat’s hippocampus (called ‘place cells’) fire up when a rat enters a particular spot in its environment. When these lace cells are activated they create a cognitive map in the

rat's brain, allowing researchers to find out where the rat is based on which neurons are active. In the study of Xu et al. (2018) rats were placed in an eight-arm maze and by measuring the place cell activity in the hippocampus, the decision of the rat which of the eight arms to visit next could be predicted. Outsmarting rodents should be applied to improve rodent management.

Big data could be used to improve rodent management actions. Especially for urban surroundings there is a lot of information available, such as when are there events, where are food-trucks, where are the sites where food-markets are, at what time it the chance of pest-rodents finding human food highest, etcetera. By combining the knowledge of the behaviour and needs of a specific pest species with this data, outbreaks of rodent-pests could be prevented. For example; when a football match ends, all visitors go home. They will leave the arena and will also leave trash including food for rodents, which should be directly be cleaned up before rodents get the chance to eat from the left-overs. Using the knowledge of species specific behaviour is not yet applied for rural areas.

Another example of outsmarting a pest-rodent could be by placing traps using tacit knowledge and logical thinking. This would be an example of a very basic, maybe even old-fashioned way to trap rodents by outsmarting them. However, nowadays there are more technical solutions available, which could decrease the labour needed to manage pests. For example there are snap-traps that are equipped with sensors that give a signal when the trap snaps. The use of these traps with electronics makes monitoring less intensive and more effective. The use of other techniques such as visualisation and/or machine learning could also improve and simplify monitoring. For example, a very useful tool to detect (pest) rodent presence, are camera traps. The use of camera traps is more precise than looking for rodent marks like footprints, droppings, or smear marks. These cameras are able to make photos and videos, which are of added value as a picture is worth a thousand words. However, these trap camera's produce large amounts of data which need to be evaluated. Luckily there are methods developed to reduce workload by partially eliminate non-target recordings without having to watch all recordings (Swinnen, Reijniers, Breno, & Leirs, 2014).

Aim of this thesis

The main aim of this thesis was to compare rodent-borne health risks in farming systems for two cultural and climatic total different continents / regions; with the Netherlands versus Bangladesh as representative countries for Europa and Asia, respectively. In order to do this, two objectives were set up. The first objective of this thesis was to **assess the prevalence of three selected zoonotic pathogen species in wild rodents in Asia and Europe**.

To meet the first objective, the following research questions were formulated:

- What is the prevalence of pathogenic *Leptospira* and *Toxoplasma gondii* in wild rodents and insectivores in the Netherlands (Chapter 2)
- To what extent are rodents from Bangladesh infected with *Toxoplasma gondii?* (Chapter 3)
- What is the prevalence of *Leptospira* infection in rodents from Bangladesh (Chapter 4)
- What is the prevalence of *Clostridium difficile* in wild rodents and insectivores in the Netherlands (Chapter 5)

By increasing the use of correct, preventive, species specific management methods based on IPM, the chance of disease spread from rodent(s) to human will decrease. Therefore the second objective of this thesis was to **assess the effect of current rodent management methods in Asia and to improve rodent management based on IPM in order to reduce the chance for the rural population to contract rodent-borne zoonoses.**

To meet this second objective, two research questions were formulated:

- What is the efficacy of rodent management and monitoring methods on post-harvest losses by rodents in Bangladesh (Chapter 6)
- Can the landscape of fear of pest species be used within rodent pest management strategies (Chapter 7)

Chapter 2

Wild rodents and insectivores as carriers of pathogenic *Leptospira* spp. and *Toxoplasma gondii* in The Netherlands

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Submitted

Abstract

Small mammals such as rodents can to carry zoonotic pathogens. Currently, there is impaired knowledge on zoonotic pathogens in rodents and insectivores in the Netherlands. This limits opportunities for preventive measures and complicates risk-assessments for zoonotic transmission to humans. *Leptospira* spp. and *Toxoplasma gondii* are present on a list of prioritized emerging pathogens in the Netherlands and were therefore the focus of this study. Both pathogens have the ability to survive under moist environmental conditions. In total, a group of 379 small mammals (rodents & insectivores) were tested on pathogenic *Leptospira* spp., and 312 on *Toxoplasma gondii*. Rodents and insectivores were trapped at various sites, but mostly on pig and dairy farms throughout the country. Over five percent of the animals (5.3%, n=379) tested positive for *Leptospira* DNA, and five of the animals (1.6%, n=312) tested were positive for *Toxoplasma gondii* DNA. The animals positive for *T.gondii* were all brown rats and the ones for *Leptospira* spp. were various species. Our results show that insectivores and rodents might be used as an indicator for the environmental contamination and/or the contamination in wildlife for *Leptospira* spp.

Introduction

Rodents and insectivores can be potential hosts for numerous zoonotic pathogens (Meerburg, Singleton, & Kijlstra, 2009). Thus, it is essential to monitor pathogen presence in these small mammal populations. There is impaired knowledge (e.g. prevalence, geographic distribution, rodent species that are host) on rodent borne diseases in the Netherlands, which limits opportunities for preventive measures and complicates the assessment of risk of zoonotic transmission to humans. In order to increase the understanding on rodent-borne pathogens in the Netherlands, we set up a study to assess pathogen presence in common rodent and insectivore species from the Netherlands. Two important pathogens were selected from a list of prioritized emerging pathogens relevant for the Netherlands; (I) *Leptospira* spp., and (II) *Toxoplasma gondii*. Both pathogens are able to infect a wide range of species (Acha & Szyfres, 2003; Bharti *et al.*, 2003; Levett, 2001; Newell *et al.*, 2010; Opsteegh, 2011). The spirochaetal bacteria *Leptospira* spp. causes leptospirosis, which is an acute febrile disease in humans occurring worldwide (Bharti *et al.*, 2003). The global burden of human leptospirosis is estimated on more than 60,000 deaths and over 1 million of severe leptospirosis cases in studies led by the World Health Organisation (Costa *et al.*, 2015; Torgerson *et al.*, 2015; WHO, 2011). The bacterium is generally transmitted via direct or indirect contact with spirochetes secreted in the environment via the urine of infected reservoir animals (Hartskeerl, Collares-Pereira, & Ellis, 2011). Hosts can be divided into reservoir and accidental hosts. Reservoir hosts are animal species which do not show symptoms after infection, and act as infection reservoir by lifelong shedding of leptospires in their urine and via parent-offspring transmission (Foley & Straub, 2017; Mwachui, Crump, Hartskeerl, Zinsstag, & Hattendorf, 2015; Hartskeerl & Terpstra, 1996). Accidental hosts shed only for a relative short period leptospires in their urine after infection, and these hosts develop severe or even lethal disease after infection (a.o. humans) (Fraga, Carvalho, Isaac, & Barbosa, 2015; Mwachui *et al.*, 2015) Shedded leptospires have the ability to survive for prolonged periods of time in moist environments (Levett, 2001). Moreover, contaminated water is a serious risk for infection (Haake *et al.*, 2002). One of the most important wildlife reservoir hosts are rodents (Faine, 1994; Terpstra, 1989). In the Netherlands brown rats (*Rattus norvegicus*) and voles (*Microtus arvalis)* are the most important reservoir species and infection sources for human leptospirosis (Fernandes *et al.*, 2016; Guernier *et al.*, 2016; Himsworth, Parsons, Jardine, & Patrick, 2013; Obiegala *et al.*, 2017; Zilber *et al.*, 2016; Zuerner, 2015). A publication from 1996 (Hartskeerl & Terpstra) from the Netherlands showed that other rodents species and some insectivores can also be reservoir hosts (hedgehogs (*Erinaceus europeanus*), muskrats (*Ondatra zibethicus*), house shrews (*Crocidura russula*), and house mice (*Mus musculus*). Nevertheless, there is hardly

scientific information available on the presence of *Leptospira* spp. in rodents and insectivores in the Netherlands. In 1934 a report was published on Leptospirosis in the Netherlands, mentioning a prevalence of *Leptospira* spp. in brown rats of 11-56% (n= 60), emphasizing the differences between test-locations (Schüffner, 1934). Research from 1992 on muskrats in the Netherlands found 7% (n=327) positive on *Leptospira interrogans* (Steinen, Schuurman, Gravekamp, Korver, & Terpstra, 1992). More recently, a study on *Leptospira* spp. in brown rats found a prevalence of 42% (n= 150), with prevalences varying between geographic areas within the Netherlands (range of 33-57%) (Maas *et al.*, 2018). The National Institute for Public Health and the Environment in the Netherlands (RIVM) tested 189 mice on *Leptospira* spp. from between 2007 and 2015*,* and found 45.5% of *Apodemus sylvaticus* (n=55), 73.3% of the tested *Microtus arvalis* (n=60), and41.8% of the tested *Myodus glareolus* (n=74) mice positive for *Leptospira* spp. (van den Broek *et al.*, 2016).

Besides potential carriage of *Leptospira* spp., rodents and insectivores can also be infected with *Toxoplasma gondii*, a protozoan parasite (Dubey, 2014; Dubey, 2016; Kim & Weiss, 2008; Krijger, Cornelissen, Wisselink, & Meerburg, 2014; Robert-Gangneux & Dardé, 2012). Rodents have been suggested to be reservoirs of infection for cats, pigs, and dogs (Dubey & Frenkel, 1998; Kijlstra *et al.*, 2008). Felid species are the only hosts that are able to shed *Toxoplasma gondii* oocysts in the environment (Dubey, 2016) (Nicolle & Manceaux, 1908). However, the parasite is present in a wide range of warm blooded animals, first as tachyzoites and later as bradyzoites, also called tissue cysts (Dubey, 2016). When a cat consumes an infected intermediate host, the parasite can complete its lifecycle (Dubey, Miller, & Frenkel, 1970). There are a couple publications on *T. gondii* in rodent and insectivore species in the Netherlands. In 2012 250 small mammals were tested and found 4% positive for *T.gondii* (Meerburg, De Craeye, Dierick, & Kijlstra, 2012). Another study form the Netherlands found 11.9% of the rodents and insectivores (n=101) positive for *T. gondii* (Kijlstra et al., 2008). Research from 2014 on common moles (insectivore) from the Netherlands found a prevalence of 2.3% (n=86)(Krijger *et al.*, 2014). It is interesting to see that the prevalence varies per species and even per location.

Because rodents can be host to both zoonotic pathogens *Leptospira* spp. (definitive host) and *T. gondii* (intermediate host), and since the current status of its prevalence in the Netherlands is unknown, rodents and insectivores from several geographically spread areas in the Netherlands were tested on presence of those two zoonotic pathogens.

Methods

Rodent trapping was conducted from November 2016 until January 2017 on 10 conventional pig farms and one cow farm, distributed over 4 provinces in the Netherlands; Limburg, Noord-Brabant, Gelderland and Overijssel (Figure 1) by professional and certified rodent management companies. Each farmer was surveyed and asked about the presence of cats and/or stray cats on their farm. All locations were visited and screened for rodent tracks. Snap-traps were then placed accordingly by a certified pest-manager. Traps were placed one week in pre-bait position, after which they were placed and used for 1 month. Traps were checked upon daily to ensure a maximum period between capture and storage of 24h. Trapped animals were stored in separate seal bags at –18°C.

In October 2018, rodents were trapped on three locations on recreational areas in nature reserves on the island Texel (province of Noord Holland, Figure 2.1) by a rodent manager using the EKO1000 traps, and by use of the rodenator (Meyer Industries, USA). Trapped animals were stored in separate seal bags at –18°C.

Figure 2.1 Map of The Netherlands showing rodent trapping locations. * is a pig farm, ◊ is a cow farm, and • a nature reserve.

All rodents were thawed at 4°C 24 hrs before dissection. During dissection at Wageningen Bioveterinary Research (WBVR, Lelystad, The Netherlands) each animal was identified to species level and sexed and of each rodent randomly one kidney and the brains were collected. Samples were stored at -20ºC until further analysis. All rodent samples were tested for *Leptospira* spp. (n=379), whereas the samples from rodents trapped on pig farms and Texel were besides *Leptospira* spp. also tested on *Toxoplasma gondii* (n=312).

Leptospira spp. diagnostics

From each kidney sample a small transversal slice (≤ 25 mg) was cut (Figure 2.2) and treated for DNA extraction (QIAamp DNA Mini Kit, QIAGEN, Hilden Germany). All tissues were processed for DNA extraction according to the manufacturer's protocol with some modifications; tissues were digested by using 360 µl of buffer ATL (QIAGEN, Hilden Germany) and 40 µlof proteinase K (QIAGEN), mixed and incubated for 3 hours at 56°C, were heated at 70°C for 10 minutes after adding AL buffer, after which ethanol was added. All DNA samples were stored at –20°C until further testing by PCR.

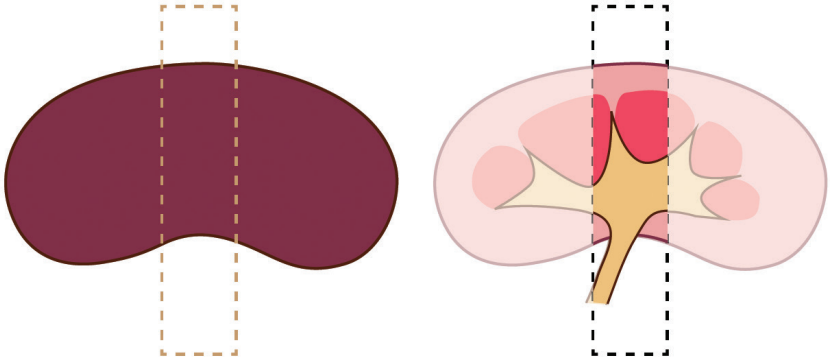

Figure 2.2 Schematic overview of the transversal slice of the kidney.

Quantitative polymerase chain reaction (RT-qPCR) for Leptospira spp. detection and speciation

Each DNA sample was diluted (1:10) with UltraPure DNase/RNase-free distilled water (Invitrogen, UK) and tested in triplicate. The SYBR Green real-time qPCR targetting *secY* gene was used (Ahmed, Engelberts, Boer, Ahmed, & Hartskeerl, 2009). Reactions of in total 25 µl were set up with 10 µl sample to be examined, 12.5 µl of SYBR Green Supermix (Bio-Rad, UK) of 2x stock reagent (100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM of each dNTP, 50 units/ml iTaq DNA polymerase, 6 mM $MgCl_2$, 20 nM fluoresein and stabilizers), 1 µl SecYIVF (400 nM) as forward primer, and 1 µl SecYIV (400 nM) as reverse primer, 0.5 µl UltraPure DNase/RNase-

free distilled water (Invitrogen, UK). For the negative control 10 µl sterile UltraPure water was used as template. A Bio-Rad CFX96 real-time PCR fast detection system was used to perform the reactions by a first cycle of 5 min of activation at 95°C with subsequent dissociation steps consisting of: 95°C/5 s; 54°C/5 s; 72°C/15 s for 40 cycles. The programme finished with 95°C/1 min and a cooling at 20°C/1 min and the dissociation was measured stepwise, every 0.5°C.

Amplicon specificity was checked by conducting a melting curve analysis which was also used to determine the *Leptospira* species; a sample was classified positive when Ct value ≤ 35 cycles and Tm between 78.5-84.5°C. Samples were tested in triplicate and classified as positive when ≥2 runs resulted positive. A retest in trifold was conducted on samples that gave only one amplification curve. Samples were classified as positive if the repeat run resulted in ≥1 positive reaction and if the amplification melting curve was conform set values.

Toxoplasma gondii diagnostics

The brain tissue was thawed at 4°C. Samples were homogenised for 30 seconds by an ultra turrax homogeniser after adding 1 ml DPBS. DNA was extracted from 250 µl of the homogenised brain tissue with the DNeasy Blood & Tissue kit (Qiagen GMBH, Hilden, Germany). The manufacturers protocol was slightly adjusted; 50-100 pc glass homogeniser beads were added to each sample and the samples were mixed by vortexing for 10 minutes at 1400 rpm to facilitate lysis. Hereafter, lysis buffer was added and samples were then incubated for 2.5 hr at 56°C, after which another vortexing cycle of 10 minutes at 1400 rpm took place. During the addition of ethanol, we added 1.5µl HCl 35%, and used only 50 µl AE buffer to elute the DNA. DNA samples were stored at –20°C until tested by Real-Time PCR. Of each sample 5 µl DNA was tested by a RT-qPCR using SYBR Green (Applied Biosystems) in an ABI 7500 Real-Time PCR system (PE Applied Biosystems). A standard reaction mixture contained 12.5 µl of SYBR Select Master Mix, 1 µl (10 µM) of the primers, 5 µl of DNA template and 5.5 µl PCR grade water. The primers (529-F: AGG AGA GAT ATC AGG ACT GTA G and 529-R: GCG TCG TCT CGT CTA GAT CG) are complementary to the 529-bp repeat element (GenBank AF146527). The cycling profile involved an initial PCR activation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and primer annealing and extension at 60°C for 60 s. Following amplification, a melt curve analysis was performed to verify the specificity of the amplified products by their specific melting temperatures (Tm). For quantification of the amount of *T. gondii* DNA in the samples, a standard curve of DNA extracted from cultured tachyzoites from the *T. gondii* RH strain was used. Data acquisition and analysis of the results were performed using the 7500 System SDS Software (Applied Biosystems). Samples with Ct-value <37.5 and Tm-value between 81.9 and 83.5°C were considered as positive.

Statistical analysis

To compare frequency between sex the Chi-square test was was used, to analyse between provinces a one way ANOVA was used, for further analyses descriptive statistics were used. Results were considered statistically significant with a p-value of $p<0.05$. Statistical analyses were performed by using SPSS, version 23 (IBM SPSS Statistics Inc).

Results

In total 379 rodents and insectivores were trapped, 351 on livestock farms (Limburg, Brabant, Gelderland and Overijssel), and 28 in nature reserves (Noord-Holland). The trapped animals consisted out of three insectivore and seven different rodent species. About half of the number of animals were black rats (*Rattus rattus*, 49.6%), second predominant species was the house mouse (*Mus musculus*, 22.2%). All trapped animals were tested for pathogenic *Leptospira* spp.. Twenty were found positive (*Leptospira* species *Interrogans* (n=15) and *Kirschneri* (n=5)) thus showing an overall incidence of 5.3% (Table 2.1). The prevalence of *Leptospira* spp. amongst wild rodents and insectivores differs significantly per province (P=0.006), with Gelderland being the province with the highest incidence (Table 2.2). There was no significant association between rodent sex and *Leptospira* spp. infection (P=0.85).

Table 2.1 Infection percentage of rodent species with *Leptospira* and *Toxoplasma gondii*.

Mammal species	Rodent or insectivore	No. positive/total (%)	
		Toxoplasma gondii	***Leptospira***
Wood mouse *(Apodemus sylvaticus)*	Rodent	0/19 (0)	2/19 (10.5)•
Harvest mouse *(Micromys minutus)*	Rodent	0/1 (0)	0/1 (0)
Common vole *(Microtus arvalis)*	Rodent	0/8 (0)	2/8 (25.0)*
Common house mouse *(Mus musculus)*	Rodent	0/84 (0)	5/84 (6.0)§
Muskrat *(Ondatra zibethicus)*	Rodent	0/1 (0)	1/1 (100)•
Brown rat *(Rattus norvegicus)*	Rodent	5/36 (13.8)	5/66 (7.6)•
Black rat *(Rattus rattus)*	Rodent	0/151 (0)	1/188 (0.5)*
Greater white-toothed shrew *(Crocidura russula)*	Insectivore	0/2 (0)	0/2 (0)
Common shrew *(Sorex araneus)*	Insectivore	0/9 (0)	4/9 (44.4)§
Crowned shrew *(Sorex coronatus)*	Insectivore	0/1 (0)	0/1 (0)
Total		**5/312 (1.6%)**	**20/379 (5.3%)**

• Species *Leptospira interrogans*
* Species *Leptospira kirschneri*
§ Both species *Leptospira interrogans* (*Mus musculus* n=4, *Sorex araneus* n=3) and *kirschneri* (*Mus musculus* n=1, *Sorex araneus* n=1)

Five animals were found positive for *T. gondii* (1.6%, Table 2.1), of which 3 female and 2 male rats. All five were brown rats from Texel (Noord Holland). With 28 brown rats (17 females, 11 males) trapped on Texel, the prevalence of this group of rodents from this specific island comes to 17.9% (Table 2.2).

Table 2.2 *Leptospira* infection percentage of the tested small mammals per province.

Province	No. tested animals	No. positive	Prevalence
Limburg	219	4	1.8%
Noord-Brabant	66	7	10.6%
Overijssel	40	5	12.5%
Gelderland	26	4	15.4%
Noord Holland	28•	0	0%
Total	**379**	**20**	**5.3%**

•On Texel (Noord Holland), only brown rats were trapped (n=28)

Discussion

Although research is conducted, still little is known about the presence and risks of zoonotic pathogens carried by rodents and/or insectivores zoonoses in the Netherlands. This knowledge gap limits opportunities for preventive measures and confounds the approximation of the potential transmission to humans. Until now, there is still little known and published about the presence of *Leptospira* spp. in rodents and insectivores in the Netherlands and other European countries. Therefore we tested rodents and insectivores from several geographically spread areas in the Netherlands on presence of those two zoonotic pathogens. In total, 5.3% of the animals (n=379) tested positive for *Leptospira* DNA, and 1.6% of the animals (n=312) tested were positive for *Toxoplasma gondii* DNA. Our results show that insectivores and rodents might be used as an indicator for the environmental contamination and/or the contamination in wildlife for *Leptospira* spp.

Most studies focus on *Rattus norvegicus* only, because these animal carriers are recognized as important infection sources for humans (Aviat *et al.*, 2009; Runge *et al.*, 2013; Terpstra, 1989) and are often present near shores of lakes, canals and rivers. In this way, they pose a serious threat for surface water contamination. A study from France (Aviat *et al.*, 2009) found 34.7% of the trapped brown rats (n=36) positive for pathogenic *Leptospira* spp., and a study in Germany found 21% of the 586 brown rats positive (Runge *et al.*, 2013). A recent study from the Netherlands reported an infection range of 33-57% in brown rats (Maas *et al.*, 2018). It is known that the infection rate amongst rats is highly variable in time and place (Kuiken, 1990; Kuiken, van Dijk, Terpstra, & Bokhout, 1991), which is also underpinned by the recent study in the Netherlands (Maas *et al.*, 2018). We found a lower infection percentage in the small mammals tested (5.3%) than these European studies. This difference could be due to multiple factors, such as difference in diagnosis methods used, or trapping year, or season. Although the majority of publications use serological methods, it is important to use molecular detection, like in the current study. A serious disadvantage of using serological methods for diagnosis is that it only detects the pathogens presence when there are sufficient levels of anti-*Leptospira* spp. antibodies present (Ahmed, Grobusch, Klatser, & Hartskeerl, 2012; Musso & La Scola, 2013). Using serological assays could therefore might lead to incorrect results. However, the main reasons for the difference in infection percentages found is that studies mentioned above focus on *R. norvegicus* only, in contrast to our study which includes more animal species. Another important reason for the difference in infection percentages is the location where the mammals were trapped. The studies above all researched mammals trapped nearby water. The animals from our study are from farms and nature reserve areas and not on

locations linked to water or water rich spaces such as rivers, canals, or recreation lakes.

Although brown rats are considered the most important hosts spreading the bacterium to humans, almost every mammal might be reckoned as potential bearer and disseminator of *Leptospira* spp. (Hartskeerl, 2006; Mwachui *et al.*, 2015) {Hartskeerl, 1996 #99;Hartskeerl, 2006 #235}. Therefore, the current study was set up to test more animal species than brown rats only. In this study it is indicated that, even though with a lower abundance, pathogenic *Leptospira* spp. are also widely distributed in other small mammals; the prevalence of *Leptospira* spp. in the tested rodents and insectivores ranged between 1-15%, with an average of 5.3%. This is confirmed by literature from European countries which report on the occurrence of *Leptospira* spp. in small rodents and shrews. A study on *Leptospira* spp. in small rodents from Croatia tested 7% of the rodents positive (n=227) (Turk *et al.*, 2003). Research from Germany on small mammals found an incidence of 5.7% (n=736) (Obiegala *et al.*, 2017), which is in line with our findings. Another study from Germany (Obiegala *et al.*, 2016) found an overall infection percentage of 9.7% (n=2961). A Swiss study from 2002 found leptospiral DNA in 12.6% of 190 small mammals (Adler, Vonstein, Deplazes, Stieger, & Frei, 2002). Czech research showed 11.6% of the trapped small mammals (n=429) positive for pathogenic *Leptospira* spp., with infection ranges varying from 0-20% between species (Treml, Pejcoch, & Holesovska, 2002). We found both *L. interrogans* and L. *kirschneri* in the rodents population tested. It is remarkable that *L. kirschneri* was found in the *Rattus rattus* (black rat). This black rat is worldwide associated with Icterohaemorrhagiae infections which belong to *L. interrogans* (Kuiken, 1990) although it harbours also *L. kirschneri* in Brazil and Mayotte (Desvars *et al.*, 2012; Moreno *et al.*, 2016). It can be concluded that besides seasonal, geographic, and temporal factors, the host species also plays a role in the infection rate.

When looking at *T. gondii* in the trapped animals, all rodents and insectivores caught on the pig and cow farms tested negative for this parasite. This is not in line with the expectations since previous studies conducted on farms in the Netherlands found rodents as well as insectivore species carrying *T. gondii;* rodents and insectivores trapped on organic farms in the Netherlands in 2004 gave an infection rate with *T. gondii* of 4% (n=250) amongst species; house mice (9.0%), common voles (4.2%) and white-toothed shrews (2.0%) (Meerburg *et al.*, 2012). Research from 2008 in the Netherlands on rodents from pig-farms, found a prevalence of 11.9% (n=101) in rodents and insectivores (Kijlstra *et al.*, 2008). Prevalences differed amongst animal species, in descending order: 14.3% of *Apodemus sylvaticus* (n=7) tested positive for *T. gondii,* 13.6% of the *Crocidura russula* (n=22), 10.3% of the *Rattus norvegicus*

(n=39), and 6.5% of the trapped *Mus musculus* (n=31) (Kijlstra *et al.*, 2008). As well in the study from Meerburg et al. (2012) as in the study by Kijlstra et al. (2008), it was noted that cats were present on the participating farms. Being the definitive host for *T. gondii,* cats could become infected by predation of infected intermediate hosts such as wildlife, or via ingestion of oocysts from the environment (Afonso, Thulliez, & Gilot-Fromont, 2006; Afonso, Thulliez, Pontier, & Gilot-Fromont, 2007; Hejlíček & Literak, 1998). In the current study however, all farms were free of cats, which might explain the absence of *T. gondii* in the small mammals tested. This is in contrast to the situation on the island Texel (NL) where there is a problem with stray cats (News, 2018; Spek, 2015). The presence of wild cats on this island (≈460km^2) could explain the relatively high prevalence of 17.9% amongst the trapped rodents (brown rats) from Texel.

Our study had some limitations, as the rodents and insectivores came from five provinces whilst there are twelve provinces in the Netherlands. A suggestion for further research would be to collect (more) rodents and insectivores from over the whole country, including all provinces to get insight in high and low frequency areas. Another 'limitation' of the study is that the samples were tested using primers which could not detect mixed infections (Moseley *et al.*, 2018), leading to a conclusion of presence of maximal one *Leptospira* species per infected animal, whilst the animal could potentially be infected with multiple *Leptospira* species. For future research, the primers for testing mixed infections should be tested and if they work as described, they should be used.

In conclusion, the results of this study indicate that *Leptospira* spp. and *T. gondii* are present in the population of wild small mammals in the Netherlands, indicating the importance of the studies for these infectious agents. Presence of *Leptospira* spp. in rodents and insectivores living around farms, could lead to transmission of the bacterium to human food (livestock) of humans itself.

Presence of *T. gondii* in small rodents present around farms could be a risk factor as rodents tend to visit barns. Theoretically production animals such as pigs could then get acquire infection, leading to potential risk for human infection as the infected meat ends on our table, potentially raw or undercooked (Guo *et al.*, 2015; Kijlstra & Jongert, 2009). Another very important risk factor for *T. gondii* is the presence of (stray) cats. A suggestion for further research would be to study the prevalence of *T. gondii* in (stray) cats in the Netherlands. For *Leptospira* spp. it is an interesting and important finding that not only brown rats, but both rodent ánd insectivore species are carriers, and therewith could be considered as potential sources for human leptospirosis in the Netherlands. Consequently, rodents and insectivores could be

good indicator species for monitoring of presence of these zoonotic pathogens in the environment.

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Chapter 3

Evidence of *Toxoplasma gondii* in rodents from Bangladesh

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Abstract

Rodents contribute to the life cycle of the protozoan parasite *Toxoplasma gondii* as an intermediate host and key prey animal of cats, the definitive host. As there is limited scientific knowledge available about the incidence and prevalence of *T. gondii* in commensal rodents in many Asian countries, we tested rodents from a commercial rice mill and eight local villages in Bangladesh for the presence of *T. gondii* DNA using rodent brain material preserved in ethanol. Overall, 10 of 296 (3.4%) rodent samples tested positive for *Toxoplasma* DNA. Our results indicate that rodents present in food-production and food storage facilities may carry *T. gondii*.

Introduction

Felids are definitive hosts for the protozoan parasite *Toxoplasma gondii* and are able to excrete *T. gondii* oocysts for several weeks following acute infection. The presence of excreted oocysts in the environment enables *T. gondii* to be taken up by numerous warm-blooded animal species acting as intermediate hosts. Humans become infected by ingesting undercooked food, contaminated water, soil or unwashed fruits and vegetables (Dubey, 2004; Dubey & Beattie, 1988). Infection may also occur via infected organ transplants, or prenatally if a mother contracts toxoplasmosis whilst pregnant. In healthy persons, toxoplasmosis is usually asymptomatic. However, congenital infections can lead to numerous foetal manifestations or neurological or ocular problems later in life (Dubey & Jones, 2008).

Rodents facilitate completion of the parasite life cycle as prey for cats. They may harbour *T. gondii* encysted within various body tissues including muscle and brain tissue (Dubey *et al.*, 1995). In many developing countries cats are often kept around households, farms, granaries and mills to control rodents. Hence, *T. gondii* transmission is possible (Brown & Khamphoukeo, 2010). In such situations, environmental contamination with oocysts increases, facilitating more intermediate host infection. Rodents can also transmit *T. gondii* directly to humans as rodents are a common food source in many countries (Khiem & Van Chien, 2003; Suwannarong & Chapman, 2014). In Thailand a seroprevalence of 4.6% of the collected rodents (n=461) was found (Jittapalapong *et al.*, 2011). Other studies have shown rodent infections may be as high as 73%, depending on rodent species, geographic region, and season (Gotteland *et al.*, 2014; Morand *et al.*, 2015; Tenter, Heckeroth, & Weiss, 2000). In humans, parts of South-East Asia are areas of high seroprevalence (Pappas, Roussos, & Falagas, 2009). In studies from 2002 and onwards, seroprevalences in pregnant women from Asia ranged from 42-49% (Borkakoty, Borthakur, & Gohain, 2007; Nissapatorn, 2007; Torgerson & Mastroiacovo, 2013). However, the situation varies per country and area as research from Vietnam reports a seroprevalence of 11.2% in pregnant women (Buchy *et al.*, 2003), and research from 2012 reports a seroprevalence of 10.3% in Japanese pregnant women (Sakikawa *et al.*, 2012). From Bangladesh, there is little information available. Here, we report the outcome of a study in 2016 and 2017 on the presence of *T. gondii* in rodents from Bangladesh using a qPCR on brain tissue stored in 98% ethanol. The potential effects of tissue storage time in ethanol has not been investigated for PCR detection of *T. gondii*.

Materials and Methods

From January 2016 to December 2017, rodents were trapped in ten locations, of which 8 were local villages (Lakhshmipur, Manaharpur, Comalla, Kadamtoli, Maurali, West-Maruali, Nagarkandi, and Baro Char) and two were rice mills in Comilla (Chittagong, all within 10kms of 23°27'23.0"N 91°10'20.6"E, Bangladesh, Figure 3.1). All villages are smallholder lowland farming communities and typically rely on rainfed rice production with limited irrigated rice production in some areas, with no discernible differences in ecology or cultural practices. The selected villages consisted of between 35 and 100 households.

Figure 3.1 Map of Bangladesh with the rat silhouette indicating the study site.

There were no discernible patterns or changes in the ratio of species between these two habitats. Rodent trapping took place in the rice storage area of the selected households and mills and was conducted every 14 days, for a period of period of three months per location per year (so traps were placed 18 nights per location in 2016 and also in 2017). Each trapping session consisted of three consecutive days (24h) with kill traps (Big snap-e; Kness, Albia, IA, USA) and live cage traps (purchased on local Bangladesh markets). Traps were baited with banana and placed in the evening and checked for captures the next morning. Rodents trapped in live traps were euthanized by cervical dislocation. Each trapped animal was identified to species, gender and maturity level, and thereafter dissected to collect brain tissue. Due to the lack of cold storage in rural areas, samples were stored in ethanol (98%) for a period of 11 to a maximum of 35 months. Samples were shipped to a laboratory in The Netherlands for further testing. Randomly, one of the two brain halves was taken from each sample and put in 20 ml Dulbecco's Phosphate-Buffered Saline (DPBS), which was refreshed after 4 hours to rehydrate overnight. Twenty-four hours later, samples were homogenised in 1 ml fresh DPBS for 30s with an ultra-turrax homogeniser. DNA was extracted from 250 µl of the homogenated brain tissue with the DNeasy Blood & Tissue kit (Qiagen GMBH, Hilden, Germany). Some adjustments to the manufacturer's protocol were made; glass beads (50 to 100pc – diameter of 0.4mm) were added to each sample and vortexed 10 minutes at 1400 *rpm* RT to facilitate lysis, where after lysis buffer was added according to the manufacturer's protocol. Samples where then incubated at 56°C for 2 hrs, vortexed 10 minutes at 1400 *rpm* at 56°C, and 1.5µl HCL 35% was added together with the ethanol for optimal DNA binding. Then, the manufacturer's protocol of the DNeasy Blood & Tissue kit was followed. During the final step, samples were eluted in 50µl AE buffer. DNA samples were stored at –20°C until tested by qPCR. Samples were tested in original DNA concentration and at a 1:5 dilution. Tachyzoite samples (*Toxoplasma gondii* parasites, RH-type, starting concentration 3*10^8 /50µl) were used as a positive control in different concentrations to determine the limit of detection (LOD), and H_2O was used as the negative control. To determine the test sensitivity, a series of 10-fold dilutions of tachyzoites starting from 3*10^7/ml was tested. DNA was tested for the 529 bp fragment of *T. gondii* (Homan, Vercammen, De Braekeleer, & Verschueren, 2000) by quantitative real-time PCR using a SyberGreen PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in an ABI 7500 Real-Time PCR system (PE Applied Biosystems, Foster City, CA, USA). A final reaction volume of 25µl was used, consisting out of 12.5 µl of two× QuantiTect SYBR Green PCR Master Mix, 10.5 µl PCR grade water, 1 µl (10 µM) of both primers (*Toxoplasma* amplification primers Tox-9, and Tox-11 (Reischl, Bretagne, Krüger, Ernault, & Costa, 2003)), and 1 µl of DNA template. The PCR procedure started with an activation step of 10 min at 95°C, followed by 50 amplification cycles consisting out of 94°C/15 s, 59°C/30s;

72°C/30s. Dissociation was measured every 0.5°C and fluorescence was measured at the end of each cycle. A melting curve analysis was performed to check the specificity of the amplicons by their specific melting temperatures (Tm). All samples were tested with an analytic test-sensitivity of 3 tachyzoites/ml (Ct 14.4 = 3*10^6 tachyzoites/assay; Ct 31.4 = 3 tachyzoites/assay). Samples were classified as positive when Ct values were <37.5 cycles, and with a Tm value between 81.9-83.5°C.

Results

The overall trap success was 40.3% in the villages and 65% in the mills. In total, 296 commensal rodents were trapped of which 49 *Bandicota bengalensis*, 8 *Bandicota indica*, 95 *Mus musculus*, 5 Mus terricolor, 15 *Rattus exulans*, and 124 *Rattus rattus*., Each animal was dissected and tested for *T. gondii* presence. The mean storage time of all samples was 21.3 months. Ten samples (3.4%) were found positive (Table 3.1), 6 of which being samples from *R. rattus* (4.8%), 2 from *B. bengalensis* (4.08%), one *M. terricolor* (20.0%), and one *M. musculus* (1.05%).

There was no statistically significant difference in prevalence between rodent species found (p=0.18), and there were no differences in the positivity rates according to sex (p=0.69). No differences in incidence were found over the trapping period or seasons.

Table 3.1 Overview of the species, sample storage time, and Ct-values of the samples that tested positive with the real time-PCR detection of *Toxoplasma gondii*.

Sample no.	Species	Storage time (months)	Ct value
1	*Bandicota bengalensis*	21	35.71
2	*Bandicota bengalensis*	17	34.37
3	*Mus musculus*	18	33.17
4	*Mus terricolor*	25	37.33
5	*Rattus rattus*	28	22.72
6	*Rattus rattus*	28	37.36
7	*Rattus rattus*	16	36.57
8	*Rattus rattus*	24	37.31
9	*Rattus rattus*	23	24.28
10	*Rattus rattus*	13	26.03

Discussion

To our knowledge, no research on *T. gondii* infection in Bangladesh rodents has been carried out before. In this study a percentage of 3.4% rodents trapped in or around food storage facilities tested positive for *T. gondii* DNA. This is in line with the results from Thai rodents in 2011, where a seroprevalence of 4.6% (n=461) was found (Jittapalapong *et al.*, 2011). However, rodent infection rates can vary depending on the species researched, the location, and climate (Gotteland *et al.*, 2014; Morand *et al.*, 2015; Tenter *et al.*, 2000). In Serbia, for example, a higher percentage of rodents was found to be positive; 10.4% of the 156 tested rodents (*Rattus norvegicus* and *M. Musculus*) were positive for Toxoplasma DNA using PCR (Vujanić *et al.*, 2010). Research from The Netherlands showed 11.9% of 101 wild rodents and shrews positive for *T. gondii* DNA (Kijlstra *et al.*, 2008), and a study from 2012 in The Netherlands found that 4% of rodents and shrews (n=250) were positive using DNA detection (Meerburg, De Craeye, Dierick, & Kijlstra, 2012), which again is more in line with the findings of our study in Bangladesh. In Brazil, wild feral rodents (Capybara (*Hydrochaeris hydrochaeris*)) were tested for *T. gondii* DNA and showed a prevalence of 15.4% (n=26) (Truppel *et al.*, 2010). In China, a PCR study to detect *T. gondii* DNA showed 22.3% of *M. musculus* to be positive (n=31) and 23.9% of the *R. norvegicus* trapped to be positive (n=92) (Yan *et al.*, 2014), which are relatively high percentages compared to other DNA studies on rodents.

Factors that could have influenced the difference in observed prevalences in the different rodent species from the selected trapping locations in Bangladesh could be the species-specific behavioural patterns, their ecology and ethology, and also the presence or absence of cats could have influenced the observed results. None of the locations had cats as pets, however, there were stray cats around which could lead to rodent infection.

Reports on *T. gondii* infection in rodents from other Asian countries are mostly on *T. gondii* detection by serologic tests (Herbreteau, Bordes, Jittapalapong, Supputamongkol, & Morand, 2012; Jittapalapong *et al.*, 2011; Salibay & Claveria, 2005) but these have several disadvantages, i.e. false negatives (Dubey, Shen, Kwok, & Thulliez, 1997). Thus, serology alone may be insufficient to determine rodent prevalence (Dubey & Frenkel, 1998). PCR is more sensitive to detect *T. gondii*, but its use may be limited by cost and lack of experience (Nimir & Linn, 2011). It is recommended to use either fresh samples or to store the samples at ≤ -20°C when carrying out PCR analyses because autolysis and/or degradation of DNA may occur when tissue samples are not immediately frozen or properly stored (Wastling & Mattsson, 2003). In our study it was not possible to use fresh or frozen

samples and, therefore, brain tissue was preserved in ethanol. The use of ethanol as a preservation method is applied in research on many other pathogens, e.g. stool samples for research on the protozoan Giardia (Wilke & Robertson, 2009), rodent ear biopsies and whole ticks for research on the bacterium *Borrelia burgdorferi s.l.*, and rodent kidneys for research on *Leptospira interrogans*. The percentage of the ethanol is critical as ethanol drives out water from tissue and cells (thus dehydrates the tissue and so preserves DNA), therefore it was decided to work with 98% ethanol. The potential effects of tissue storage time in ethanol has not been investigated for PCR detection of *T. gondii*. However, we found out that isolation of *T. gondii* brain cysts after ethanol fixation is not possible by percoll gradients, which might have been a feasible DNA isolation procedure of the purified *T. gondii* cysts (Cornelissen, Overdulve, & Hoenderboom, 1981). Some of our samples were stored for 35 months in 98% ethanol, which might have led to prolonged dehydration and subsequent DNA degradation (Prendini, Hanner, & DeSalle, 2002). Furthermore, the high spread in the Tm values by amplicons found in the field samples gives an additional limitation of the long-term storage conditions in alcohol. It is possible that in this study more animals harboured *T. gondii* DNA than the ten found positive, due to degradation of sample quality. This is an important limitation of the study, thus the results need to be interpreted only as evidence for *T. gondii* infection. The results could underestimate the prevalence of *T. gondii* infection in Bangladesh rodents. However, to confirm this, several storage conditions should be compared (i.e. fresh samples, frozen storage, ethanol storage, formalin, chemical matrices)(Lou *et al.*, 2014). A suggestion for further research would be to also include DNA testing of heart-material to minimize the chance on false negatives (Kijlstra *et al.*, 2008)

Because *T. gondii* prevalence in rodents is influenced by environmental conditions (Meerburg, Singleton, & Kijlstra, 2009) and could lead to infection of domestic cats, it is of essence that food stores and food processing facilities prevent rodent pests and limit the use of cats for rodent control. Further research is recommended to gain more insight in the prevalence of *T. gondii* in the rodent population across the food value chain in Bangladesh. A suggestion would be to study the presence of cats in the area, the prevalence of infection in cats, and the extent of rodent predation by cats. Other research which could add value to the current knowledge, is researching the specific genotypes of *T. gondii* in Bangladesh to get a better understanding of the genetic population structure in Asia (Chaichan *et al.*, 2017; Shwab *et al.*, 2014).

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Chapter 4

Prevalence of *Leptospira* infection in rodents from Bangladesh

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Abstract

Worldwide, *Leptospira* infection poses an increasing public health problem. In 2008, leptospirosis was recognised as a re-emerging zoonosis of global importance with South-East Asia being one of the most significant centres of the disease. Rodents are thought to be the most important host for a variety of *Leptospira* serovars. Because Bangladesh offers a suitable humid climate for the survival of these pathogenic bacteria, the presence of rodents could be a serious risk for human infection, especially in peri-urban areas or locations where food is stored. In order to gain more understanding of the multi-host epidemiology, a prevalence study was conducted in Comilla, Bangladesh to determine the presence of pathogenic *Leptospira* species in rodents. Real-time Polymerase Chain Reaction (qPCR) and sequencing showed that 13.1% (61/465) of the trapped rodents were infected with pathogenic *Leptospira*. Sequencing of the qPCR products identified the presence of three species: *Leptospira interrogans*, *Leptospira borgpetersenii*, and *Leptospira kirschneri*. Rodents of the genus, *Bandicota*, were significantly more likely to be positive than those of the genus, *Rattus* and *Mus*. Our results confirm the importance of rodents as hosts of pathogenic *Leptospira* and indicate that human exposure to pathogenic *Leptospira* may be considerable, also in places where food (rice) is stored for longer times. This study emphasizes the need to improve rodent management at such locations and to further quantify the public health impacts of this neglected emerging zoonosis in Bangladesh.

Introduction

Commensal rodents are known to cause substantial pre- and postharvest losses. It is estimated that rodents contribute to 5% to 10% of the losses to rice production in Asia (Grant R Singleton, 2003). Besides causing direct loss to stored food, rodents also cause indirect loss: Their gnawing makes stored produce more prone to insect or fungal attacks and they contaminate a large percentage of produce with their droppings, urine, and saliva, which could possibly harbour pathogens (Belmain, Htwe, Kamal, & Singleton, 2015; Hussain & Iqbal, 2002; Meerburg, Singleton, & Kijlstra, 2009; Mushtaq-ul-Hassan *et al.*, 2008; Singla, Singla, Parshad, Juyal, & Sood, 2008). A review by Meerburg *et al.* (2009) points out the links between food security and rodents as rodents are potential reservoir hosts for over 60 zoonotic pathogens (Battersby, 2015; Meerburg & Kijlstra, 2007; Mills & Childs, 1998). Asia is predisposed for (infectious) disease emergence (Coker, Hunter, Rudge, Liverani, & Hanvoravongchai, 2011) and there are numerous infection pathways of rodents with viruses, bacteria, and protozoans in Asia (Bordes *et al.*, 2013; Hartskeerl, 2006; Morand *et al.*, 2015; Ratnam, 1994; Vinetz, 2001). However, there is a limited number of studies available on the prevalence of rodent-borne diseases in many regions of Asia. This raises the need to determine pathogen prevalence, especially at locations where rodents come in close contact with humans or their stored food.

We studied a specific rodent-borne zoonotic pathogen, *Leptospira*, which is known to cause high disease burdens in Asia. This emerging spirochaetal bacteria occurs around rice agro-ecosystems with serious impacts on human health (Grant R Singleton, 2003). South-East Asia is mentioned as the most significant centre of the disease (Pappas, Papadimitriou, Siozopoulou, Christou, & Akritidis, 2008). Studies led by the World Health Organization (WHO) on the global burden of human leptospirosis estimated more than 1 million severe cases with over 60,000 deaths annually (Mwachui, Crump, Hartskeerl, Zinsstag, & Hattendorf, 2015; Torgerson *et al.*, 2015; WHO, 2009, 2010, 2011). Leptospirosis alone affects rural communities in most countries of Asia negatively, an endemic area for leptospirosis (Bahaman & Ibrahim, 1988; LaRocque *et al.*, 2005; Light, Nasution, & Van Peenen, 1971; Pappas *et al.*, 2008; Van *et al.*, 1998; Victoriano *et al.*, 2009). For example, in the rural areas of Bangladesh, there are innumerable ponds and shallow waters which facilitate the survival and transmission of the *Leptospira* to both maintenance hosts as well as dead-end hosts, such as humans.

In Thailand, the cases of human leptospirosis markedly increased over the 1995 to 2000 period. In 2000, leptospirosis was associated with 320 deaths reported among rice farmers (G. R. Singleton, 2003). This line is also seen in Malaysia, where the

number of reported cases has multiplied over 14 times between 2004 (248 cases reported) and 2012 (3604 cases reported) (Benacer, Thong*, et al.*, 2016). This is even likely to be an underestimation because of the lack of awareness of leptospirosis symptoms due to the wide variety of these (Hartskeerl, 2003; Hartskeerl *et al.*, 2001; Vieira, Gama-Simões, & Collares-Pereira, 2006). Moreover, it is expected that the global disease burden will increase due to climatic change in combination with population growth, the expansion of urban areas, and floods (Antesberger *et al.*, 2004; Hochrainer & Mechler, 2011; Lau, Smythe, Craig, & Weinstein, 2010; Meerburg *et al.*, 2009; Senior, 2008; UnitedNations, 2014).

Leptospira is classified into 22 species, encompassing over 300 serovars (Adler & de la Peña Moctezuma, 2010; Cerqueira & Picardeau, 2009), and in Bangladesh, at least 12 serovars have been observed (Morshed, Konishi, Terada, Arimitsu, & Nakazawa, 1994). Leptospirosis is maintained through chronic infection in the renal tubules of reservoir hosts, which shed *Leptospira* in their urine. The majority of mammalian species are natural hosts of pathogenic leptospires. Especially, small mammals can transmit infection directly or via contaminated water and food to domestic (farm) animals and humans (Bharti *et al.*, 2003; Levett, 2001; WHO, 2003). Almost every rodent species may carry and excrete leptospires (Faine, 1994). Rodents are thought to be the most important reservoir host for a variety of serovars, and serovar prevalence varies between rodent species (Levett, 2001). Rats serve mostly as reservoir of the serovars, Icterohaemorrhagiae and Copenhageni, whereas serovars of the Ballum serogroup can be found in house mice (*Mus musculus*) (Bharti *et al.*, 2003; Bolin, 2000; Levett, 2001; Thiermann, 1981). *Leptospira* serovars usually do not cause disease in reservoir hosts, but do cause disease in the dead-end host, which in this case is the human (Ko, Goarant, & Picardeau, 2009). Humans can acquire infection by contact with infected animals, animal tissue, animal excretions, or by contact of abrasions, cuts in the skin, or conjunctiva with contaminated water (Waitkins, 1987). Leptospirosis causes feverish illness in humans, and when severe it can result in Weil's disease (Faine, 1994). Weil's disease is characterized by jaundice, acute renal failure, and bleeding (McBride, Athanazio, Reis, & Ko, 2005), and is often mistaken for several diagnoses (Hartskeerl, Collares-Pereira, & Ellis, 2011; Holt, Davis, & Leirs, 2006; Laras *et al.*, 2002; Levett, 2001).

An effective strategy to minimize infection is to limit contact between humans and commensal reservoir hosts. In Asian food production systems and storage, however, the risk of contact is almost unavoidable. Thus, it is essential to monitor infection prevalence of the reservoir, in order to target control of the reservoir host at times and places where the risk is highest (Begon, 2003). Therefore, we aimed to gain

insight in the prevalence of leptospirosis occurring in and potentially transmitted by rodents in Bangladesh.

Granaries offer a rich feed source for rodents, as well as suitable circumstances for the survival of leptospires. As several *Leptospira* reservoir hosts (e.g., rodents) live in the same locations as people work and produce food, granaries harbour a potential epidemiological niche for pathogen transmission to humans (Natarajaseenivasan, Boopalan, Selvanayaki, Suresh, & Ratnam, 2002). Information on leptospirosis and its consequences is extremely limited in many regions in Asia, amongst which is Bangladesh (G. R. Singleton, 2003). To our knowledge, this study is the first one conducted in Bangladesh on the infection of rodents with *Leptospira* spp. This knowledge gap results in a lack of precautions taken when handling commensal rodents or when preparing and consuming potential contaminated food (G. R. Singleton, 2003), which requires actual prevalence rates of *Leptospira* in rodents living in and around food storages.. The objective of this study was to assess the presence and infection rate of pathogenic *Leptospira* in commensal rodent species in rural Bangladesh.

Materials and Methods

Study Area

From March 2015 until March 2016, the first part of this study was conducted in a rice mill in the South-East region of Bangladesh. Two rice milling stations and 8 villages were selected for further research in 2016 and 2017. The villages were visited for 6 months per year: June, July, August, and October, November, December. Both rice mills were visited for a period of nine months, from August 2016 until March 2017. The selected rice mills and villages are situated in the Chittagong division, Comilla district (all within a 10 km circle from 23°27’23” N 91°10’20” E). Rodent trapping was conducted in the rice storage areas of the milling stations, where the paddy rice from the fields is stored in jute bags. The owners of the rice mills participated in the project and agreed to the use of their property and buildings for this study. In the villages, rodent trapping was conducted in the rice storage area of households. No ethics approval was required because the rodents that were trapped are pest species and the Bangladesh government has no regulations on animal ethics concerning pest species. However, all local staff were trained to work with the animals according to the Netherlands code of scientific practice and the participating researchers completed the Laboratory Science Course as required by European Directive 2010/63/EU and the revised Dutch Act on Animal Experimentation. Although the animals used were not laboratory animals, the

NCad opinion on 'Alternative methods for killing laboratory animals' was followed, as provided by the Netherlands National Committee for the protection of animals used for scientific purposes. Moreover, the guidelines of the American Society of Mammologists were followed during the study. The above procedure was also mentioned in the original project proposal and approved by the donor (Netherlands Organization for Scientific Research, NWO-WOTRO).

Rodent Trapping

During the research period, every 14 days, rodent trapping was conducted for 3 consecutive periods of 24 h. Rodent trapping was performed by the use of 10 kill traps (14 × 7 cm; Big snap-e; Kness, Albia, IA, USA) and 10 life traps (purchased at local Bangladesh markets, Figure 4.1). Traps were placed during evening time at locations where rodent damage was observed and tracks were seen. Traps were checked by research staff the next morning. Rodents trapped in life traps were immediately euthanized by cervical dislocation and after identification of the species, gender, and life stage (juvenile/mature), each animal was dissected. Of each rodent, one kidney was randomly taken and stored in 98% ethanol and shipped to the OIE and National Collaborating Centre for Reference and Research on Leptospirosis (NRL), Amsterdam, The Netherlands for PCR testing.

Figure 4.1 Example of (a) the placement of a life-trap in a rice milling station, and (b) a rodent trapped in a locally purchased life trap.

Laboratory Diagnostics

Processing of the kidney samples for testing on pathogenic *Leptospira* was conducted at the NRL. From each kidney, a small transversal sample (up to 25 mg) was taken and processed for DNA extraction using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). A slightly adjusted protocol was applied; the amount of ATL buffer, proteinase K^+, AL buffer, and absolute ethanol were doubled to

ensure complete tissue lysis. Hereafter, DNA extraction took place according to the manufacturer's protocol. The extracted DNA samples were stored at –20 °C until tested by real-time quantitative polymerase chain reaction (RT-qPCR). DNA samples were 1:10 diluted using UltraPure DNase/RNase-free distilled water (Invitrogen, UK) and tested in triplicate using SYBR Green real-time qPCR, targeting the *secY* gene (Ahmed, Engelberts, Boer, Ahmed, & Hartskeerl, 2009) The reactions were set up to a final volume of 25 μL containing 10 μL of DNA sample; 1 μL of both forward and reverse primers, SecYIVF and SecYIV, at a final concentration of 400 nM each; 0.5 μL UltraPure DNase/RNase-free distilled water (Invitrogen, UK); and 12.50 μL of SYBR Green Supermix (Bio-Rad, UK) of 2 x stock reagent containing 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM of each dNTP, 50 units/mL iTaq DNA polymerase, 6 mM $MgCl_2$, 20 nM fluoresein, and stabilizers. As negative control, 10 μL of sterile UltraPure water was used. The reaction was performed and the result was analysed on a CFX96 real-time PCR detection system (Bio-Rad). The amplification protocol consisted of a first cycle of 5 min of activation at 95 °C, followed by 40 cycles of amplification (95 °C/5 s; 54 °C/5 s; 72 °C/15 s). The programme finished with 95 °C/min and cooling at 20 °C/1 min, and the amplified product was melted (70–94 °C) with plate readings set at 0.5 °C. A melting curve analysis was conducted to check the amplicon specificity. Samples were classified as positive when Ct values were ≤35 cycles with a Tm value between 78.5 and 84.5 °C. Samples were tested in triplicate and classified as positive when ≥2 runs resulted as positive. Samples that showed one amplification curve were retested in triplicate and classified as positive if the repeat run resulted in ≥1 positive reaction, and if this reaction showed an amplification melting curve that conformed to the set values. All products' real-time PCR analysis were sequenced (Macrogen, Seoul, Korea), regardless of the outcome of the PCR and blasted to double-check PCR-results. Blast results were accounted for as decisive.

Statistical Analysis

Descriptive statistics were used, and for comparisons between rodent species, Fisher's exact test was conducted. Results were considered statistically significant with a *p*-value of $p < 0.05$. Statistical analyses were performed by using SPSS, version 23 (IBM SPSS Statistics Inc, College Station, TX, USA).

Results

Rodent Trapping

In total, 465 rodents were trapped in the rice mills and villages. Most trapped rodents were identified as *Bandicota bengalensis* (*n* = 140), *Rattus rattus* (*n* = 191), followed by *Mus musculus* (*n* = 97), *Rattus exulans* (*n* = 23), *Bandicota indica* (*n* = 9), and *Mus terricolor* (*n* = 5). In the dry season, more rodents were trapped (*n* = 292) compared to the rainy season (*n* = 173).

Laboratory

No anomalies were found in the animals during the dissections. Out of 465 rodents, 177 rodents showed a positive qPCR result. Sequencing of the samples from the qPCR run revealed 61 samples which showed sequence data of the partial *secY* gene upon alignment. Table 4.1 shows these real-time PCR and sequencing results (more detailed information on all 61 positive samples can be found in Appendix A).

Table 4.1 Number of positive and negative tested kidney tissue samples from six commensal rodent species from Bangladesh for two types of tests to identify *Leptospira*, and the total number. Positive numbers are followed by their relative number (%).

Rodent Species	qPCR		DNA Sequencing		Total
	+	-	+	-	
Bandicota bengalensis	44 (31.4)	96	26 (18.6)	114	*140*
Bandicota indica	6 (66.7)	3	7 (77.8)	2	9
Mus musculus	53 (54.6)	44	5 (5.2)	92	*97*
Mus terricolor	0 (0.0)	5	0 (0.0)	5	5
Rattus exulans	19 (82.6)	4	8 (34.8)	15	*23*
Rattus rattus	55 (28.8)	136	15 (7.9)	176	191
Total	**177 (38.1)**	**288**	**61 (13.1)**	**404**	**465**

In total, 13.1% (61/465; SD = 0.33) of the tested rodents were infected with pathogenic leptospires (Table 4.2). Of the five *Mus terricolor* animals that were trapped, none were positive for *Leptospira.* The other five rodent species tested did show positive samples, showing a significant difference between infection rates per species ($p < 0.000$). The highest infection rate was found in *B. indica* (77.8%, 95% CI = 0.43–1.1), followed by *R. exulans* (34.8%, 95% CI = 0.14–0.55), *B. bengalensis* (18.6%, 95% CI = 0.12–0.25), *R. rattus* (7.9%, 95% CI = 0.04–0.11), and *M. musculus* (5.5%, 95% CI = 0.01–0.09).

When looking at gender, in total, the kidney samples of 33 (14.2%) of 233 (Standard Error = 0.02) female rodents and 28 (12.1%) of 232 (SE = 0.02) male rodents were

positive for pathogenic leptospires by sequencing and qPCR. Only female *R. rattus* were significantly more likely to be positive for *Leptospira* compared to male *R. rattus* (a *p*-value of 0.01, Pearson Chi-Square).

Significant differences were found when analysing for infection probability between species: *B. indica* showed significantly higher infection rates with *Leptospira* than all other species ($p < 0.05$, two tailed Pearson Chi-Square, Figure 4.2), and *B. bengalensis* and *R. exulans* both showed significant higher infection rates than *M. musculus* and *R. rattus*.

Table 4.2 Prevalence of Leptospira infection (positive/total number and relative number in % in parentheses) determined by sequencing and qPCR among six different rodent species per gender and total.

Rodent Species	Prevalence		Total
	Female	Male	
Bandicota bengalensis	13/67 (19.4)	13/73 (17.8)	26/140 (18.6)
Bandicota indica	5/6 (83.3)	2/3 (66.7)	7/9 (77.8)
Mus musculus	2/58 (3.4)	3/39 (7.7)	*5/97 (5.5)*
Mus terricolor	0/4 (0.0)	0/1 (0.0)	0/5 (0.0)
Rattus exulans	1/5 (20.0)	7/18 (38.9)	8/23 (34.8)
Rattus rattus	12/93 (12.9)	3/98 (3.1)	15/191 (7.9)
Total	**33/233 (14.2)**	**28/232 (12.1)**	**61/465 (13.1)**

The obtained sequence data indicated three different types of pathogenic *Leptospira* strains present in the rodents: *Leptospira interrogans* (n = 15, GenBank accession no: CP020414.1), *Leptospira borgpetersenii* (n = 11, GenBank accession no: CP015814.2), and *Leptospira kirschneri* (GenBank accession no: LSSQ00000000.1). No significant link between the rodent species and encountered *Leptospira* strains was found (Table 4.3).

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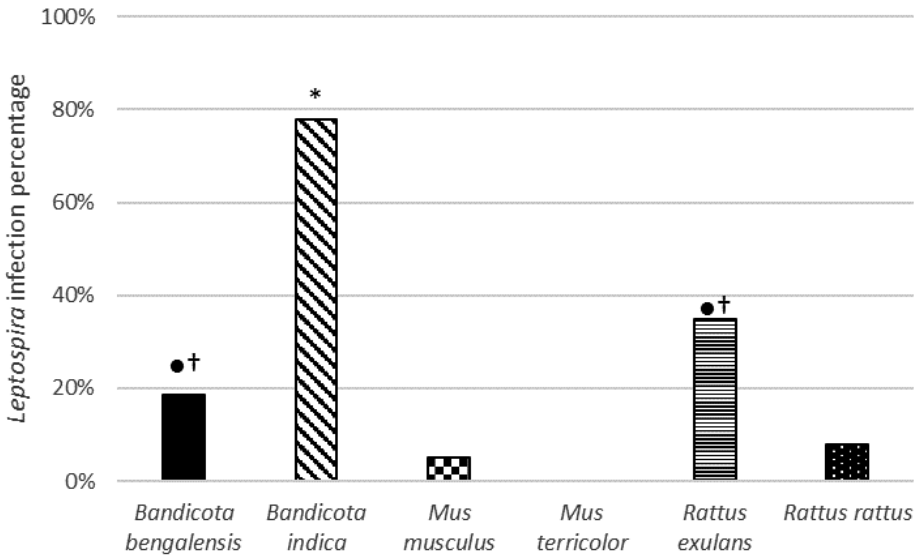

Figure 4.2 Infection percentage of six commensal rodent species from Bangladesh with *Leptospira*. * Significant difference from all ($p < 0.05$), † significant difference from *M. musculus* ($p < 0.005$), ● significant difference from *R. rattus* ($p < 0.005$)..

Table 4.3 Number of rodents found positive for six rodent species and three *Leptospira* species using qPCR and sequencing.

Rodent Species (*n*)	**Number of Rodents Positive**		
	L. borgpetersenii	***L. interrogans***	***L. kirschneri***
Bandicota bengalensis (140)	10	16	0
Bandicota indica (9)	2	5	0
Mus musculus (97)	1	4	0
Mus terricolor (5)	0	0	0
Rattus exulans (23)	1	6	1
Rattus rattus (191)	5	10	0
Total	**19**	**41**	**1**

When considering the two seasons (wet and dry), a significant difference was found for the effect of season (wet/dry) on the chance of infection with *Leptospira* (p = 0.019, two tailed Pearson Chi-Square, Table 4.4), with a higher chance of infection in the dry season.

Table 4.4 Prevalence of *Leptospira* infection (number infected/total and %) determined by sequencing and qPCR among six different rodent species and total divided into the dry and wet seasons.

Species	Dry Season	Wet Season
Bandicota bengalensis	19/77 (2.5)	7/63 (11.1)
Bandicota indica	7/9 (77.8)	0/0 (0)
Mus musculus	3/64 (4.7)	2/33 (6.1)
Mus terricolor	0/4 (0)	0/1 (0)
Rattus exulans	7/12 (58.3)	1/11(9.1)
Rattus rattus	10/126 (7.9)	5/65 (7.7)
Total	**46/292 (15.7)**	**15/173 (8.7)**

Discussion

Pathogenic *L. interrogans, L. borgpetersenii,* and *L. kirschneri* species were identified in the rural Bangladesh rodent population. Almost 1 out of every 7 (61/465) rodents trapped had *Leptospira* bacteria in their kidneys and where thus potentially capable of shedding leptospires in and around food storage. In our study, no damage or anomalies were found during the dissections, which confirms the role of the animals as a natural reservoir. These results indicate that the risk of human exposure to pathogenic *Leptospira* is likely to be substantial for the workers of the Rice Milling Station of Comilla, and also for local people, since people can acquire leptospirosis via direct or indirect contact with the urine of an infected host, which in this case can also lead to a risk for the consumers of rice. However, although handling the rice may be dangerous, the risk of contracting leptospirosis via food consumption is limited if the rice is properly cooked.

The prevalence of leptospirosis in humans in Thailand, Malaysia, and India has been reported for some areas with infection rates between 15% and 35% (Bharadwaj *et al.*, 2002; Herbreteau, Bordes, Jittapalapong, Supputamongkol, & Morand, 2012; Jena, Mohanty, & Devadasan, 2004; Karande *et al.*, 2005; Kumar *et al.*, 2012; Manocha, Ghoshal, Singh, Kishore, & Ayyagari, 2004; Samsudin *et al.*, 2015; Sehgal, Murhekar, & Sugunan, 1995; Thaipadungpanit *et al.*, 2007; Wangroongsarb, Petkanchanapong, Yasaeng, Imvithaya, & Naigowit, 2002). Despite

the occurrence of leptospirosis in South-East Asia, only a few studies have been performed on *Leptospira* prevalence in rodents, and to our knowledge, no studies from Bangladesh have been published before. Moreover, there are only scarce and frequently dated reports about the epidemiology of leptospirosis among citizens in Bangladesh. Research from 1994 showed a human seroprevalence of 38% (*n* = 89) in a rural district of Bangladesh close to rivers that regularly flood (Morshed *et al.*, 1994). However, no link with risk factors (such as rodents) was made. In 2001, febrile patients (*n* = 1297) from two hospitals in Dhaka, Bangladesh were tested on leptospirosis and 63 patients (4.8%) were found to be positive (LaRocque *et al.*, 2005). More recent research from Bangladesh showed that over 13% of febrile people (*n* = 584) were serologically positive for *Leptospira* organisms (Kendall *et al.*, 2010). One study on the prevalence of *Leptospira* in Bangladesh looked at dairy cows in Chittagong and showed that almost 50% of the samples were positive for *Leptospira* organisms (Parvez, Prodhan, Rahman, & Faruque, 2015), which poses a high infection risk to people working on cattle farms.

A study from Cambodia (2012) on *Leptospira* in rodents showed an overall infection of 11.1% (*n* = 642) (Ivanova *et al.*, 2012) and a study from Malaysia found 11.0% (*n* = 357) (Benacer, Mohd Zain*, et al.*, 2016), which correlates with our findings (13.1%), although we did not use a serological assay but a molecular assay. A serological study from Vietnam showed that 22% of trapped rodents host *L. interrogans* and all rodents were trapped in urban areas close to the South-China Sea and in Hanoi City, a city along the Red River (Koma *et al.*, 2013). Research from 2003 conducted on the Andaman Islands showed a seroprevalence of 7.1% in *R. rattus* (*n* = 85) (Sharma, Vijayachari, Sugunan, & Sehgal, 2003), which again is in line with our findings (7.9%). Research on rodents from a suburban area in India showed 14.3% of the trapped *R. rattus* (*n* = 28), and 16.1% of the *B. bengalensis* (*n* = 58) were serological positive for *Leptospira* (Saravanan *et al.*, 2000). The findings specifically on *R. rattus* infections in India by Saravanan et al. were higher than the 7.9% found in the *R. rattus* (*n* = 191) from the current study in Bangladesh. This difference could be caused by the sensitivity and specificity of the used testing methods (serology vs. molecular diagnostic), or differences in the environment, to the ratio in which rodent species occurred and possibly also to the trapping easiness (or shyness) of each species. Meta-studies on *Leptospira* prevalence in rodents in South-East Asia (Table 5) showed that *B. bengalensis, B. indica, B. savilei,* and *R. exulans* were infected with *Leptospira*, whereas investigated species of the *Mus* genus appeared to be uninfected (Singleton *et al.*, 2003; Tangkanakul & Kingnate, 1998; Thaipadungpanit *et al.*, 2007; Wangroongsarb *et al.*, 2002). In contrast, we found a *Leptospira* prevalence of 5.5% in trapped *M. musculus*.

For *B. bengalensis* and *R. rattus*, the ratio of males and females trapped was almost evenly distributed (≈1:1). For *R. exulans* (although we trapped only 23 animals of this species), more males than females were trapped (18 out of 23). For the other three species from this study, *B. indica, M. musculus*, and *M. terricolor*, more female specimens were collected. Research in 2006 from Pakistan found 40% of the trapped *B. bengalensis* to be female (*n* = 167). This male:female ratio can, in comparison to our results, be explained due to the fact that rodents trapped in this study in Bangladesh were trapped at indoor locations only, whilst in the study of Rana et al. (2006) they were trapped over multiple crop fields, which reflects the difference between male and female behaviour more. Males are more active in their explorations and depredation on food crops, whereas females may be more (trap) shy or are more concealed, or were retracted prior to breeding periods (Rana *et al.*, 2006). Moreover, in our study, there was no correlation between *Leptospira* prevalence and the gender of the animals for five of the six rodent species, which was also demonstrated in other earlier studies (Krøjgaard *et al.*, 2009; Nuttall, 1929). Some studies have shown that in Norway rats (*R. rattus*), both in South-East Asia and in the United States of America, male specimens have a higher infection rate than females (Benacer, Mohd Zain, Amran, Galloway, & Thong, 2013; Cosson *et al.*, 2014; Easterbrook *et al.*, 2007). However, in the current study, it was found that female *R. rattus* were significantly more prone to a *Leptospira* infection than males. Apparently, in our situation, the habitat's use of rats creates a mechanism where both sexes were more or less equally exposed to infection.

When looking at the results from the rodent population from our study sites, it showed *R. exulans* (*n* = 23) were present in lower numbers compared to both *B. bengalensis* (*n* = 140) and *R. rattus* (*n* = 191), while the population of *R. exulans* consisted mainly of males. This raises the question of whether *R. exulans* competes with one of the other rodent species present. It is not unusual that species compete with each other. An early report from Bombay and Calcutta (1966) showed that *B. bengalensis* increased in population size enormously and displaced the *R. rattus* in urban areas (Seal & Banerji, 1966). One of the underlying mechanisms was the high reproductive capacity of *B. bengalensis* (Parrack & Thomas, 1970; Smiet, Fulk, & Lathiya, 1980; Thitipramote, Suwanjarat, & Breed, 2009), and the aggressive behaviour that dominant males exhibit (Parrack & Thomas, 1970; Smiet *et al.*, 1980). Other research from India reported *B. bengalensis* to be the most aggressive field rodent (Mann, 1973) and that the females confine most of their time to hoarding food and are less active than males (Parrack & Thomas, 1970). Furthermore, although bandicoots are generally nocturnal, the *B. bengalensis* is known to also become active during daytime when conditions are undisturbed (Parrack & Thomas, 1970). This could be advantageous in comparison to other rodent species. Unfortunately,

almost no scientific studies are published on the competition of *B. bengalensis* with other rodent species. We found only one report from India that claimed that female *R. rattus* are submissive to *B. bengalensis* (Sridhara, Narasimham, & Krishnamoorthy, 1980). Sridhara et al. (1980) postulate that there is less aggression in closely related rodent species (e.g., within species from the *Rattus* genus) compared to the violent interaction of rodents more distantly related from each other (e.g., *Rattus* genus vs. *Bandicota* genus). Unfortunately, rodents of the *Rattus exulans* species were not researched. This finding might explain the higher number of *Bandicota* specimens and the higher *Leptospira* infection rate compared to *Rattus* specimens trapped in our study. *R. rattus* is amongst the most omnipresent rodents in the world, and has a strong potential to displace other (native) rodent species (Cox, Cox, & Warren, 2000; Harper, Dickinson, & Seddon, 2005). In Australian ecosystems, it was shown that the invasive *R. rattus* was dominant over the native *R. fuscipes* (Stokes, Banks, Pech, & Spratt, 2009; Stokes, Banks, Pech, & Williams, 2009). On Madagascar, *R. rattus* competes for resources with the native rodents and replaces native rodent species (Goodman, 1995). Because *B. bengalensis* and *R. rattus* are larger than *R. exulans* (Aplin, Brown, Jacob, Krebs, & Singleton, 2003), we expect *B. bengalensis* and *R. rattus* to be superior to *R. exulans*. In New Caledonia, Perez et al. (2011) most frequently trapped *R. rattus* specimens (>60%, n = 140) and very rarely trapped *R. exulans* rodents (<5%, n = 11), which supports our hypothesis that *R. rattus* is dominant over *R. exulans*.

In Bangladesh, *Leptospira* prevalence (in %) in rodents was significantly higher in the dry season than in the rainy season. These findings are not in line with the findings from Malaysia and also from Cambodia, where rodents showed a lower infection rate in the dry (6.3%) than in the wet (26.7%) season (Benacer, Mohd Zain*, et al.*, 2016; Ivanova *et al.*, 2012). In a study on febrile patients (n = 1297) from Dhaka, a peak in the occurrence of *Leptospira* in patients was found in October and November, shortly after the monsoon season in Bangladesh (LaRocque *et al.*, 2005). These findings confirm the relation between floods or excessive rainfall and Leptospirosis outbreaks (Lau *et al.*, 2010; Sharma *et al.*, 2003; WHO, 2009). Besides this seasonal influence on human infection, the risk of *Leptospira* infection also depends on the geographic location, as well as on other risk factors, such as the risk of flooding, contaminated surface waters, and proximity to rubbish dumps (attractive for rodents) (Halliday *et al.*, 2013; Reis *et al.*, 2008; Sarkar *et al.*, 2002). Easterbrook et al. (2007) stated that seasonal fluctuations in *Leptospira* infections in rodents do not occur due to the fact that once infected, the antibodies remain in the animal and the animal will test positive. This can explain our results and findings from other countries in South-East Asia, which show that rodent species living in

households have a stable infection level, regardless of the geography and season (Cosson *et al.*, 2014; Ivanova *et al.*, 2012).

Our study has some limitations. The extracted DNA from the kidneys was diluted before being added to the PCR mix in order to obtain better results by reducing potential inhibitors present in the samples. Inhibitors reduce the activity of the DNA polymerase enzyme and as a result a false negative result will be observed. As pathogenic *Leptospira* colonize the kidney of the rodent, using this tissue as a source for leptospires DNA is critical; however, very high concentrations of host DNA are present. The abundant DNA of the host in the PCR reaction could lead to false-positive results as SYBR green dye is able to bind to any double-stranded DNA present in a very high concentration, thus rodent DNA can also be bound. By sequencing all samples, it was possible to filter out all false positive qPCR results. Furthermore, the effect of potential residual inhibitors in the samples could possibly result in false negative qPCR results. Because of this, we consider the 13.1% prevalence to be a prudent estimate of the actual prevalence in the rodent population in the study areas in Comilla. However, the asset of this study is that molecular diagnostics were used instead, which gives an indication of the carriership of the animal. Serologic methods used by most of the other studies conducted on *Leptospira* in South-East Asia indicate that the animal did have contact with leptospires, but it remains unclear whether the animal is still a possible carrier and therefore a potential reservoir (G. R. Singleton, 2003; Wangroongsarb *et al.*, 2002).

We found *L. interrogans* as well as *L. borgpetersenii* DNA in the samples from all five rodent species that tested positive (*B. bengalensis, B. indica, M. musculus, R. rattus, R. exulans)*. Other studies confirm the relation between *R. exulans* and *L. interrogans* (Cosson *et al.*, 2014; Perez *et al.*, 2011), and in Thailand, *R. exulans* was also found to be infected with both *L. interrogans* and *L. borgpetersenii* (Kositanont, Naigowit, Imvithaya, Singchai, & Puthavathana, 2003; Wangroongsarb *et al.*, 2002). These findings are in line with the fact that *Leptospira* species, *borgpetersenii* and *interrogans*, contribute a great deal to human disease in Asia (Benacer *et al.*, 2013; Cosson *et al.*, 2014; Laras *et al.*, 2002; Thaipadungpanit *et al.*, 2007). Also, in Europe, *L. borgpetersenii* and *L. interrogans* are the most observed *Leptospira* genomospecies present in rodents; however, in Europe, a third genomospecies is

Table 4.5 Rodent species investigated for *Leptospira* species in South-East Asia, with an x indicating the presence of the *Leptospira* species in that specific rodent species.

Rodent Species	Infection % (#)	Leptospira Species					Test Method				Country
		Borg-petersenii	*Interrogans*	*Kirschneri*	*Weilii*	*Inadai**	*Serology*	DNA (kidney)	Culture	DNA (isolates)	
Bandicota bengalensis	12% (n = 42)					x	x		x		India (2000)
Bandicota bengalensis	17% (n = 42)	x	x				x		x		India (Gangadhar *et al*., 2000)
Bandicota bengalensis	18.6% (n = 140)	x	x					x			Bangladesh †
Bandicota bengalensis	16.6% (n = 58)		x				x				India (Saravanan *et al*., 2000)
Bandicota indica	4% (n = 75)	x *	x *			x	x		x		India (Gangadhar *et al*., 2000)
Bandicota indica	23% (n = 75)						x		x		India (Gangadhar *et al*., 2000)
Bandicota indica	3.7% (n = 27)		x					x			Thailand, Lao PDR, Cambodia (Cosson *et al*., 2014)
Bandicota indica	2.7% (n = 36)		x						x	x	Thailand (Wangroongsarb et al., 2008)
Bandicota indica	3.5% (n = 170)	x	x				x				Thailand (Kositanont *et al*., 2003; Wangroongsarb et al., 2002)
Bandicota indica	10.8% (n = 65)	x	x					x			Vietnam (Loan *et al*., 2015)
Bandicota indica	77.8% (n = 9)	x	x					x			Bangladesh †

Bandicota savilei	1.9% (n = 52)					x			x				Thailand, Lao PDR, Cambodia (Cosson et al., 2014)
Bandicota savilei	0% (n = 2)									x	x		Thailand (Wangroongsarb *et al*., 2008)
Bandictoa savilei	2.3% (n = 175)	x	x				x						Thailand (Kositanont *et al*., 2003; Wangroongsarb et al., 2002)
Rattus exulans	0.45% (n = 220)		x						x				Thailand, Lao PDR, Cambodia (Cosson *et al*., 2014)
Rattus exulans	18.2% (n = 11)		x					x		x			New Caledonia (Perez *et al*., 2011)
Rattus exulans	34.8% (n = 23)	x	x	x				x					Bangladesh †
Rattus exulans	38% (n = 19)						x					x	Malaysia (Benacer, Mohd Zain, *et al*., 2016)
Rattus exulans	0% (n = 1)							x		x			Malaysia (Azhari *et al*., 2018)
Rattus exulans	6.8% (n = 322)	x	x				x						Thailand (Kositanont *et al*., 2003)
Rattus exulans	6.9% (n = 317)	x	x				x	x		x			Thailand (Wangroongsarb *et al*., 2002)
Rattus exulans	0% (n = 3)									x		x	Thailand (Wangroongsarb *et al*., 2008)
Rattus exulans	14.3%		x				x						Vietnam (Loan *et al*., 2015)
Rattus rattus	17.8% (n = 129)		x					x		x			New Caledonia (Perez *et al.,* 2011)
Rattus rattus	7.9% (n = 191)	x	x					x					Bangladesh †
Rattus rattus	14.3% (n = 28)		x				x						India (Saravanan *et al*., 2000)

Table 4.5 continued

Rattus rattus	7.1% (n = 85)	x	x	x	x				Andaman Islands (Sharma *et al*., 2003)
Rattus rattus	7% (n = 285)	x	x		x			x	Malaysia (Benacer, Mohd Zain, *et al*., 2016)
Rattus rattus	70% (n = 20)	x	x				x	x	Malaysia (Benacer *et al*., 2013)
Rattus rattus	11.9% (n = 59)	x	x	x		x	x		Malaysia (Azhari *et al*., 2018)
Rattus rattus	5% (n = 464)	x	x		x	x	x		Thailand (Wangroongsarb *et al*., 2002)
Rattus rattus	4.7% (n = 492)	x	x		x				Thailand (Kositanont *et al*., 2003)
Mus musculus	5.5% (n = 97)	x	x			x			Bangladesh †
Mus musculus	0% (n = 4)								Thailand (Kositanont *et al*., 2003)
Mus terricolor	0% (n = 5)					x			Bangladesh †

* All isolates were tested with Microscopic Agglutination Test (MAT) assay on a reference panel of 21 well-known serovars, from the *L. interrogans* and *L. borgpetersenii* group, but also on 1 serovar from *L. noguchii*, *L. biflexa*, and *L. satarosai* groups—unfortunately, no specification on which serogroups tested positive is mentioned. † This study.

also commonly found in rodents: *L. kirschneri* (Mayer-Scholl *et al.*, 2014; Turk *et al.*, 2003). It is interesting to note that we also found one rodent sample infected with *L. kirschneri* from a *R. exulans* sample. Our findings are the first to confirm the presence of *L. kirschneri* in the rodent species, *R. exulans*, in Bangladesh.

Bandicota is an Asian genus of rodents, consisting of three species: *B. bengalensis*, *B. indica*, and *B. savilei* (Aplin, Frost, Tuan, Lan, & Hung, 2003; Carleton & Musser, 2005; Corbet & Hill, 1992; Musser & Brothers, 1994; Wilson & Reeder, 2005). The few reports on *Leptospira* prevalence in the *Bandicota* genus indicate that all three species are potential carriers of the same leptospira species (*L. interrogans, L. borgpetersenii, L. weilli, L. inadai*). Our study found that the probability of an infection with *Leptospira* was significantly higher for rodents of the *Bandicota* genus. Thus, *Bandicota* rats could be an important host in the epidemiology of leptospirosis in Comilla. Previous studies on urban rodents identify *R. rattus* to be the main reservoir host for human pathogenic *Leptospira* (Johnson *et al.*, 2004; Ko *et al.*, 1999; Sarkar *et al.*, 2002). However, due to the limited information available, it is not possible to link the strain or serovar infection to a specific host species. This is unfortunate, as such information could give insight into a possible co-evolution of serovars with specific rodent species. Other studies from countries with a similar climate and cropping season as Bangladesh have used mostly serological and culturing methods, and unfortunately no specification is made into specific serogroups for each rodent species. Therefore, the only comparison possible is to see which strains are found and if this correlates with the findings in Bangladesh (Table 4.5).

Cosson et al. (2014) postulate that *Leptospira* species show an ecological niche; they found *L. borgpetersenii* to be more abundant in rodents from dry habitats (non-floodable lands) than *L. interrogans,* which implies that the infection of rodents can be linked to ecology. The *B. indica* (n = 172) is more common in the field (74%) than in or around houses (Cavanaugh, Ryan, & Marshall Jr, 1969; Herbreteau, Gonzalez, Andrianasolo, Kittayapong, & Hugot, 2005), and in Vietnam, the *B. bengalensis* was found only in grass habitats (1969). The fact that the *Bandicota* species from Bangladesh were found to be infected with *L. interrogans* (Table 4.5) is thus in line with expectations.

Our study provides new data on rodent species as carriers of pathogenic *Leptospira* in South-East Asia. From our results and the literature research (Table 4.5), we can state that *L. interrogans* and *L. borgpetersenii* are the most common species found in rodents in South-East Asia. However, to find out whether specific strains/serovars adapt to specific reservoir hosts in specific habitats, more in-depth research with different diagnostics needs to be conducted. Although our results confirmed the

importance of *Bandicota* spp. and *Rattus* spp. as hosts of leptospires for human health and our findings indicate that human exposure to pathogenic *Leptospira* may be considerable in Comilla, the impact of leptospires on human health continues to be under recognised. In many Asian human populations, including populations in Bangladesh, the burden of undifferentiated feverish illness is substantial (Victoriano *et al.*, 2009). One way to minimise this problem of recognising the disease is to conduct broader diagnostic tests to determine the cause of these illnesses and to inform people on the preventive measures they can take to prevent leptospirosis. Our findings highlight the complex multi-host epidemiology of leptospirosis and the importance of considering the role of rodents, and other animal hosts in the maintenance and transmission of infection when evaluating human risks. One of the key actions to minimise the public health impacts of leptospirosis in Bangladesh is to improve rodent management. A key question is the percentage to which the rodent population should be reduced to and which species should be diminished to prevent infection. In any case, preventive measures should be taken for rodent control, such as preventing rodents from accessing domestic areas and food storage to prevent pathogen transmission to humans. Rodents could be denied access to food and drinking water by taking rodent-proofing measures to existing buildings or by constructing rodent-proof warehouses. Furthermore, rodents could be discouraged from visiting domestic areas by keeping the environments clean, removing potential nesting sites, and by installing adequate sanitation and waste disposal.

With more knowledge about the rodent species present, leptospirosis, and its consequences, local people can be informed about the need for better rodent management practices. This could lead to a reduction of the impact of rodent-borne zoonotic disease in Bangladesh

Conclusions

The objective of this study was to assess the presence and infection rate of pathogenic *Leptospira* in commensal rodent species in rural Bangladesh. In order to do so, 465 rodents were collected from a total of six different species, in descending order of appearance: *Bandicota bengalensis*, *Rattus rattus*, *Mus musculus*, *Rattus exulans*, *Bandicota indica*, and *Mus terricolor*.

Pathogenic *Leptospira* was found in 13.1% of all rodents, and three *Leptospira* species were identified: *Leptospira interrogans, Leptospira borgpetersenii,* and *Leptospira kirschneri.* Significant differences were found for the infection probability between species: *B. indica* showed significantly higher infection rates with *Leptospira* than all other species, and *B. bengalensis* and *R. exulans* both showed significantly higher infection rates than *M. musculus* and *R. rattus*.

Rodents trapped carrying *Leptospira* bacteria in their kidneys (13.1%) are potentially capable of shedding leptospires in and around food storage. These findings indicate that the risk of human exposure to pathogenic *Leptospira* is likely to be substantial for local people, since people can acquire leptospirosis via direct or indirect contact with the urine of an infected host, which in this case can also lead to a risk for the consumers of rice. We can conclude that our findings highlight the complex multi-host epidemiology of leptospirosis and the importance of considering the role of rodents, and other animal hosts, in the maintenance and transmission of infection when evaluating human risk. One of the key actions to minimise the public health impacts of leptospirosis in Bangladesh is to improve rodent management.

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Baker, Badruddoza Bappy, and Saiful Haque. The authors sincerely thank the owners of the rice mills for their kind cooperation.

Table A1. Detailed information of all positive samples for Leptospira infection determined by sequencing and qPCR among six different rodent species, in order of trapping date.

Date (dd.mm.yy)	Species	Location (Mill or Village Name)	Sex (m/f)	Season
22.04.15	*Bandicota bengalensis*	Modern rice mill	M	Dry
08.05.15	*Bandicota bengalensis*	Modern rice mill	F	Dry
09.05.15	*Bandicota bengalensis*	Modern rice mill	M	Dry
10.05.15	*Bandicota bengalensis*	Modern rice mill	M	Dry
10.05.15	*Bandicota bengalensis*	Modern rice mill	M	Dry
25.05.15	*Bandicota bengalensis*	Modern rice mill	F	Dry
29.06.15	*Bandicota bengalensis*	Modern rice mill	M	Wet
15.07.15	*Bandicota bengalensis*	Modern rice mill	M	Wet
16.07.15	*Bandicota bengalensis*	Modern rice mill	M	Wet
17.07.15	*Mus musculus*	Modern rice mill	F	Wet
18.08.15	*Mus musculus*	Modern rice mill	M	Wet
19.08.15	*Rattus rattus*	Modern rice mill	M	Wet
20.08.15	*Rattus rattus*	Modern rice mill	F	Wet
04.09.15	*Rattus exulans*	Modern rice mill	M	Wet
04.09.15	*Bandicota bengalensis*	Modern rice mill	F	Wet
05.09.15	*Bandicota bengalensis*	Modern rice mill	M	Wet
22.09.15	*Bandicota bengalensis*	Modern rice mill	F	Wet
12.11.15	*Bandicota bengalensis*	Modern rice mill	F	Dry
28.11.15	*Bandicota bengalensis*	Modern rice mill	F	Dry
29.11.15	*Bandicota bengalensis*	Modern rice mill	F	Dry
29.11.15	*Rattus rattus*	Modern rice mill	F	Dry
01.01.16	*Bandicota bengalensis*	Modern rice mill	M	Dry
02.01.16	*Bandicota bengalensis*	Modern rice mill	M	Dry
18.01.16	*Bandicota bengalensis*	Modern rice mill	F	Dry
04.02.16	*Bandicota bengalensis*	Modern rice mill	M	Dry
05.02.16	*Bandicota bengalensis*	Modern rice mill	F	Dry
13.07.16	*Rattus rattus*	Lakhmsipur	M	Wet
23.11.16	*Bandicota indica*	Maruali	F	Dry
29.11.16	*Bandicota indica*	Maruali	F	Dry
14.12.16	*Bandicota indica*	Maruali	F	Dry
27.12.16	*Bandicota indica*	Maruali	M	Dry
28.12.16	*Bandicota indica*	Maruali	M	Dry
01.01.17	*Rattus rattus*	Maruali	M	Dry

01.01.17	*Rattus rattus*	Maruali	F	Dry
04.01.17	*Rattus rattus*	Maruali	F	Dry
08.01.17	*Rattus rattus*	Maruali	F	Dry
09.01.17	*Rattus rattus*	Maruali	F	Dry
09.01.17	*Bandicota bengalensis*	Maruali	F	Dry
09.01.17	*Rattus rattus*	Maruali	F	Dry
09.01.17	*Bandicota indica*	Maruali	F	Dry
12.01.17	*Bandicota indica*	Maruali	F	Dry
12.01.17	*Rattus rattus*	Maruali	F	Dry
15.01.17	*Rattus rattus*	Maruali	F	Dry
15.01.17	*Bandicota bengalensis*	Maruali	F	Dry
02.02.17	*Rattus rattus*	Sonali rice mill	F	Dry
08.02.17	*Bandicota bengalensis*	Sonali rice mill	F	Dry
15.02.17	*Rattus exulans*	Sonali rice mill	M	Dry
15.02.17	*Rattus exulans*	Sonali rice mill	M	Dry
16.02.17	*Rattus exulans*	Sonali rice mill	M	Dry
22.02.17	*Rattus exulans*	Sonali rice mill	F	Dry
22.02.17	*Rattus exulans*	Sonali rice mill	M	Dry
23.02.17	*Rattus exulans*	Sonali rice mill	M	Dry
28.02.17	*Mus musculus*	Sonali rice mill	F	Dry
28.02.17	*Mus musculus*	Sonali rice mill	M	Dry
01.03.17	*Bandicota bengalensis*	Sonali rice mill	F	Dry
07.03.17	*Rattus exulans*	Sonali rice mill	M	Dry
15.03.17	*Bandicota bengalensis*	Sonali rice mill	M	Dry
22.06.17	*Rattus rattus*	Kadamtoli	F	Wet
05.07.17	*Rattus rattus*	Kadamtoli	F	Wet
18.07.17	*Bandicota bengalensis*	Manoharpur	M	Wet
15.11.17	*Mus musculus*	Maruali	M	Dry
22.04.15	*Bandicota bengalensis*	Modern rice mill	M	Dry
08.05.15	*Bandicota bengalensis*	Modern rice mill	F	Dry

Chapter 5

Clostridium difficile in wild rodents and insectivores in the Netherlands

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Abstract

With wild rodents and insectivores being present around humans and their living, working, and food production environments, it is important to gain knowledge of the zoonotic pathogens present in these animals. The enteropathogen *Clostridium difficile,* an opportunistic anaerobic bacteria, can be carried by both animals and humans, and is distributed globally. It is known that there is genetic overlap between human and animal sources of *C. difficile.* In this study, the aim was to assess the presence of *C. difficile* in rodents and insectivores trapped on and around pig and cattle farms in The Netherlands. In total 347 rodents and insectivores (10 different species) were trapped and 39.2% tested positive for presence of *C. difficile*. For all positive samples the ribotype (RT) was determined, and in total there were 13 different RTs found (in descending order of frequency: 057, 010, 029, 005, 073, 078, 015, 035, 454, 014, 058, 062, 087). Six of the ribotypes isolated from rodents and insectivores are known to be associated with human CDI; RT005, RT010, RT014, RT015, RT078, and RT087. The presence of rodents and insectivores in and around food production buildings (e.g. farms) could contribute to the spread of *C. difficile* in the human environment. In order to enable on-farm management for pathogen control, it is essential to comprehend the role of wild rodents and insectivores that could potentially affect the ecology of disease agents on farms.

Introduction

The opportunistic anaerobic bacteria *Clostridium difficile* is an enteropathogen for both humans and animals that is distributed globally (Freeman *et al.*, 2010). There are more than 800 ribotypes of *C. difficile* known and this gram-positive bacteria can be found in the intestinal tract of many animal species, but also in water, soil, and on meat (Al Saif & Brazier, 1996; de Boer, Zwartkruis-Nahuis, Heuvelink, Harmanus, & Kuijper, 2011; Fawley, 2018; Songer *et al.*, 2009). *C. difficile* infection (CDI) is one of the most frequently observed sources of mucosal injury and inflammation in hospital patients, leading to diarrhoea or inflammation of the colon (Kelly & LaMont, 1998). However, it is also described in patients who did not visit the hospital (Chernak *et al.*, 2005). CDI is an emerging disease, both in human patients and in animals used for food (Balsells *et al.*, 2018; Crobach *et al.*, 2018; Keessen, Gaastra, & Lipman, 2011; Rodriguez Diaz, Seyboldt, & Rupnik, 2018). The bacterium *C. difficile* not only causes disease in humans, it is also able to cause enteric disease in several animal species, such as horses, piglets, calves, and other domestic animals (Båverud, 2002; Kecerova, Cizek, Nyc, & Krutova, 2019; Rupnik, 2007; Rupnik, Wilcox, & Gerding, 2009). This finding suggests that animals and humans may share a common source (Rupnik, 2007), and it has been shown that there is substantial overlap of *C. difficile* strains present in humans and animals (Keessen *et al.*, 2011; Rodriguez Diaz *et al.*, 2018). This overlap of *C. difficile* types could indicate zoonotic spread amongst animals and humans. With wild rodents being present around humans and their living, working, and food production environments, it is important to gain knowledge of the zoonotic pathogens present in these commensal rodents (Himsworth *et al.*, 2014; Meerburg, 2010; Meerburg, Singleton, & Kijlstra, 2009) and insectivores. There are few studies published on the presence of *C. difficile* in rodents (Andrés-Lasheras *et al.*, 2017; Burt, Meijer, Burggraaff, Kamerich, & Harmanus, 2018; Burt, Siemeling, Kuijper, & Lipman, 2012; de Oliveira *et al.*, 2018; Himsworth *et al.*, 2014) and even fewer in insectivores (Jardine, Reid-Smith, Rousseau, & Weese, 2013). Therefore the aim of this study was to assess the presence of *C. difficile* in rodents and insectivores trapped on and around pig and cattle farms in The Netherlands. *C. difficile* spores in rodent droppings are able to survive in the environment for prolonged periods, which leads to numerous options for host-to-host exposure and transmission (Knetsch *et al.*, 2018; Leffler & Lamont, 2015). In order to enable pathogen control on farms, it is essential to understand the role of wild rodents and insectivores that could potentially affect the ecology of disease agents on farms (Rothenburger, Rousseau, Weese, & Jardine, 2018).

Materials and Methods

Small mammal trapping was conducted from November 2016 until January 2017 on 10 conventional pig farms and one dairy farm in The Netherlands distributed over the country. Rodents and insectivores were trapped using snap-traps as part of standard pest-control activities (cadavers were otherwise destined for disposal). The period between capture and storage was kept as short as possible to prevent for overgrowth (max 24 hrs). Trapped animals were stored in separate bags at –18°C. All specimens were thawed at 4°C 24hrs before dissection. During dissection at the Wageningen Bioveterinary Research Instutue, each animal was identified to species level and sexed. Samples of 2-4 droppings were collected from the ileum of each animal. Samples were stored at -20ºC until further analysis.

Analysis and ribotyping of the samples

Analysis of the rodent gut content for *C. difficile* was conducted following the procedure of Hopman et al (Hopman *et al.*, 2011), except for two alterations; (I) *C. difficile* enrichment broth was used (CDEB, Mediaproducts, Groningen, The Netherlands) in the enrichment phase, and (II) samples were incubated for 7 days in CDEB before plating out on agar (selective agents in CDEB were moxalactam and norfloxacine). Samples were classed as positive for *C. difficile* if they produced colonies of Gram-positive rods with a characteristic odour of horse manure and typical morphology (grey colonies with an uneven edge). Isolates were further identified and characterized at the National Reference Laboratory at Leiden, The Netherlands by capillary ribotyping (Bidet *et al.*, 2000) following the consensus protocol as described by Fawley *et al*.(2015).

Statistical analysis

The results of the *C. difficile* analysis were compared between the genders of the rodents and insectivores caught, using an independent samples T-test using IBM SPSS statistics software, version 23.

Results and discussion

In total 347 rodents and insectivores were trapped with snap traps and tested for the presence of *C. difficile* (Table 5.1). Ten different species were analysed, three of which were insectivores; the greater white-toothed shrew (*Crocidura russula),* the common shrew *(Sorex araneus),* and the crowned shrew *(Sorex coronatus*). Rodents were caught in greater numbers than insectivores, with the black rat (*Rattus rattus*) being predominant (53.6%), followed by the house mouse (*Mus musculus*, 24.2%). It was found that 39.2% (n=347) of the trapped rodents tested positive for *C. difficile*. This percentage is in line with a previous study on *C. difficile* in rodents from The Netherlands, in which 35% of the rodents were positive (Burt *et al.*, 2018). Similar to other previous studies on *C. difficile* in rodents (Burt *et al.*, 2018; Himsworth *et al.*, 2014), there was no association between gender and occurrence of *C. difficile* in the present work. This is in contrast to many other pathogens, for which male rodents have been shown to be more prone to infection (Meerburg *et al.*, 2009).

Table 5.1 Overview of results of *C. difficile* analysis per rodent and insectivore species and gender.

		Number of animals (no. positive for *C. difficile* between brackets)			
Species	**Type**	**Female**	**Male**	**Total**	**%**
Wood mouse *(Apodemus sylvaticus)*	Rodent	10	9 (1)	19 (1)	5.3
Greater white toothed shrew *(Crocidura russula)*	Insectivore	1	1	2	0
Eurasian harvest mouse *(Micromys minutus)*	Rodent	1	0	1	0
Common vole *(Microtus arvalis)*	Rodent	4	4 (1)	8 (1)	12.5
House mouse *(Mus musculus)*	Rodent	36 (17)	48 (13)	84 (30)	35.7
Muskrat *(Ondatra zibethicus)*	Rodent	0	1 (1)	1 (1)	100
Brown rat *(Rattus norvegicus)*	Rodent	18 (3)	18	36 (3)	8.3
Black rat *(Rattus rattus)*	Rodent	100 (56)	86 (43)	186 (99)	53.2
Common shrew *(Sorex araneus)*	Insectivore	3	6 (1)	9 (1)	11.1
Crowned shrew *(Sorex coronatus)*	Insectivore	0	1	1	0
Total		**Total**	**174 (59)**	**347 (136)**	**39.2**

The ribotype for all samples of rodent and insectivore intestinal content was determined, and found 13 different RTs in total (in descending order of frequency: 057, 010, 029, 005, 073, 078, 015, 035, 454, 014, 058, 062, 087, Table 5.2). The black rat (*Rattus rattus*) and house mouse (*Mus musculus*) are species with the highest diversity in RTs, 8 and 7 types, respectively. The ribotype most frequently isolated

was RT057, which was only found in black rats and house mice. Although present at such high percentages, no references to RT057 could be found in the literature. However, RT057 is also frequently found in humans and characterized as producing toxin A and B (unpublished data of the Dutch National Reference Laboratory for *C. difficile* infections). The fact that no literature was found on this ribotype could be due to the possibility that RT057 does not result in clinical symptoms in humans.
Three insectivore species were tested, of which one (*S. araneus*) was found to carry *C. difficile* (RT005). Unfortunately, literature on *C. difficile* in shrews or other insectivores such as moles or hedgehogs is scarce. Only one published report could be found: a study in Canada assessed *C. difficile* in wild mammals, including two short-tailed shrews (*Blarina brevicauda*) from around a dairy farm, one of which was found positive for *C. difficile* (Jardine *et al.*, 2013).

It is known that there is genetic overlap between human and animal sources of *C. difficile* (Crobach *et al.*, 2018; Knight & Riley, 2016; Rodriguez Diaz *et al.*, 2018). In this study, 6 ribotypes that are known to be associated with human CDI were isolated from rodents; RT005, RT010, RT014, RT015, RT078, and RT087. Below, we describe the four which were found in more than one of our samples.

In Europe, RT005 is a source of CDI in humans (Freeman *et al.*, 2015; Reil *et al.*, 2012) and is also associated with rodents. In a recent study from New York, RT005 was isolated from *Mus musculus* (Williams *et al.*, 2018). RT005 has also been described in pest species around pig farms (*Mus musculus, Rattus* sp.) in Spain (Andrés-Lasheras *et al.*, 2017), in a Norway rat (*Rattus norvegicus)* in Canada (Himsworth *et al.*, 2014), and in an urban mouse in The Netherlands (Burt *et al.*, 2018).

In Europe, RT014 has also been found to cause CDI in humans (Freeman *et al.*, 2015), and occurs prominently in Dutch CDI patients (Bauer *et al.*, 2011; Hensgens, Goorhuis, Notermans, van Benthem, & Kuijper, 2009) as well as in other European countries (Arvand *et al.*, 2014; Indra *et al.*, 2015). Of the isolated RT types, RT 014 occurs as most often reported type in the database of Dutch National Reference Laboratory for C. difficile infections since 2006 (see Supporting information, Table S1). RT014 is commonly found in pigs (Knight & Riley, 2016; Knight, Squire, & Riley, 2015; Martin, Monaghan, & Wilcox, 2016). In previous studies, ribotype 014 was found in rodents as well (Burt *et al.*, 2018; de Oliveira *et al.*, 2018; Himsworth *et al.*, 2014). Cats and dogs have been found to carry RT014 (Andrés-Lasheras *et al.*, 2018; Rabold *et al.*, 2018), which could be linked to the rodents; as cats commonly hunt small rodents, *C. difficile* can possibly be transferred from rodent to cat.

A third ribotype isolated from the rodents/insectivores, which is known to be associated with human CDI, is RT078. This is a known causative agent for human CDI in Europe (Goorhuis, Bakker*, et al.*, 2008; Hensgens, Goorhuis, Notermans, Bethem, & Kuiper, 2010) and the most common ribotype present in pigs, causing diarrhoea in these animals (Debast *et al.*, 2009; Goorhuis, Debast*, et al.*, 2008; Keel, Brazier, Post, Weese, & Songer, 2007). RT078 is the third-most frequently found PCR ribotype in Dutch hospitals and in hospitals in several other European countries (Bauer *et al.*, 2011; Hensgens *et al.*, 2009). A study from 2012 (Burt *et al.*, 2012) showed that *M. musculus* from a pig farm and other pest species present on the farm (insects, birds, rodent droppings, and bird droppings) carried RT078. In Spain, RT078 was also found in rodents (*Rattus* sp. and *Mus musculus*) on Spanish pig farms (Andrés-Lasheras *et al.*, 2017).
Another well-known human ribotype is RT010, which was recently also found in dogs (Álvarez-Pérez *et al.*, 2015; Rabold *et al.*, 2018) and in rabbits (Drigo *et al.*, 2015). The occurrence of this strain in animals and humans suggests at least a common source of infection.

Evidence for zoonotic transmission of *C. difficile* (strain RT078) has only recently been reported by Knetsch et al. (2014; 2018), and for strain RT014, evidence was found for zoonotic transmission between pigs and humans (Knight, Squire, Collins, & Riley, 2017). This transmission potential between animals and humans leads to a zoonotic risk, not only between humans and farm animals, but also pets and humans, and (indirectly) rodents and humans. This study concludes that wild rodents and insectivores are a reservoir for several *C. difficile* ribotypes, some of which are associated with human CDI. The presence of rodents and insectivores in and around food production buildings (e.g. farms) could contribute to the spread of *C. difficile* in the human environment. An interesting question to address during future research is whether the ribotypes found in these small mammals are also present in the environment if rodents and insectivores are absent. If so, this could mean that small mammals acquire infection from the environment and are then able to distribute the pathogen further throughout their habitat.

Table 5.2 *Clostridium difficile* ribotypes confirmed in samples of the intestinal contents of wild rodents and insectivores in The Netherlands.

Ribotype (RT)	No. of isolates	Species
005•	10	*M. musculus, R. rattus, S. araneus*
010•	12	*R. rattus*
014•	1	*R. rattus*
015•	2	*M. Musculus, R. norvegicus*
029•	12	*A. sylvaticus, M. arvalis, M. musculus*
035	2	*M. musculus*
057	81	*M. musculus, R. rattus*
058	1	*R. rattus*
062	1	*R. rattus*
073	6	*M. musculus*
078•	5	*M. musculus, O. zibethicus, R. rattus*
087•	1	*R. norvegicus*
454	2	*R. rattus*

•RT associated with CDI in humans

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Supporting Information Legend

Table S1. Occurrence of the *Clostridium difficile* ribotypes from this study in the Dutch human database since 2006 (unpublished data of the Dutch National Reference Laboratory for C. difficile infections).

Ribotype (RT)	Number of times reported in Dutch National Reference Laboratory
005•	663
010•	116
014•	2291
015•	515
029	143
035	13
057	58
058	1
062	33
073	17
078•	1496
087•	180
454	11

•RT associated with CDI in humans

All RT types were isolated from human faeces sent to the Dutch National Reference Laboratory for *C. difficile* infections. The fact that an RT type is isolated does not necessarily mean that the person has CDI as we do not have insight in clinical information on the samples.

Chapter 6

Efficacy of rodent management and monitoring methods on post-harvest losses by rodents in Bangladesh

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Submitted

Abstract

The presence of pest rodents around food production and storage sites is one of many underlying problems contributing to food contamination and loss, particularly influencing food and nutrition security in low-income countries. By reducing both pre- and post-harvest losses by rodents, millions of food-insecure people would benefit. Studies on the impact of rodents is particularly lacking in post-harvest systems. As there is limited quantitative data on post-harvest rice losses due to rodents in Asia, we assessed stored rice-losses in local households from eight rural communities and two rice milling factories in Bangladesh in order to monitor the effect of different rodent control strategies. Four treatments were applied, (i) untreated control (ii) use of domestic cats, (iii) use of rodenticides, (iv) use of snap-traps. In total, over a two year period 210 rodents were captured from inside people's homes, with Rattus rattus trapped most often (n= 91), followed by Mus musculus (n=75) and Bandicota bengalensis (n=26). In the milling stations, 68 rodents were trapped, of which 21 M. musculus, 19 R. rattus, 17 B. bengalensis, 8 Rattus exulans, and 3 Mus terricolor. In 2016, losses from rice-baskets within households were between 13.6-16.7%. In 2017, the losses were lower, ranging from 0.6-2.2%. Daily rodent removal trapping proved to be most effective to diminish stored produce loss. The effectiveness of domestic cats was limited.

Introduction

The fight against hunger persists with the number of undernourished people continuing to rise. In 2017 about 820 million people were undernourished globally (2018). FAO defines undernourishment as the daily energy intake of a person being too low to meet their daily minimum dietary energy requirements (kcal/day per person). Southern Asia has the highest undernourishment rate with an estimated number of over 275 million people suffering from hunger (FAO *et al.*, 2018). In Bangladesh the proportion of undernourished in 2017 was around 15% of the total population, which is almost 25 million people (FAO *et al.*, 2018). Asia produces more than 90% of global rice production with rice accounting for approximately 60% of the daily caloric intake on average across Asia (Singleton, 2003). One contributing factor to food insecurity is the presence of rodents. On yearly basis, rodents cause 5-10% loss to rice production in Asia, which leads to a worldwide estimated loss of 11 kg of food per person per year (Singleton, 2003). By sustainably reducing pre- and post-harvest losses by rodents, nearly 280 million undernourished people could meet their daily energy requirements (Meerburg, Singleton, & Kijlstra, 2009).

For this study, Bangladesh was selected as study case. In 2018, Bangladesh produced 53.6 million tons of paddy rice (FAO, 2018), where a loss of 10% of post-harvest rice loss due to rodents parallels to an annual loss of 5.36 million tons. Singleton (2003) states that reports of up to 20% post-harvest grain losses due to rodents are not uncommon. Unfortunately, there is little quantitative data on Asian post-harvest losses to cereals due to rodents (Belmain, Steven R, Htwe, Kamal, & Singleton, 2015; Htwe, Singleton, & Maw, 2016; Mian, Ahmed, & Brooks, 1987; Parshad, 1999; Singleton, 2003). From previous studies it is known that rodents do play a significant role in post-harvest losses in Asia, but only a few recent publications (Belmain, Steven R *et al.*, 2015; Brown, P. R., McWilliam, & Khamphoukeo, 2013; Htwe *et al.*, 2016) provide information on the magnitude of the post-harvest rice losses of villages in Southern Asia. Research by Belmain et al. (2015) indicated an annual household loss of over 70 kg of stored rice. The study of Htwe *et al*. (2016) in Myanmar calculated that the total amount of grain that was lost due to rodents, came down to enough rice to feed local households for 1.6–4 months. Belmain *et al.* (2015) showed that farmers without rodent management on average lose 2.5% of their stored rice stocks, but when applying rodent management they reduced the loss to 0.5% (Belmain, Steven R *et al.*, 2015). Therefore, the first objective of the current study was to assess how large post-harvest losses in Bangladesh are in local households and in rice milling stations.

With the knowledge that proper rodent management leads to decrease of stored-produce loss (Taylor *et al.*, 2012), the need to implement or improve rodent management strategies becomes clear. However, it is crucial to study rodent ecology to understand their patterns of behaviour and feeding for effective ecologic rodent pest management (John, 2014; Krijger, Belmain, Singleton, Groot Koerkamp, & Meerburg, 2017). For example, measuring the actual impact of rodent presence on stored rice is difficult as these animals do not only eat rice (Ognakossan *et al.*, 2016), but also are able to remove grain from storage facilities to another location (Mdangi *et al.*, 2013) and to contaminate the grain. Besides, rodents usually forage in a different habitat than where they nest (Maqbool, 2011; Pye, Swain, & Seppelt, 1999), which makes it difficult to make a realistic estimation of the density of a rodent population in and around grain stores.

Although pest rodent presence is considered a problem across many rural farming communities (e.g. by damaging clothes, blankets, eating and contaminating stored rice), rodent management is usually applied too late (Aplin, K., Brown, Singleton, Douangboupha, & Khamphoukheo, 2006; Brown, P. R. *et al.*, 2008). Rodent control is mostly practised once damage to crops or stored produce becomes visible (Belmain, Steven R. *et al.*, 2006; John, 2014), whilst rodent control in rural Asian environments relies mainly on the use of rodenticides (Mathur & Prakash, 1984; Parshad, 1999). Other management methods which can be applied are trapping, habitat management (e.g. proofing, sanitation) and biocontrol (e.g. wild or domestic predators, rodent pathogens) (Brown, P. R. *et al.*, 2008; Capizzi, Bertolino, & Mortelliti, 2014). As a second objective of the study, we wanted to assess the efficacy of different rodent management methods capable of reducing post-harvest losses under local contexts.

Materials and methods

Study locations

Research was conducted over 2016 and 2017 in the South-East region of Bangladesh. In total, two rice milling stations and eight villages participated in the study. The selected villages are: A: Lakhshmipur, B: Comalla, C: Kadamtoli, D: Monahpur, E: Maurali, F: West-Maruali, G: Nagarkandi, and H: Baro Char, and the rice mills are situated in the Chittagong division, Comilla district, Comilla sadar upazila (all within 10kms of 23°27’23.0”N 91°10’20.6”E). Bangladesh has a subtropical monsoon climate, which is characterized by broad variations in rainfall, temperatures and humidity per season. The selected sites all experienced the same climate and monsoon rainfall cycles between June and October. In Bangladesh there are two to three crops per year (depending on the climate and irrigation possibilities during the

dry period), with most farmers planting rice. The size of the villages selected was between 75 and 150 households per village. Ethical approval was obtained through project partner AID-Comilla, which explained activities in the local language, gaining consent from each household involved. The owner of the rice mills were part of the project team and agreed to use their properties and buildings for this study.

For every village, ten households were randomly selected, with the pre-condition that the household stored paddy rice for several months after each harvest and consented to participate in the study. Paddy was stored inside the house in jute sacks or baskets made from woven reeds, bamboo, and/or wood, which are usually left uncovered and positioned in bedrooms or other living areas. Sometimes, rice is stored in plastic barrels or steel drums, often without a lid. Storage trials took place during both the wet (June, July, August) and dry seasons (October, November, December), with one replication (2016 and 2017, Figure 6.1). In order to reduce the possibility of trial activities influencing rodent populations, data collection in the wet season took place in four different villages (A-D) from those involved during the dry season (E-H).

	Season	
	Wet	Dry
2016 & 2017	June - July - August	October - November - December
	Villages and treatment	
	A = Trapping B = Cats C = Rodenticides D = Control	E = Trapping F = Cats G = Rodenticides H = Control

Figure 6.1 Study design villages Bangladesh.

Assessment of stored rice losses

To assess the rice losses due to rodents, the method developed by Belmain et al. (2006) was used as basis for both the rice milling stations and households. Baskets made from woven reeds and bamboo were purchased on a local market. The baskets were 20 cm deep, had a base diameter of 21 cm and a diameter of 41 cm at the open top (Figure 6.2). Each basket was filled with five kg threshed paddy rice, and each selected household received one rice basket to determine the loss due to rodents (n=10 per village) for a period of 3 consecutive months. Every fortnight the baskets were weighed and moisture content was measured using a portable grain

moisture meter (Model GMK 303RS; G-WON HITECH Co. LTD, Korea). In the two rice milling stations, 10 rice baskets filled with threshed rice were randomly placed in the paddy rice storage warehouse for a period of 9 months (July 2016-March 2017). The rice baskets in the mills were also weighed and moisture content was measured every fortnight. In contrast to the procedure described in Belmain et al (2006), baskets were not restocked after weight measurements.

Weight losses of the rice in the baskets could be influenced by potential moisture changes. To correct for those changes in moisture content, the following formula was used:

$$Wa = Wi * (100 - MCi)/(100 - MCf)$$

With Wa being the adjusted weight, Wi initial weight, MCi =initial moisture content [%], and MCf = the final moisture content [%]. All results are reported as adjusted weights.

Figure 6.2 Baskets made from woven reeds and bamboo filled with rice to assess the rice losses due to rodents.

Monitoring rodent presence

Rodent presence was monitored before, during, and after the treatments in both the rice milling stations and in the villages for two consecutive days each fortnight using Giving up Densities (GUD) and tracking tiles. To measure GUDs, open plastic trays of 30 * 20 * 8 cm were filled with approximately 4 cm local sand within which 25 peanuts were randomly buried. The sand was sieved the next morning in order to count the peanuts eaten, and all trays were restocked to repeat the procedure over two consecutive nights every fortnight. Each household received one tray, which was placed in an area near obvious signs of rodent presence (faeces, holes, damage to storage structures). Tracking tiles (Figure 6.3) were used to passively monitor rodent activity and consisted of white ceramic wall tiles (20 x 30 cm) that were blackened with soot using a smoking paraffin lamp. Two blacked tiles were placed in each household for two consecutive days each. The percentage area marked by rodent footprints was determined by placing a transparent plastic sheet marked into 16 cells- on top of the tile (Figure 6.3.B). The number of cells with rodent footprints was expressed as a percentage of the total number of cells. By calculating the percentage of the tile covered with footprints the relative amount of rodent activity could be measured (Hacker *et al.*, 2016). After each count, tiles were re-blackened.

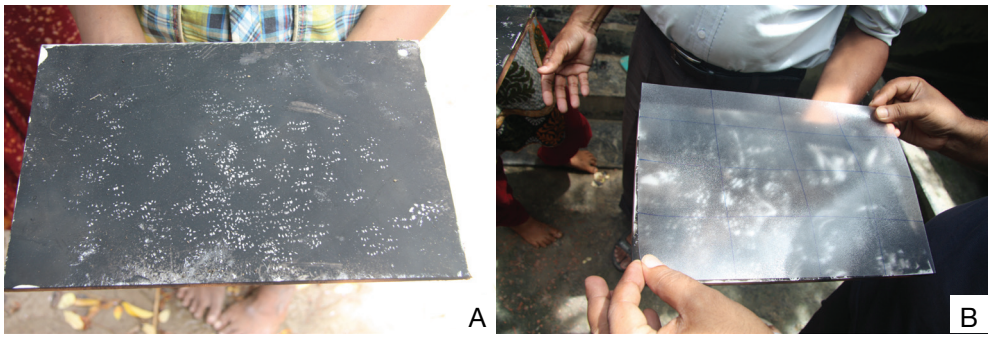

Figure 6.3 Tracking tiles A) With rodent footprints B) Determining the percentage area marked by rodent footprints by placing a transparent plastic sheet marked into 16 cells- on top of the tile.

Rodent control measures in villages

Ethical approval and permissions for the work were secured through the owners of the mills and the individuals involved in all the communities. All staff followed international guidelines on the handling of wild mammals in field research (Sikes & Gannon, 2011) and according to the Netherlands code of scientific practice. Although the animals used were not laboratory animals, the NCad opinion on

'Alternative methods for killing laboratory animals' was followed, as provided by the Netherlands National Committee for the protection of animals used for scientific purposes (NCad, 2015). There were four treatments assessed: (I) control (no treatment), (II) place 20 domestic cats per village, (III) anticoagulant rodenticides (locally purchased, containing 56% Aluminum phosphate, and Zn phosphide, Figure 6.4), three bait stations per household with weekly bait replacement, and (IV) daily rodent trapping with 4 traps per household (using snap traps 14 x 7 cm; Big Snap-E, Kness, Albia, IA, USA, and locally made life traps measuring 10 * 15 * 33cm). The staff placed traps in the afternoon and visited the trap locations the next morning to check for captures. Rodent species were identified according to Aplin et al. (2003; 2003). Each measurement period took 3 months (June-August, and October-December), in which 4 villages were visited, receiving one of the four management methods. In the first month, rice losses were assessed and rodent presence was monitored. In month 2 and 3 the rodent control treatments were conducted alongside the monitoring activities.

Figure 6.4 Locally purchased rodenticides, containing 56% Aluminum phosphate and Zn phosphide.

Rodent control measures in rice mills

Two rice mills were studied. At mill one, rodent control was conducted using domestic cats. At mill two, no rodent-management was applied (control). After two months of monitoring losses and rodent presence, 20 cats were placed at rice milling station no. 1; thereafter the rice loss and rodent monitoring was continued for 6 more months (both locations, August 2016-January 2017). During months 8 and 9 (January & February 2017) in both mills, rodent presence was measured by rodent trapping for two consecutive days each fortnight (20 traps, 10 snap and 10 live traps). Rodent trapping was conducted in the warehouse of each milling station where paddy rice is stored in jute bags.

Data analysis

Frequency tables of number and species of captured rodents inside households are given. The efficacy of the treatments in the villages was assessed through data collected on: (I) the amount of rice eaten per day, (II) the percentage of tiles marked with rodent footprints per night, and (III) GUDs (the fraction of peanuts consumed per night). The amount of consumed rice during a measurement interval (t1, t2) (usually t2= t1+14 days) was calculated as the difference in weight of remaining rice + basket at t1 and t2. The difference was divided by the number of days, resulting in the average amount of rice eaten per day. For statistical analysis this variable was log-transformed as y = log(amount per day+1), with the value one added to avoid problems near zero, leading to approximate normality and constant variance of residuals in later analysis. On the two mornings consecutive to the day of the rice weight measurements, tiles on two locations per household were scored for footprints. On the same two mornings the GUD was determined (number of eaten peanuts out of twenty-five peanuts). For all three response variables, measurements were taken repeatedly on the same experimental unit, potentially leading to correlated responses. Therefore, generalized linear mixed models (glmm) were used for statistical analysis (more specifically random coefficient models, see e.g. (Qiu, Gort, Torricelli, Takken, & van Loon, 2013)) that assume a normal distribution for transformed values of daily rice losses and binomial distributions for fractions tile marking and GUD. For the amount of rice eaten per day, experimental baskets were the experimental units. Each basket, with ever decreasing amounts of remaining rice, was followed over time. For the tracking tiles and GUDs, the experimental units were fixed locations of tiles and trays within households.

In the random coefficient model, each experimental unit has its own (random) quadratic (or higher order) time trend for the response. Other identifiable sources of variation include village (with ten households per village), year (the same households

were observed in 2016 and 2017), season (four villages were observed during the wet season, and four different villages during the dry season), and location within a house (for tracking tiles and GUDs). In the fixed part of the glmm quadratic or, if needed, cubic time trends for the four treatments were included, and these time trends were allowed to be different for the four year by season combinations. The random part of the glmm consisted of the random quadratic (or cubic) time trend per experimental unit, random effects for village by year combinations (largely, allowing for differences in rodent populations between villages per year) and village by year by time combinations (allowing for random deviations from a quadratic time trend at village level). To handle possible overdispersion of the binomial fractions random effects were included.

At the start of the study each household was observed under control conditions for 2-4 weeks, i.e. without application of the treatment, leading to 2-3 repeated measurements per household. Calling the timepoint of treatment application is termed t0, the observations prior to t0 are used to estimate the variability between villages, allowing to separate effects of treatments and villages. An implicit assumption here is that the variability between villages does not depend on the treatment. The fixed part of the glmm is a piecewise regression model: until time t0 a single quadratic (or cubic) regression line (corresponding to the control) is specified (per year-season combination). After t0 the regression lines becomes treatment dependent. Cubic models are chosen if found to be significantly better than the quadratic models ($P<0.05$).

After fitting the glmm the treatment time trends were compared between year-season combination using F-tests. Next, within year-season combinations time trends between treatments were compared using F-tests. If time trends were different between treatments, treatments were compared at specific timepoints, namely at timepoints when rice measurements were taken. Overview plots of the data are produced, split by year and season, with lines connecting observations from the same experimental units, using different colours for treatments, followed by plots showing the fitted regression models (only for year-season combinations with significant treatment effects). In order to quantify the strength of the (linear) relationships between the three monitoring methods, Pearson correlation coefficients for the three response variables were calculated. To this end for tiles and GUDs the fractions from two consecutive observation days were averaged. All statistical analyses were performed using R version 3.4.3 (R Core Team, Vienna, Austria); glmm's were fitted using package lme4 (Bates, 2010) and compared using package pbkrtest (Halekoh & Højsgaard, 2014); user-defined contrasts were made using package car (Fox *et al.*, 2012); treatment comparisons were made

using package emmeans (Lenth, Love, & Hervé, 2017); plots were produced using package ggplot2 (Wickham, 2010).

The efficacy of the presence of cats as rodent management in the mills was assessed using descriptive statistics only due to no replication of the cat treatment, i.e. only two mills were followed over time: one without cats and one with cats.

Results

In total, 210 rodents were captured from inside people's homes (Table 6.1). *Rattus rattus* was present in almost all villages, and trapped most often (43.3%), followed by the *Mus musculus* (35.7%) and *Bandicota bengalensis* (12.4%)

Table 6.1 Number and species of rodents captured inside households of the participating villages in Bangladesh, over the wet and dry seasons of 2016 and 2017.

Season	Village, year	Treatment	Total no. captured	*Bandicota bengalensis*	*Bandicota indica*	*Mus musculus*	*Mus terricolor*	*Rattus exulans*	*Rattus rattus*
	Laksmipur	Trapping							
	2016		64	10	0	24	0	0	30
	2017		21	0	0	13	0	5	3
	Comalla	Cats							
Wet	2017		8	1	0	2	0	0	5
	Kadamtoly	Rodenticides							
	2017		5	0	0	0	1	0	4
	Monahpur	Control							
	2017		23	11	0	3	0	0	9
		Subtotal	121	22	0	42	1	5	51
	Maruali	Trapping							
	2016		42	0	6	20	0	2	14
	2017		39	3	3	10	1	0	22
	West Maruali	Cats							
Dry	2017		3	0	0	2	0	0	1
	Nagar Kandi	Rodenticides							
	2017		2	1	0	1	0	0	0
	Baro Char	Control							
	2017		3	0	0	0	0	0	3
		Subtotal	89	4	9	33	1	2	40
		Total	**210**	**26**	**9**	**75**	**2**	**7**	**91**

Adjustments to the original planning were made, in 2016 village A-D were visited 9 times, E-H 5 times. For 2017, all villages (A-H) were visited 6 times. In 2016, all villages experienced similar losses, ranging from 677.9 grams loss per basket per month to 846.5 grams loss per month (13.6-16.9)% loss from the basket stored within the household (Table 6.2). In 2017, the losses were lower, ranging from 29.1 grams per month to 107.9 grams eaten per month (0.6-2.2%).

Table 6.2 Average amount of stored rice-loss in Bangladesh households per interval (14 days), n=10 baskets per village.

				Average losses (g) per village 2016				
Interval	**Laksmipur**	**Comalla**	**Kadamtoly**	**Monahpur**	**Maruali**	**West Maruali**	**Nagar Kandi**	**Baro Char**
1	1046.83	690.00	1051.37	963.95	224.23	255.48	227.24	270.36
2	557.88	294.02	554.12	781.22	459.88	330.89	374.97	367.18
3	290.89	889.15	291.49	495.70	408.37	349.07	434.42	398.31
4	400.90	433.28	398.00	274.42	572.46	465.68	319.16	451.86
5	417.06	451.75	416.53	206.46				
6	299.71	262.13	270.07	173.60				
7	111.94	159.81	101.33	219.55				
8	61.30	162.76	71.04	270.89				
				2017				
	Treatment	**Total no. captured**	***Bandicota bengalensis***	***Bandicota indica***	***Mus musculus***	***Mus terricolor***	***Rattus exulans***	***Rattus rattus***
1	60.50	60.10	54.05	61.70	32.37	24.51	22.48	27.80
2	36.82	42.62	38.40	38.70	15.22	14.69	11.78	11.20
3	60.19	47.14	41.73	50.96	17.96	15.41	13.42	13.91
4	78.59	45.48	40.91	51.79	14.27	12.64	14.15	14.62
5	44.68	51.72	42.94	53.03	11.59	8.57	10.12	11.62
6	43.13	51.29	44.21	32.73	14.79	11.23	16.61	13.45

In the selected villages, rodent management methods were performed to compare their effectivity. When comparing the three monitoring methods, the GUDs and tracking tiles corresponded the strongest (ρ= 0.73), followed by the correlation between the loss and tracking tiles (ρ= 0.54), and the least strong correlation was found between the results of the rice losses and GUDs (ρ= 0.44).

Stored rice losses

Villages

The treatment time trends of daily rice loss show significant differences among year-season combinations (Figure 6.5, comparing all four year-season combinations simultaneously gives P<0.0001; all individual pairwise comparisons result in P<0.0001 too).

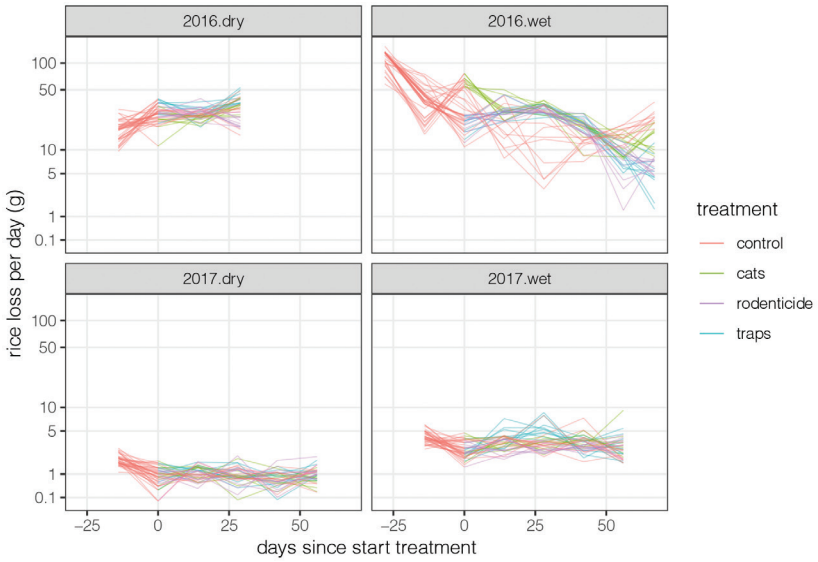

Figure 6.5 Overview plot of the data on rice loss per day, split by year and season. Lines connect observations from the same experimental units (baskets). Each treatment has its own colour; red = control, green = cats, purple = rodenticide, and blue = traps.

When looking per season, only within the wet season of 2016 (Figure 6.6) significant time trend differences between treatments are found (P=0.0017).

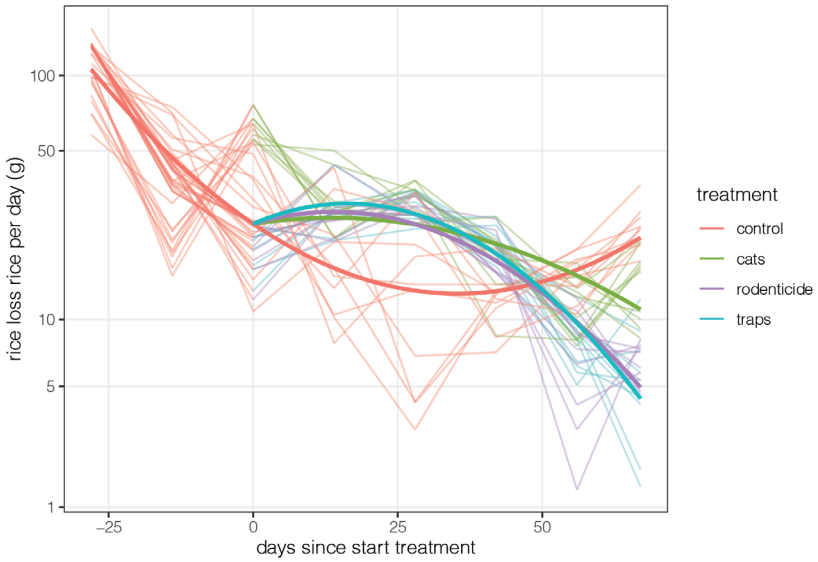

Figure 6.6 Plot of rice loss per day in the wet season of 2016, with lines connecting observations from the same experimental units (baskets) and estimated time trends per treatment.

When comparing the responses on rice loss between the treatments at specific time points, significant differences are found. At 14 days after the start of the treatments, the control group differs from the three treatment types and shows unexpectedly significant less loss of rice per day (Figure 6.6). Over time, this difference vanishes and finally, more loss of rice is found in the control groups. Looking at 67 days after the start of the treatment (the final measurement day) significantly more rice is eaten per day in the control group than in the rodenticide and trap treatment groups, while the difference between the control and cat group is not significant. At this time point, there are also differences between the three treatments found; the use of rodenticides and traps result in less rice loss per day compared to the use of cats, but no significant difference between rodenticide and traps are found.

Tracking tiles

The treatment time trends of fractions of cells of tracking tiles tripped upon (placed in the villages) differ significantly amongst year-season combinations (Figure 6.7; $P<0.0001$ comparing all four year-season combinations simultaneously; pairwise comparisons also give $P<0.0001$ for all pairs).

For the tracking tiles there are time trend differences between treatments in both wet seasons (2016 $P<0.0001$, and 2017 $P<0.0001$). When comparing the fractions

Figure 6.7 Overview plot of the fraction of cells tracking tiles tripped upon (averaged over two locations and two consecutive observation days), split by year and season. Lines connect observations from the same households.

of cells of tiles tripped upon between the treatments in the wet season of 2016, at 56 and at 67 days (final measurement day) after the start of the treatment, significant differences can be found as the control group shows significantly more rodent activity than do tiles in the treatment villages (Figure 6.8).

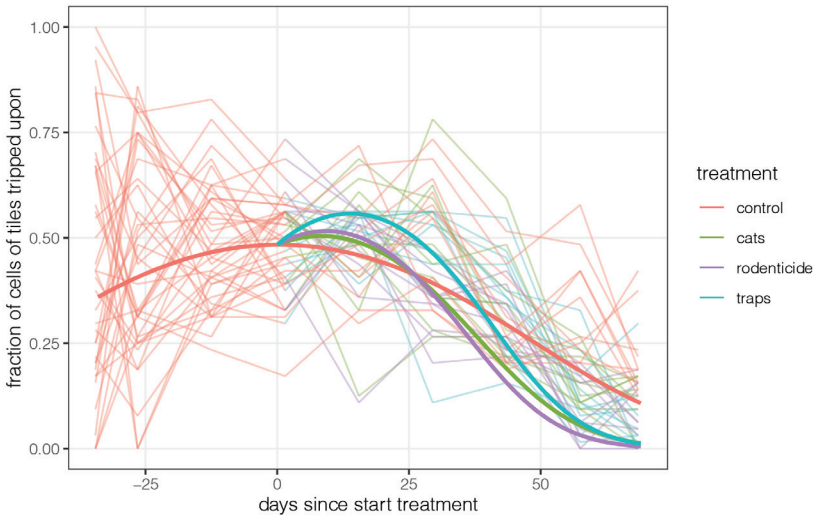

Figure 6.8 Plot of the fraction of cells of tracking tiles tripped upon by rodents in the wet season of 2016, with lines connecting observations from the same household and estimated time trends per treatment.

Looking at the wet season of 2017, the model finds significant difference in the activity on the tracking tiles for 14, 28 and 42 days after the start of the treatments. The most effective method is the use of rodenticide which is significantly different from the control.

Giving Up Densities

The treatment time trends of the GUDs in the village households differ significantly amongst year-season combinations (P<0.0001, Figure 6.8), with the exception of the two seasons in 2017 (p=0.219).

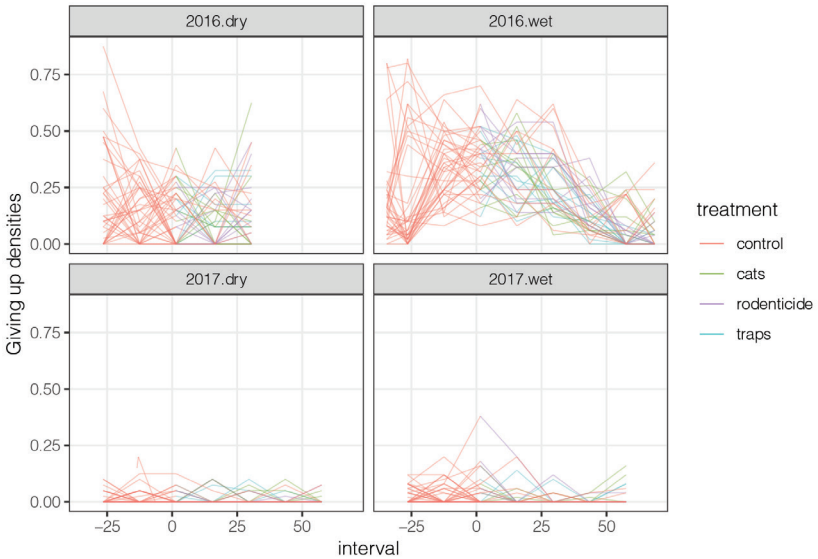

Figure 6.9 Overview plot of the GUDs in fraction of eaten peanuts per day (averaged over two consecutive observation days), split by year and season. Lines connect observations from the same household.

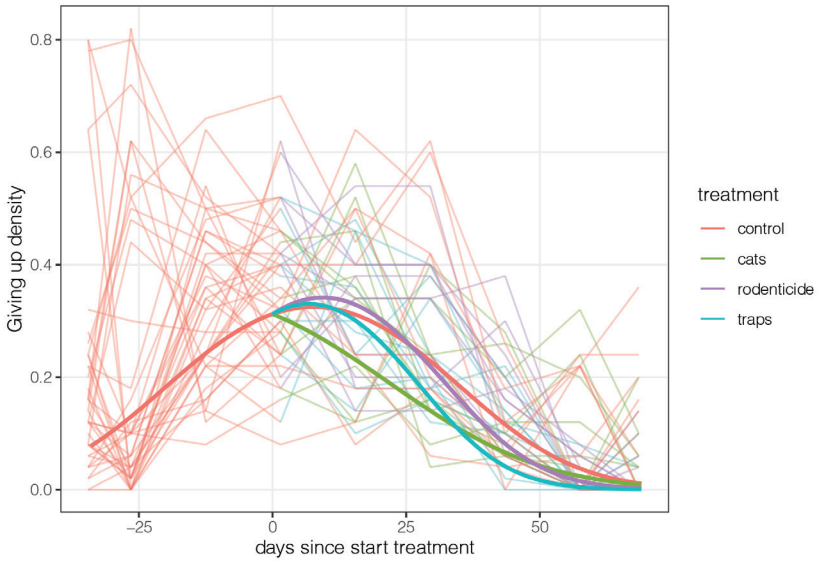

Figure 6.10 Plot of the GUDs in fraction of eaten peanuts per day in the wet season of 2016, with lines connecting observations from the household and estimated time trends per treatment.

For the GUDs significant time trend differences between treatments are found only in the wet season of 2016 (Figure 6.8, P=0.011). At 56 and 67 days after the start of the treatments, the control group shows significantly higher GUDs than the trap and rodenticide treatments, (Figure 6.10).

Rice milling stations

In the rice mills, there was no effect of the placement of cats observed (Figure 6.11). However, when looking at the graph, it seems that shortly after the placement of the cats, cat presence has a slight positive effect on the weight loss per basket.

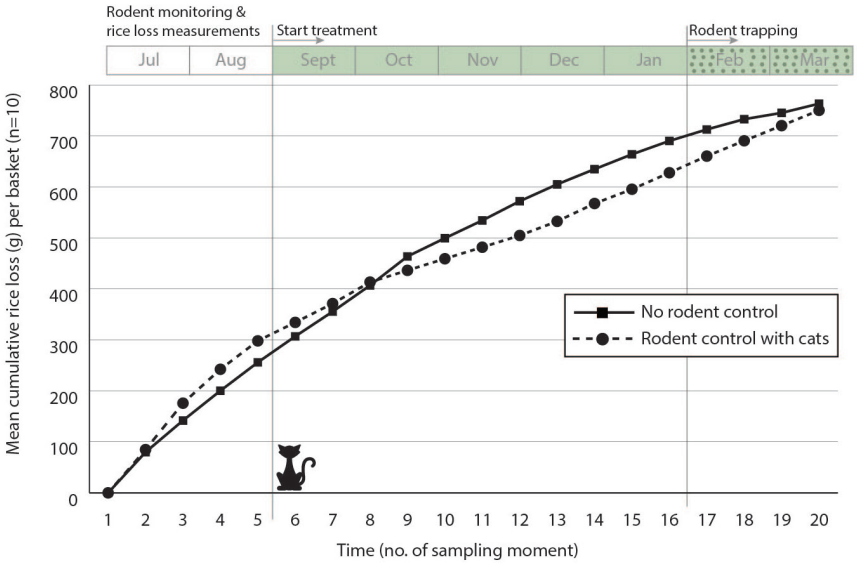

Figure 6.11 Cumulative loss of stored rice removed by rodents form baskets placed in two rice mills (one without rodent control and one with rodent control by cats) in Bangladesh from July 2016-March 2017.

The tracking tiles and the GUDs show no clear patterns and show no effect after introducing the cats in one of the two mills. During the trapping phase in the end of the study, more rodents were trapped in the control mill (n=48) than were in the mill with cats as rodent management (n=20) (Table 6.3).

Table 6.3 Rodents trapped in two rice mills in Bangladesh.

	Rice mills	
Species	**Control**	**Cats**
Rattus rattus	12	7
Mus musculus	16	5
Bandicota bengalensis	13	4
Rattus exulans	7	1
Mus terricolor	0	3
Total	**48**	**20**

Discussion

In order to assess post-harvest losses to stored rice, losses were monitored in multiple households and two rice milling stations. It was found that all villages experienced similar losses, ranging from 13.6-16.9% loss per month from the basket stored within the household in 2016, and in 2017 the losses were lower, ranging from 0.6-2.2% per month. In the FAOSTAT database it is stated that in 2013 the annual mean consumption of rice per person per month in 2013 was 14.3 kg, which comes down to almost 500 grams per day. Looking at the data for 2016 with an average loss of 796.6 gram/month, it means that the amount of lost rice could feed one person for almost 2 days. Research of Htwe et al. (2016) in Myanmar also found differences in mean loss of grain between locations and time; in 2013 they observed losses of 14% and 8.2% and a year later in 2014 they observed a loss of 4% and 1.2%. A study in Laos on rice loss by rodents found that losses were higher in the dry than in the wet season (respectively 10.% and 7.4%)(Brown, P. R. *et al.*, 2013), which is similar to what we expected to find in the current study. However, we did not found significant differences in rice loss due to rodents between the rainy and dry season.

With respect to rodent management method we found that the main rodent pests in village households were *Rattus rattus*, followed by *Mus musculus,* and *Bandicota bengalensis*. This is in line with findings from Bangladesh (Chakma *et al.*, 2018), India (Santra & Manna, 2008), Pakistan (Rehman *et al.*, 2019), and Myanmar (Brown, P. R. *et al.*, 2008; Htwe *et al.*, 2016), where *R. rattus* and *B. bengalensis* were also found to be the main rodent pests. During the dry season, fewer rodents were trapped (n=89) than during the wet season (n=121). It was expected that there were stronger declines in the amount of rice in the baskets in the locations where a treatment was conducted compared to control households. However, this was only seen in one of the four seasons. Only in the wet season of 2016 were time trend differences found between treatments. In order to make solid statements about which rodent management method is most efficient, the research periods should have been at least one month longer as we began to see an effect at the last day of the research period (at 67 days after starting the treatments). Here it was found that the use of rodenticides and daily trapping resulted in less rice eaten per day. As no difference between those two management methods was found, we suggest to use daily trapping as a pest management tool as this is a non-toxic, sustainable method. Furthermore, the use of rodenticides could have a negative impact on the environment as non-target species can be affected (Elmeros *et al.*, 2019; Smith & Shore, 2015). Other studies on rodent management methods also found daily rodent removal trapping to be effective (Belmain, Steven R *et al.*, 2015;

Eisen *et al.*, 2018; Mari Saez *et al.*, 2018). A study from Uganda showed that the effect of trapping disappears shortly after its cessation (Eisen *et al.*, 2018). Therefore, it is suggested to keep trapping in order to keep the rodent pest population as low as possible.

For both the villages and the rice mills there was no significant effect of the placement of cats on the amount of rice loss. However, when looking at the data from the mills, it seems that cat presence has a slight effect on the weight loss per basket shortly after the placement of the cats. Although the cats were fed daily to keep them in and around the households and mill, we think the cats all strayed away during the research period which could have influenced the results. For further research we suggest to use radio tracking of the cats to show how active they are, where they forage and dimensions of their home range. Despite the fact that some rodent species in Bangladesh are larger than cats, the presence of predators could be effective on the presence of rodent pests (Davies *et al.*, 2017; Mahlabla, Monadjem, McCleery, & Belmain, 2017); however, more research should be conducted to make statements on the effect of domestic cats on rodent presence. The research of Mahlaba et al. (2017) in Africa found no effect when using cats alone as a rodent management method in homesteads in Africa; however, when combined with dogs there was significant proof for diminished rodent activity.

In this study the baskets with rice were not topped up after each measurement period, which could have influenced the results because this would mean that the baskets get less attractive over time (e.g. by droppings and urine, and by depletion of rice present). Furthermore, it needs to be taken into account that the selected villages were different from each other (different location, different size of ponds, distance to the road, presence of natural predators such as raptors e.g. ospreys). To correct for these confounding elements, at the start of the study all households were observed under control conditions for 2-4 weeks, i.e. without application of the treatment, leading to 2-3 repeated measurements per household.

When comparing the three rodent monitoring methods we found that the GUDs and tracking tiles showed similar results. Based on our results we would suggest to use tracking tiles for monitoring, rather than GUDS or using the rice loss method as tracking tiles correlate very well with rodent abundance (Hacker *et al.*, 2016). Moreover, the benefit of using tracking tiles is that the rodent activity measured as a passive monitoring method. When using open rice baskets or GUDs one could state that these method are attracting rodents and other pest species. However, GUDs can still be used to measure behaviour instead of rodent presence, as this is also where the method was designed for. GUDs provide insights into the feeding

behaviour and habitat preferences of animals by giving an index of the costs of foraging in a given patch, in our case the trays with peanuts per study site. The more food left in a patch after the departure of an animal, the higher the GUD, indicating high costs (Brown, J. S., 1988). In the wet season of 2016 significant time trend differences between treatments were found; the control group showed a higher fraction of peanuts eaten (thus lower GUD) than at the trap and rodenticide treatments. This could indicate that both trapping and rodenticide placement increases the perceived fear of the rodents present.

In conclusion, post-harvest losses due to pest rodents in rural communities are significant and create food security and food safety issues for rural farming communities. Pest rodent management through daily trapping, and improvement of food storage in households are recommended to reduce losses and increase human health.

Chapter 7

The need to implement the landscape of fear within rodent pest management strategies

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Abstract

Current reactive pest management methods have serious drawbacks such as the heavy reliance on chemicals, emerging genetic rodenticide resistance, and high secondary exposure risks. Rodent control needs to be based on pest-species ecology and ethology to facilitate development of ecologically-based rodent management (EBRM). An important aspect of EBRM is a strong understanding of rodent pest species ecology, behaviour, and spatiotemporal factors. Gaining insight in the behaviour of pest-species is a key aspect of EBRM. The landscape of fear is a mapping of the spatial variation in the foraging cost arising from the risk of predation and reflects levels of fear a prey species perceives at different locations within its home range. In practice, the landscape of fear (LOF) is a mapping of habitat use as a result of perceived fear, which shows where bait or traps are most likely to be encountered and used by rodents. Several studies link perceived predation risk of foraging animals with quitting-harvest rates or giving-up densities (GUDs). GUDs have been used to reflect foraging behaviour strategies of predator avoidance, but to our knowledge very few papers have directly used GUDs in relation to pest management strategies. An opportunity for rodent control strategies lies in the integration of the LOF of rodents in EBRM methodologies. Rodent management could be more efficient and effective by concentrating on those areas where rodents perceive the least levels of predation risk.

Introduction

Putting integrated pest management (IPM) into practice with respect to rodents has often failed to recognise that rodent control needs to be based on a solid understanding of species-specific behaviours, biology and the phenology of damage caused by different rodent species affecting agricultural production. In the past, there has been more attention for insect pests compared to rodent pests, and especially in developing countries it is therefore often thought that the 'I' in IPM stands for 'Insect'.(Singleton, 1997) A result is that IPM strategies for rodent pests still lag seriously behind IPM strategies for insect pests. Effective rodent management in an agricultural landscape consists of four general elements: (I) prevention, (II) monitoring, (III) implementation of a combination of control methods, and (IV) community involvement in management.(Meerburg *et al.*, 2004; Singleton, 1997)

Ecologically-based rodent management

Ecologically-based rodent management (EBRM) builds on IPM; the reduction of the impact of rodent pests by using specific knowledge about rodent species behaviour, ecology, biology and damage to sustainably manage rodent pests. EBRM proceeds on the basis that integrated rodent management strategies can be developed from a sound ecological basis (e.g. rodent pest species' habitat use and population dynamics) in order to reduce the economic and social impact of rodent pests in cost-beneficial ways that do not adversely affect the environment.(Singleton, Leirs, Hinds, & Zhang, 1999; Smith & van den Bosch, 1967) EBRM was promoted due to a growing demand for more effective and species-specific rodent control strategies that were not entirely recognised by early IPM practitioners who overly relied on chemical rodenticides.(Singleton *et al.*, 1999) Moreover, rodenticide use has become less acceptable because of increased genetic resistance(Meerburg, van Gent-Pelzer, Schoelitsz, & van der Lee, 2014; Rost *et al.*, 2009) and because of heightened animal welfare concerns.(Meerburg, Brom, & Kijlstra, 2008)

Generally, traditional forms of pest management are reactive; rodent control is mostly practiced once damage to crops or stored produce becomes visible.(John, 2014) Several Asian studies have shown EBRM to be highly effective in diminishing rodent damage(Jacob, Singleton, Herawati, & Brown, 2010; Palis *et al.*, 2011; Singleton & Brown, 2003; Singleton, Sudarmaji, Jacob, & Krebs, 2005) and have reduced farmers' reliance on rodenticides.(Brown & Khamphoukeo, 2010; Brown *et al.*, 2006; Palis *et al.*, 2011; Singleton *et al.*, 2005) For EBRM to be effective it is also important to recognise that less than 10% of all rodent species are pest species, and many current rodent control methods do not sufficiently discriminate between

pest and non-pest species.(Singleton, Brown, Jacob, & Aplin, 2007) Moreover, it is often not known what proportion of the population of a pest species needs to be culled for a significant reduction in economic damage.(John, 2014; Singleton *et al.*, 2007) Thus more knowledge (i.e. monitoring) on the species present, their behaviour, and the consequences of their presence is essential for effective control.

Progression from dominance of rodenticides to integrated rodent management

In 1944, the accidental discovery of anticoagulant rodenticides occurred in the USA by accident through the detection of dicoumarin (warfarin) in spoiled sweet clover hay fed to cattle that subsequently suffered from internal bleeding.(Hadler & Buckle, 1992; Link, 1944) Because rodents do not immediately feel ill after eating bait laced with warfarin, warfarin and its modern-day anticoagulant analogues have become THE definitive tool for controlling rodents. Until the late 1980s, their efficacy and relative safety certainly contributed to stifling other research avenues on rodent pest management such as developing more ecologically sound methods of rodent management.(Hadler & Buckle, 1992) Rodent control practices in agricultural environments are still mostly based on the use of rodenticides.(Arora, Srivastava, & Pandey, 1984; John, 2014; Mathur & Prakash, 1984; Parshad & Malhi, 1995) However, incorrect application of such chemicals fast tracks the development of rodenticide resistance (reported from 1966 onwards for several rodent species) and increases the risk of both primary and exposure of predators.(Jackson & Kaukeinen, 1972)

State of the art of EBRM use on pest rodents

An important aspect of EBRM is the use of spatio-temporal factors in the context of the population dynamics of rodent pests and the agricultural resource to be protected. As an example, it is more effective to cull far fewer animals during the early stages of rice production than to kill many later on in the season to reduce crop damage.(Singleton *et al.*, 2007) The EBRM spatio-temporal aspect is often applied in cropping systems to reduce pre-harvest losses, but there have been few studies on EBRM to reduce post-harvest losses. Fluctuations in the population abundance of peri-urban and urban rodent species (rodent species that are continuously present in the neighbourhood of humans and cause losses to stored products and increased risks of disease transmission) may be less than those of field rodent species, but the spatio-temporal aspect of EBRM is still important. For example, if rodent numbers are managed before agricultural produce is put into a storage facility, the population growth of rodent pests and negative consequences to stored grain can be significantly curtailed. Especially in the post-harvest situation, rodent management should focus more on the behaviour of the pest rodent species than on the current reactive methods. A behaviour all animals have in common is the search

for provisions. So what happens when one focusses on species-specific foraging behaviour to gain more knowledge to enable managing those pest-species?

Search for provisions

The optimisation of foraging behaviour of animals addressing what food type should be included in the diet was first published by Pianka and MacArthur(MacArthur & Pianka, 1966) and Emlen.(1966) Charnov developed in 1976 the first optimal patch use model, which is known as the Marginal Value Theorem (MVT).(Charnov, 1976) This theorem hypothesizes that animals foraging assume that nutrition products occur in clusters, and that their food consumption decreases linearly (but not constant) with the time spent on that exact location. When making foraging decisions, animals balance the benefit of energy rewards and the price of predation.(Brown, 1988) The MVT predicts that animals foraging in a patch will decide whether to depart is not based on depletion of a food patch, but rather on the assessment of costs of foraging and the yield rate of the current patch versus the yield rate of another 'new' food patch.(Charnov, 1976; Milinski & Heller, 1978) By creating food patches and assessing the amount of food left after foraging, the giving-up density (GUD) of a food source becomes a measurable unit.(Brown, 1988; Brown, Kotler, & Mitchell, 1997; Brown, Kotler, Smith, & Wirtz II, 1988) The GUD reflects the perceived costs of foraging on that location. The more food left in a patch after the departure of an animal, the higher the GUD, indicating high costs.(Brown, 1988) GUDs provide insights into the feeding behaviour and habitat preferences of animals.(Brown, 1988; Ylönen, Jacob, Davies, & Singleton, 2002) Furthermore, GUDs also reveal the balance between food and safety; the metabolic costs of a foraging animal, its perceived predation risk during foraging, and the missed opportunity costs (MOC) of the forager by not engaging in activities other than foraging.(Brown, 1988; Brown & Kotler, 2004) With feeding rate being a direct function to food density, GUDs can be used as an index of the forager's quitting harvest rate.(Makin, Payne, Kerley, & Shrader, 2012; Schmidt, Brown, & Morgan, 1998)

Perceived predation risks

Because rodents can serve as prey for many different species of reptiles, birds and mammals, they avoid places where the relative risk of predation is high. Both indirect cues (e.g. vegetation cover, weather conditions, light intensity) as well as direct cues (e.g. sound, odours, urine, or other excrements from potential predators) enable rodents to assess predation risk during foraging.(Orrock, Danielson, & Brinkerhoff, 2004) A study on the effect of owl predation on rodents' search for provisions in America showed that adjustments in foraging behaviour as

a response to perceived predation risk are predominantly based on an awareness of the presence of a predator, rather than on the actual capture or killing of prey by the predator.(Brown, 1988{Verdolin, 2006 #162; Verdolin, 2006) Brown(1988) postulates that prey animals 'manage risk' according to H = C + P + MOC, where H is harvest rate, C the metabolic costs, and P stands for the costs of risk of predation. Research on foraging and predation risk trade-off has been used in many different animal contexts, from aquatic to terrestrial systems.(Werner & Hall, 1988) A review in 2013 on GUD methodologies discussed its use, practical benefits and drawbacks and gave insight into the many species that have been studied (mule deer (*Odocoileus hemionus*), red fox (*Vulpes vulpes)*, voles (*Microtus* spp. and *Myodes* spp., gerbils (*Gerbillus allenbyi*), gold fish (*Carassius auratus*), squirrels (*Tamiasciurus hudsonicus, Callospermophilus lateralis,* and *Sciurus niger*), mice (*Rhabdomys pumilio, Baeolophus bicolor, Acomys russatus, Acomys cahirinus* and *Peromyscus maniculatus*), possums (*Trichosurus vulpecula*), rats (*Rattus fuscipes*), chipmunks (*Tamias minimus*)).(Bedoya-Perez, Carthey, Mella, McArthur, & Banks, 2013) For all foraging animal species, the perception of safety of feeding activities includes the encounter rate with predators, the lethality of the predator, and the chance of surviving predation.(Abrams, 1993; Brown, 1999; Brown & Kotler, 2004; Lima & Dill, 1990) Prey animals continuously have to balance between demand for food and safety, e.g. reduced predation risk.(Jacob & Brown, 2000) With the costs of risk of predation (P) varying across the landscape, so will the intensity of patch exploitation. The way in which animals use their habitat during their foraging behaviour(Laundré, Hernández, & Altendorf, 2001) as a result of fear for predation is called the landscape of fear (LOF). Such a landscape is strongly based on the ecology of a particular prey species and on the ecology and hunting techniques of their predators.(Matassa & Trussell, 2011; Singleton *et al.*, 1999) In our opinion, the LOF can be seen wider than the concept introduced by Laundré *et al*.,(2001) and should include both the way foraging animals use their habitat as result of perceived fear, as well as an actual landscape. Thus besides predator-prey relations, the LOF also can be constructed on perceived fear of intra-specific relations. An intruder (e.g. rat from a different colony) will also be able to provoke fear among rats in a resident colony,(Davis, Emlen, & Stokes, 1948) however, intruders can also be in fear of residents. In this case risk of injury from interference and aggression from conspecifics will affect the LOF.

Making better use of rodents' natural behaviour

Several studies have linked perceived predation risk of foraging animals with their quitting harvest rates or GUDs (review by Brown and Kotler).(2004) The LOF reflects levels of fear of predation perceived by a prey species on different locations within its home range.(Laundré, Hernández, & Ripple, 2010) The LOF is species-specific; our assumption is that a spatial LOF will look different for the grey squirrel (*Sciurus carolinensis*) than for the Norway rat (*Rattus norvegicus)* because each species will perceive fear of predation via different cues. Furthermore, each prey-species has different aptitudes (e.g. climbing ability, speed, agility) and thus each species is vulnerable to different degrees to different predators (e.g. terrestrial or/and aerial(Makin *et al.*, 2012)), which leads to each species having different predation costs of foraging (i.e. fear). Knowledge of a species specific short-term temporal feeding patterns (e.g. night vs. day activity) could be an effective guide for trap or bait placement and offers possibilities to reduce risks for non-target animals (e.g. by making the trap inactive during times the pest species is inactive). Knowledge on species specific behaviour could also improve trap/bait placement and trapping systems. When combining the perceived risk of predation with rodent behavioural responses, spatial use patterns of individuals could be explained.(Laundré *et al.*, 2010) In applying these concepts of rodent behaviour on rodent management, some rodent species, e.g. Norway rats (*R. norvegicus*), express a degree of neophobic behaviour, which partly explains poor bait uptake when rodenticides are applied; whilst other species, e.g. house mice, show neophilia and innate curiosity for what is new in their environment.(Cowan, 1977; Macdonald, Mathews, & Berdoy, 1999)

Landscape of fear as a component of rodent management

A recent study examined the relationship between giving-up densities (GUDs) of *Rattus tanezumi* and the spatial heterogeneity of their damage to rice crops in the Philippines.(Jones *et al.*, 2016) They concluded that bait or trap placement towards the centre of rice crops that are typically <0.1 ha, would be more likely to be visited by rats. Another study in wheat crops in Australia used GUDs to assess whether house mice modified their habitat selection based on perceived predation risk. (Ylönen *et al.*, 2002) Both studies highlighted that a better understanding of factors influencing habitat use of rodent pests could aid decisions on their management. What is lacking is objective evidence on whether pest control strategies based on the habitat use of pest rodents are more effective and have a more long-term effect than reactive rodent management. We suggest that a better understanding of rodent behavioural ecology, especially the concept of the LOF, will result in more effective strategies for management of rodent pests. To be able to use the LOF in management, it is essential to identify the possible advantages and disadvantages,

and current knowledge gaps of the LOF methodology, which can point the way for further research.

Gaps and opportunities for implementation of the LOF as rodent management tool

A classic paper by Rosenzweig (1987) provides prescient advice for pest-managers to take habitat selection into account in order to improve the management results *"Pest populations may be controlled most cheaply by concentrating on their cradle habitats (although natural selection might interfere)"* (Rosenzweig, 1987), which is also stated years later by Morris.(2003) As discussed earlier, not only habitat use plays a role when developing successful management methods, but also foraging behaviours should be taken into account as they provide reliable indicators for future situations (more reliable than use of 'old' cues indicating the past).(Kotler, Morris, & Brown, 2016) We feel that GUDs are a valuable tool to measure an animal's decision making. Research on GUDs as a monitoring tool for rodent species habitat preferences in relation to population densities and food supply indicate that rodents take greater risks when foraging during periods of high animal densities and resource depletion.(Strauß, Solmsdorff, Pech, & Jacob, 2008; Ylönen *et al.*, 2002) Therefore, it is important to monitor the number of animals present; the perceived risk of an animal is lower when it lives in a large group, than when it is on its own. Moreover, competing species often create patterns in GUDs and habitat use that are convergent with predation risk.(Morris, 2009) For example, two competing prey species using the same food patches could lead to the same effect as avoidance of predation risk; the feeding rates of both prey species will deteriorate as the species use up resource levels in shared food patches. The decrease of harvest yields will lead to more effort in foraging in a food patch which by GUDs would be indicated as 'safe'.(Morris, 2009) On the other hand, research from Australia showed that with high population densities of house mice, their spatial use became more opportunistic in some habitats where food is limited, which can also lead to a different result in the GUDs.(Ylönen *et al.*, 2002) These facts indicate the need to evaluate inter-specific competition whilst measuring for predation risk behaviour of foraging animals when using GUDs.(Makin *et al.*, 2012; Morris, 2009) A low GUD indicates a 'safe place', which might result in overconsumption there, whereas uptake of bait in riskier places (high GUD) will be less. However, these dose rates might need to be adjusted to deal with the consumption rate in response to this LOF induced effect. This is only valid when a) there is no effect of density on GUDs; b) under-consumption does not deliver the required dose or c) over-consumption matters. Simple measures such as GUDs are generally cheap to conduct; however, Bedoya-Perez *et al.*(2013) indicated seven important aspects that need careful consideration when using and interpreting GUDs: (1) the relation between costs and benefits of the forager is linear but not constant (e.g. curvilinear), (2) the forager's

physical condition, (3) more than one forager can visit a food patch simultaneously and sequentially, (4) composition of the food-patch (nutritional value of the food and properties of the substrate), (5) food patch predictability, (6) the forager's behaviours to maximize fitness and overcome costs of searching for provisions, and (7) non-target species foraging from food patches.(Bedoya-Perez *et al.*, 2013) Based on these shortcomings, it can be stated that the use of GUDs to reflect foraging behaviour strategies of predator avoidance(Jacob & Brown, 2000) cannot be assumed completely sufficient. However, it is indisputably clear the GUDs are an effective tool to map a population's LOF, which could be beneficial for pest-management by providing objective information on which to base decision making, collecting clear evidence of where rodents are more or less likely to forage and how to manipulate habitats to increase fear levels.

Current rodent management in agricultural and peri-urban habitats have made little use of the LOF as an opportunity to strengthen pest management. For example, intensity of rodenticide use and trapping could decrease significantly if an understanding of the LOF is applied in the spatial placement of such control interventions in agricultural landscapes.(Jones *et al.*, 2016) This is particularly the case in developing countries where there have been few reports of studies on the spatial and foraging behaviour of major rodent pest species. Current rodent trapping sometimes includes parts of the LOF implicitly, for example the placement of traps along walls as it is known that most commensal rodents prefer to move alongside walls. Trapping studies on micro-habitat use have tried to reflect the concept of trap success depending on perceived predation risk. However, still the most effective placement of rodent traps inside and around buildings or within agricultural fields is generally based more on tacit knowledge of the pest controller rather than rigorous data on the behaviour of the targeted pest species in a landscape. Van der Merwe and Brown (2008) visualised the LOF of the cape ground squirrel via a physical map that showed the predation costs of foraging (Figure 7.1a). A map of the LOF can show valleys representing relative safety, and peaks which indicate perceived danger (Figure 7.1b). (Laundré *et al.*, 2010) In both graphics the LOF was used as a model to visualise how fear could alter the area used by prey as it tries to reduce the risk of predation, specifically during foraging.(Altendorf, Laundré, López González, & Brown, 2001; Laundré *et al.*, 2001; Laundré *et al.*, 2010) Within the LOF, animals will spend the most time in the valleys, where the perceived predation risk is the lowest. This information will enable rodent management to place traps on those specific perceived low fear locations, which we suggest will increase trapping rates and thus pest management success.

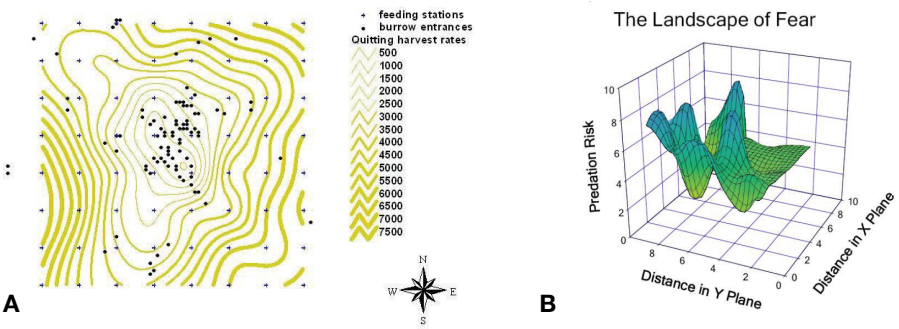

Figure 7.1 Two different ways of visualisation of the landscape of fear **A)** 2D map of the cape ground squirrel, the thicker the grey line, the more 'safe' the squirrel feels to forage (adapted from Merwe & Brown, 2008) **B)** 3D depiction of the landscape of fear, with highest giving up densities at the peaks (retrieved from Laundré et al 2010).

Rodents can alter their risk management in several ways; (I) by time allocation, e.g. shorten the exposure time and forage as fast and shortly as possible to reduce predatorily encounters, (II) by vigilance, e.g. reduce the lethality of encounters with a predator, (III) by safety in numbers by synchronised activity, and (IV) by night vs. day activity to avoid encounters with predators. Again, trapping efficiency could be substantially improved if we had mapped the LOF of the specific rodent pest species and then placed the traps accordingly (so where GUDs are lowest(Jones *et al.*, 2016) i.e. peaks of the LOF). One option would be to conduct a systematic analysis of the behaviour of pest species where their ethology may help clarify potential actors in response to GUDs for LOF and management actions for those species. Because the LOF differs among species, it also differs between target and non-target rodents, which in turn could be used for minimising unwanted effects on non-targets. In case of doubt, the LOF of the non-target species should also be mapped to prevent trapping in overlapping perceived risk valleys. To date, however, no study has systematically mapped the spatial behaviour of rodent pest species where beneficial species would be at risk of non-target poisoning. In our view, one should concentrate on the following four key points for the use of the LOF as basis for rodent management: (I) pest species with the lowest GUD will be most easiest to target, (II) species are most susceptible during times of the year when their GUDs are lowest; during these intervals management methods will be most effective, (III) species are most likely to be trapped in (micro-) habitats where their GUDs are lowest; thus concentrate rodent management where rodents perceive the least levels of predation risk, and (IV) management strategies which increase perceived risk of predation for the target pest species will lower pest damage. Measures to promote populations of appropriate predators should be taken, such as placing out nest boxes for birds of prey (e.g. owls(Brown *et al.*, 1988)) and educating local communities about the benefit of local biological predators (e.g. foxes(Lindström

et al., 1994; Saunders, Coman, Kinnear, & Braysher, 1995)). Research into the use of "biocontrol" by domestic predators (e.g. cats, dogs) as rodent management method in Africa showed that the presence of these predators affected the foraging behaviour of pest rodents.(Mahlabla, Monadjem, McCleery, & Belmain, 2017) Presence of both cats and dogs increased levels of fear (measured by increased GUDs) for local foraging rodent species, which led to diminished rodent activity. (Mahlabla *et al.*, 2017) However, reliable scientific evidence that bio-control via predation minimizes rodent population size below damage threshold levels is not yet available.

Conclusion

Connecting the LOF to rodent pest species is a novel approach with many opportunities to further enhance ecologically-based rodent pest management. Implementing the LOF into rodent management may enable the development of preventive control rather than reactive methods through better timing and habitat targeting for trapping or placement of rodenticides. It is extremely important to continuously look at alternatives for pest-management. A recent study of Mul *et al*.(2016) developed a fully automated pest monitoring tool to implement IPM effectively. This was done by focussing on the behaviour of the pest species, after which monitoring was conducted to develop a model which predicts the location and grow of the population.(Mul *et al*., 2016; Mul *et al*., in press) In conclusion, for effective management, it is essential to align management methods with the pest-species biology and behaviour. Until now, there are few studies on the behaviour of commensal and non-commensal pest species over different habitats and environments (e.g. city vs countryside) which are a necessity for composing and using the LOF. It would be best to have an overview of all species present, and whether and when they compete with each other or not. The idea to use the LOF as an EBRM tool holds promise for novel strategies and capacities for practical use as a unifying behavioural ecological concept. A study on the influence of domestic predators on pest rodent foraging behaviour by Mahlaba et al.(2017) suggest that the integration of the LOF into EBRM will provide stronger insights into the ecology of rodent pest species. The use of LOF is much stronger and broader applicable than the use of tacit knowledge, as tacit knowledge generally based on experience and can be highly subjective, and is difficult to transfer to another person by formal means The LOF concept is meant to provide a more evidence-based approach. In turn, this would enable the development of more efficient rodent management methods.

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Chapter 8

General discussion

During this thesis several topics were researched, all linked to each other by rodents as common denominator. To visualise this, a simplified hypothetical framework was developed which also shows potential interactions influencing (rodent borne) zoonoses (Figure 8.1). This scheme functions as main framework to base an action plan for public health upon, to respond to emerging rodent-borne zoonotic diseases.

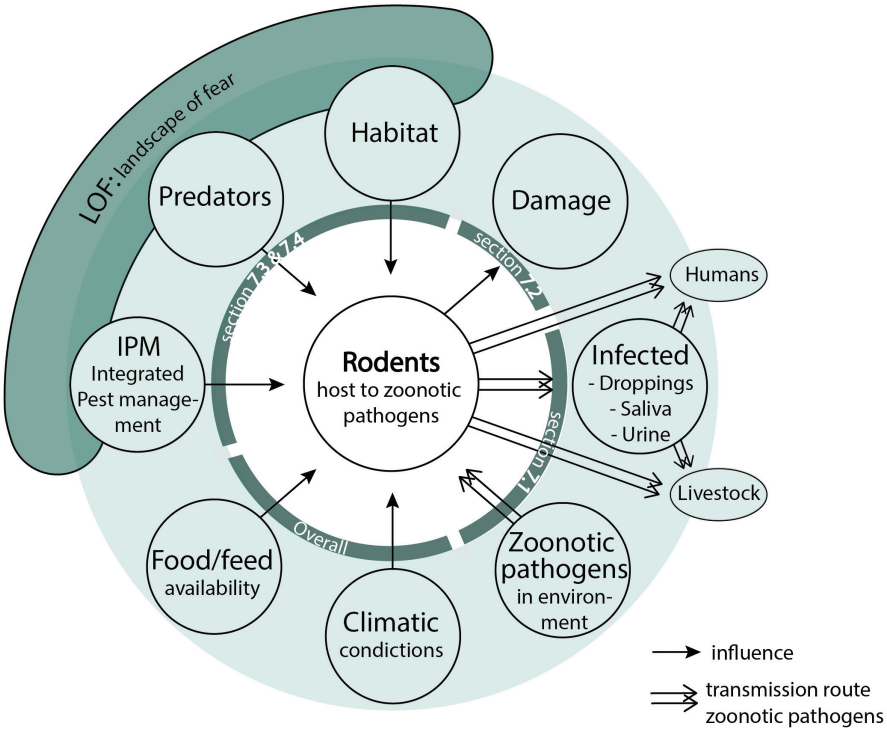

Figure 8.1 Framework showing aspects of rodent presence and density on potential transmission routes of rodent-borne zoonoses to humans or livestock. In the inner ring (dark green) the sections of the discussion on selected aspects are indicated.

The main aim of this thesis was to compare rodent-borne health risks in farming systems for two cultural and climatic total different continents; with the Netherlands versus Bangladesh as representative countries for Europa and Asia, respectively. In order to do this, two objectives were set up. The first objective of this thesis was to **assess the prevalence of selected zoonotic pathogen species in wild rodents in Asia and Europe**.

To meet the first objective, the following research questions were formulated and discussed in section 7.1:

- What is the prevalence of pathogenic *Leptospira* and *Toxoplasma gondii* in wild rodents and insectivores in the Netherlands (Chapter 2)
- To what extent are rodents from Bangladesh infected with *Toxoplasma gondii?* (Chapter 3)
- What is the prevalence of *Leptospira* infection in rodents from Bangladesh (Chapter 4)
- What is the prevalence of *Clostridium difficile* in wild rodents and insectivores in the Netherlands (Chapter 2)

By increasing the use of correct, preventive, species specific management methods based on IPM, the chance of disease spread from rodent to human will decrease. Therefore the second objective of this thesis was to **assess the effect of current rodent management methods in Asia and to improve rodent management based on IPM in order to reduce the chance for the rural population to contract rodent-borne zoonoses.**

To meet this second objective, two research questions were formulated and discussed in sections 7.2-7.4:

- What is the efficacy of rodent management and monitoring methods on post-harvest losses by rodents in Bangladesh (Chapter 6)
- Can the landscape of fear of pest species be used within rodent pest management strategies (Chapter 7)

Together the research questions contribute to answer the main aim of this thesis; assess rodent-borne health risks in farming systems for two cultural and climatic total different countries; the Netherlands (Europe) versus Bangladesh (Asia).

This chapter, the general discussion, aims to discuss the findings on an overall level, in the context of the main objectives of this thesis. The in depth discussion of the specific findings per chapter can be found in the discussion sections of the chapters itself (Chapter 2-7). Sections 7.1-7.4 are linked to the framework connecting all research topics of this thesis (Fig. 1).

7.1. Rodents and zoonoses

From a human point of view, rodents have always been associated with disease and both wild and commensal rodents can be a vector for zoonotic pathogens. Thus, rodent presence can form a threat for public health. Over 200 zoonotic diseases are recognized as threat for both human and animal health. Of all emerging infectious diseases, 60.3% is zoonotic. The majority of these emerging zoonotic diseases (70.8%) originates from wildlife (Jones, K. E. *et al.*, 2008). The results presented in this thesis show presence of three zoonotic pathogens in wild rodents and insectivores, which I will discuss below.

Toxoplasma gondii prevalence in wild small mammals

Toxoplasmosis is still one of the most common parasitic infections in the world and is caused by the protozoan parasite *Toxoplasma gondii.* In **Chapter 3** a group of 312 small mammals from the Netherlands was tested on *T. gondii.* Rodents and insectivores were trapped at various sites, but mostly on pig and dairy farms throughout the Netherlands. Five of the animals, all brown rats, (1.6%, n=312) were positive for *T. gondii* DNA. All five infected rats were caught on the island Texel (NL) (17.9%, n=28). The rodents from farms tested negative for *T. gondii*, which is not in line with the expectations since previous studies conducted on farms in the Netherlands found rodents as well as insectivore species carrying *T. gondii.* General infection rates of rodents from the Netherlands with *T. gondii* are reported between 2-14.3% (**Chapter 3**). Prevalences differ between studies and also amongst animal species. For the brown rat (*R. norvegicus*) infection percentages of 10.3% in the Netherlands are shown (Kijlstra *et al.*, 2008), but also lower and higher prevalences are reported in Europe; a prevalence of 1% (n=84) in wild brown rats from Czech (Hejlíček, Literák, & Nezval, 1997), compared to a mean prevalence of 35% (n = 235) in wild brown rats from the UK (Webster, 1994).

In studies from the Netherlands which show *T.* gondii presence in small mammals (Kijlstra *et al.*, 2008; Meerburg, B. G. *et al.*, 2012) cats were present on the participating farms. Being the definitive host for *T. gondii,* cats could become infected by predation of infected intermediate hosts such as wildlife, or via ingestion of oocysts from the environment (Afonso, Eve *et al.*, 2006; Afonso, E *et al.*, 2007; Hejlíček & Literak, 1998). In my research in the Netherlands however, all farms were free of cats, which might explain the absence of *T. gondii* in the small mammals tested. This is in contrast to the situation on the island Texel (NL) where there is a problem with stray cats (News, 2018; Spek, 2015). The presence of wild cats on this island (≈460km^2) could explain the relatively high prevalence of 17.9% amongst the trapped rodents (brown rats) from Texel.

Looking at a country in a total different climate, 296 rodents from Bangladesh **(Chapter 4)** were analysed on the presence of *T. gondii* DNA. To our knowledge, no research on *T. gondii* infection in Bangladesh rodents has been carried out before. In my study a percentage of 3.4% rodents trapped in or around food storage facilities tested positive for *T. gondii* DNA. This is in line with the results from Thai rodents in 2011, where a seroprevalence of 4.6% (n=461) was found (Jittapalapong *et al.*, 2011). However, rodent infection rates can vary depending on the species researched, the location, and climate (Gotteland *et al.*, 2014; Morand *et al.*, 2015; Tenter *et al.*, 2000). In Serbia, for example, a higher percentage of rodents was found to be positive; 10.4% (n=156, *Rattus norvegicus* and *M. Musculus*) (Vujanić *et al.*, 2010). Research from the Netherlands showed 11.9% of 101 wild rodents and shrews positive for *T. gondii* DNA (Kijlstra *et al.*, 2008), and a study from 2012 in The Netherlands found that 4% of rodents and shrews (n=250) were positive using DNA detection (Meerburg, B. G. *et al.*, 2012), which again is more in line with the findings of our study in Bangladesh. In Brazil, wild feral rodents (Capybara (*Hydrochaeris hydrochaeris*)) were tested for *T. gondii* DNA and showed a prevalence of 15.4% (n=26) (Truppel *et al.*, 2010). In China, a PCR study to detect *T. gondii* DNA showed 22.3% of *M. musculus* to be positive (n=31) and 23.9% of the *R. norvegicus* trapped to be positive (n=92) (Yan *et al.*, 2014), which are relatively high percentages compared to other DNA studies on rodents.

Factors that could have influenced the difference in observed prevalences in the different rodent species from the selected trapping locations in Bangladesh could be the species-specific behavioural patterns, their ecology and ethology, and also the presence or absence of cats could have influenced the observed results. None of the locations in Bangladesh had cats as pets, however, there were stray cats around which could lead to rodent infection.

Reports on *T. gondii* infection in rodents from other Asian countries are mostly on *T. gondii* detection by serologic tests (Herbreteau *et al.*, 2012; Jittapalapong *et al.*, 2011; Salibay & Claveria, 2005) but these have several disadvantages, i.e. high rate of false negatives (Dubey, J. *et al.*, 1997). Thus, serology alone may be insufficient to determine rodent prevalence (Dubey, J. & Frenkel, 1998). PCR is more sensitive to detect *T. gondii*, but its use may be limited by costs and lack of experience (Nimir & Linn, 2011).

The difference of the infection percentages of the tested rodents might be due to rodent species, but also to climate. Climatological aspects can influence the ecological context and balance within which pathogen hosts/vectors develop and transmit diseases (Patz, Graczyk, Geller, & Vittor, 2000). For *T. gondii* there are three

potential effects of climate change in Europe: (I) the survival of the pathogen will be affected, (II) increased precipitation will simplify/facilitate the spread of sporulated oocysts, and (III) the ecology of the hosts will be altered (Meerburg, B. G. & Kijlstra, 2009). Prevalence of *Toxoplasma* is high in moist warm areas and low in dry and hot zones, and also low in the arctic areas (Tenter *et al.*, 2000). For Europe climate change means increase in temperatures, drier summers, and wetter winters. With the mean winter temperatures increasing, it is to be expected that sporulated oocyst survival will increase (sporulated oocysts can survive at -10°C for 3.5 months, at 4°C for 54 months, at 35°C for 1 month, and at 40°C for 9 days (Dubey, J., 1998). This increase could have consequences for *T. gondii* prevalence in intermediate and final hosts (Meerburg, B. G. & Kijlstra, 2009). Because *T. gondii* prevalence in rodents could lead to infection of cats, it is of essence that food stores and food processing facilities prevent rodent pests and limit the use of cats for rodent control.

Presence of *T. gondii* in small rodents present around farms could be a risk factor as rodents tend to visit barns. Theoretically production animals such as pigs could then get acquire infection, leading to potential risk for human infection as the infected meat ends on our table, potentially raw or undercooked (Guo *et al.*, 2015; Kijlstra & Jongert, 2009).

It is clear that the answer to the research questions "What is the prevalence of pathogenic *Leptospira* and *Toxoplasma gondii* in wild rodents and insectivores in the Netherlands" and "To what extent are rodents from Bangladesh infected with *Toxoplasma gondii?"* are not defined only by the found percentages. We can conclude that rodent infection rates can vary depending on presence of cats or cat species, the rodent species researched, the location, and climate.

Leptospira spp. prevalence in wild small mammals

Until now, there is still little known and published about the presence of *Leptospira* spp. in rodents and insectivores in the Netherlands and other European countries. In **Chapter 3** a group of 379 small mammals from the Netherlands was tested on pathogenic *Leptospira* spp. Rodents and insectivores were trapped at various sites, but mostly on pig and dairy farms throughout the Netherlands. Over five percent of the animals (5.3%, n=379) tested positive for Leptospira DNA. The animals positive for *Leptospira* spp. were various species. Most studies focus on *Rattus norvegicus* only, because these animal carriers are recognized as important infection sources for humans (Aviat *et al.*, 2009; Runge *et al.*, 2013; Terpstra, 1989) and are often present near water rich areas. In this way, they pose a serious threat for surface water contamination. Studies on pathogenic Leptospira from various countries in Europe on *R. norvegicus* show mean infection percentages between 21-57% (Aviat

et al., 2009; Maas *et al.*, 2018; Runge *et al.*, 2013). Our research showed a lower infection percentage in the small mammals tested (5.3%) than the other published studies from European countries. This could be due to multiple factors, such as difference in diagnosis methods used, or trapping year, or season, or trapping location (close to water). Although the majority of publications use serological methods, it is important to use molecular detection, like I used in my research. A serious disadvantage of using serological methods for diagnosis is that it only detects the pathogens presence when there are sufficient levels of anti-*Leptospira* spp. antibodies present (Ahmed, A. *et al.*, 2012; Musso & La Scola, 2013). However, the main reason for the difference in infection percentages found is that studies mentioned above focus on *R. norvegicus* only, in contrast to my study which includes more animal species.

Although brown rats are considered the most important hosts spreading the bacterium to humans, almost every mammal might be reckoned as potential bearer and disseminator of *Leptospira* spp. (Hartskeerl, 2006; Mwachui *et al.*, 2015b){Hartskeerl, 1996 #99;Hartskeerl, 2006 #235}. In our study (**Chapter 3**) it is indicated that, even though with a lower abundance, pathogenic *Leptospira* spp. are also widely distributed in other small mammals from Europe. The prevalence of *Leptospira* spp. in the tested rodents and insectivores from the Netherlands ranged between 1-15%, with an average of 5.3%. This is confirmed by literature from European countries which report on the occurrence of *Leptospira* spp. in small rodents and shrews with a range between 5.7-20% (Adler, H. *et al.*, 2002; Obiegala *et al.*, 2016; Treml *et al.*, 2002; Turk *et al.*, 2003).

In Bangladesh a higher mean infection percentage of Leptospira in rodents was found (**Chapter 5**): qPCR and sequencing showed 13.1% (n=465) of the trapped rodents were infected with pathogenic *Leptospira.* Interestingly, rodents of the genus *Bandicota* were significantly more likely to be positive than those of the genus *Rattus* and *Mus*. Thus, *Bandicota* rats could be an important host in the epidemiology of leptospirosis in Asia. Previous studies in Asia on rodents identify *R. rattus* to be the main reservoir host for human pathogenic *Leptospira* (Johnson *et al.*, 2004; Ko *et al.*, 1999; Sarkar *et al.*, 2002). Other studies from Asia show varying numbers of infection percentages, ranging from 7.1%-22% (Benacer, Mohd Zain, *et al.*, 2016; Ivanova *et al.*, 2012; Koma *et al.*, 2013; Saravanan *et al.*, 2000; Sharma *et al.*, 2003).

Both *L. interrogans* and *L. kirschneri* were present in the rodents from Europe, and *L. interrogans, L. borgpetersenii,* and one rodent with *L. kirschneri* DNA in the samples from Asia (Bangladesh). In Europe, *L. borgpetersenii* and *L. interrogans* are the most

observed *Leptospira* genomospecies present in rodents; however, in Europe, a third genomospecies is also commonly found in rodents: *L. kirschneri* (Mayer-Scholl *et al.*, 2014; Turk *et al.*, 2003).

From our results and the literature research (**Chapter 5**), it can be stated that *L. interrogans* and *L. borgpetersenii* are the most common species found in rodents in South-East Asia. However, to find out whether specific strains/serovars adapt to specific reservoir hosts in specific habitats, more in-depth research with different diagnostics needs to be conducted. The findings of the research in Bangladesh are in line with the fact that *Leptospira* species, *borgpetersenii* and *interrogans*, contribute a great deal to human disease in Asia (Benacer *et al.*, 2013; Cosson *et al.*, 2014; Laras *et al.*, 2002; Thaipadungpanit *et al.*, 2007). For Europe, it is remarkable that *L. kirschneri* was found in the *Rattus rattus* (black rat). This black rat is worldwide associated with Icterohaemorrhagiae infections which belong to *L. interrogans* (Kuiken, 1990) although it harbours also *L. kirschneri* in Brazil and Mayotte (Desvars *et al.*, 2012; Moreno *et al.*, 2016). In this thesis *L. kirschneri* was found in a *R. exulans* sample from Bangladesh. Our findings are the first to confirm the presence of *L. kirschneri* in this rodent species (*R. exulans*).

Reports on *Leptospira* prevalence in rodents from the *Bandicota* genus indicate that all three rodent species from this genus are potential carriers of the same *Leptospira* species (*L. interrogans, L. borgpetersenii, L. weilli, L. inadai*) (Aplin, K. P., Frost, *et al.*, 2003; Carleton & Musser, 2005; Corbet & Hill, 1992; Musser & Brothers, 1994; Wilson & Reeder, 2005). However, due to the limited information available in published reports, it is not possible to link the strain or serovar infection to a specific host species. This is unfortunate, as such information could give insight into a possible co-evolution of serovars with specific rodent species.

In my study in Bangladesh, *Leptospira* prevalence in rodents was significantly higher in the dry season (15.7%) than in the rainy season (8.7%), which is not in line with the findings from Malaysia and also from Cambodia, where rodents showed a lower infection rate in the dry (6.3%) than in the wet (26.7%) season (Benacer, Mohd Zain, *et al.*, 2016; Ivanova *et al.*, 2012). It is known that there is a strong positive relation between floods or excessive rainfall and Leptospirosis outbreaks (Lau *et al.*, 2010; Sharma *et al.*, 2003; WHO, 2009) (LaRocque *et al.*, 2005). Besides this seasonal influence on human infection, the risk of *Leptospira* infection also depends on the geographic location, as well as on other risk factors, such as the risk of flooding, contaminated surface waters, and proximity to rubbish dumps (attractive for rodents) (Halliday *et al.*, 2013; Reis *et al.*, 2008; Sarkar *et al.*, 2002). Easterbrook et al. (2007) stated that seasonal fluctuations in *Leptospira* infections in rodents do

not occur due to the fact that once infected, the antibodies remain in the animal and the animal will test positive. This can explain our results and findings from other countries in South-East Asia, which show that rodent species living in households have a stable infection level, regardless of the geography and season (Cosson *et al.*, 2014; Ivanova *et al.*, 2012).

It is known that the infection rate amongst rats is highly variable in time and place (Kuiken, 1990; Kuiken *et al.*, 1991), our results confirm this. Looking at the results of my study I would like to hypothesise that different *Leptospira* species prefer different ecological niches; *L. borgpetersenii* seems to be more abundant in rodents from dry habitats (non-floodable lands) than *L. interrogans,* which implies that the infection of rodents could be linked to ecology.

In none of the rodents studied damage or anomalies were found during the dissections, which confirms the role of these animals as a natural reservoir. Because Bangladesh offers a suitable humid climate for the survival of these pathogenic bacteria, the presence of rodents could be a serious risk for human infection. The results confirm the importance of rodents as hosts of pathogenic *Leptospira* and indicate that human exposure to pathogenic *Leptospira* may be considerable, also in places where food (rice) is stored for longer times.

Looking back at the research questions on *Leptospira* spp.: " What is the prevalence of pathogenic *Leptospira* and *Toxoplasma gondii* in wild rodents and insectivores in the Netherlands" and "What is the prevalence of *Leptospira* infection in rodents from Bangladesh", it can be concluded that besides seasonal, geographic, and temporal factors, the host species also plays a role in the infection rate. Furthermore, our results showed that insectivores and rodents might be used as an indicator for the environmental contamination and/or the contamination in wildlife for *Leptospira* spp. This study emphasizes the need to improve rodent management at such locations and to further quantify the public health impacts of this neglected emerging zoonosis in Bangladesh.

Clostridium difficile prevalence in wild small mammals

In **Chapter 2** it was concluded that rodents and insectivores in and around food production buildings (e.g. farms) in the Netherlands can carry *Clostridium difficile* ribotypes associated with human *C. difficile* infection (CDI). In total 39.2% of the rodents and insectivores (n=347) tested positive for presence of *C. difficile.* Thirteen different ribotypes (RT) were present, in descending order of frequency: 057, 010, 029, 005, 073, 078, 015, 035, 454, 014, 058, 062, 087. In my study the black rat (*R. rattus*) and house mouse (*M. musculus*) were the species with the highest diversity

in RTs, 8 and 7 types, respectively. The ribotype most frequently isolated was RT057, which was only found in black rats and house mice. Although present at such high percentages, no references to RT057 could be found in the literature. However, RT057 is also frequently found in humans and characterized as producing toxin A and B (unpublished data of the Dutch National Reference Laboratory for *C. difficile* infections). The fact that no literature was found on this ribotype could be due to the possibility that RT057 does not result in clinical symptoms in humans. Furthermore, based on this finding it could be hypothesised that RT057 is a type that is predominantly found in mice and not in other animals. This would be an interesting question to further research.

Six RTs which have genetic overlap between human and animal sources of *C. difficile* were found: RT005, RT010, RT014, RT015, RT078, and RT087. There is only limited published on evidence for zoonotic transmission of *C. difficile* (strains RT078 and RT014)(Knetsch *et al.*, 2014; Knetsch *et al.*, 2018; Knight, Daniel R *et al.*, 2017). This transmission potential between animals and humans leads to a zoonotic risk, not only between humans and farm animals, but also pets and humans, and (indirectly) rodents and humans. *C. difficile* spores in rodent and insectivore droppings are able to survive in the environment for prolonged periods, leading to host-to-host exposure and transmission. Therefore it is concluded that rodent and insectivore presence on farms is a risk for zoonotic pathogen transmission of *C. difficile*.

An interesting question to address during future research is whether the ribotypes found in these small mammals are also present in the environment if rodents and insectivores are absent. If so, this could mean that small mammals acquire infection from the environment and are then able to distribute the pathogen further throughout their habitat.

As overall answer to the research question: ‘What is the prevalence of *Clostridium difficile* in wild rodents and insectivores in the Netherlands” is that rodent and insectivore presence on farms is a risk for zoonotic pathogen transmission of *C. difficile.*

Our findings highlight the complex multi-host epidemiology of rodent borne zoonoses and the importance of considering the role of rodents (and other animal hosts) in the maintenance and transmission of infection when evaluating human risks. One of the key actions to minimise the public health impacts of rodent borne zoonoses is to improve rodent management. A key question is to which extent a pest population should be reduced to prevent infection. In any case, preventive measures should be taken for (preventive) rodent control.

7.2. Rodent damage

Besides transmitting pathogens, rodents are also known to cause losses to stored human food and for causing damage to insulation and wiring due to their gnawing behaviour (Belmain, Steven R *et al.*, 2015; Hussain & Iqbal, 2002; Meerburg, B. G. *et al.*, 2009). In 2017 about 820 million people were undernourished globally (FAO). It is known that significant losses of stored rice occur mostly because current rice storage systems in Asia are not rodent-proof. Besides food loss, current storage methods also lead to damage and contamination of food by rodents, and to potential disease transmission via contamination of the food by rodent droppings, urine, and saliva. Inferior or absence of rodent management could lead to an increase of rodents living and foraging nearby households, which upsurges both undernourishment and the probability of zoonotic disease transmission. When assessing stored rice losses and rodent management methods in Bangladesh in **Chapter 6**, five rodent species were found to cause the rice losses: *Rattus rattus* (trapped most often), *Mus musculus*, *Bandicota bengalensis*, *Rattus exulans*, and *Mus terricolor.* Based on the results of the chapters screening for *T. gondii* (**Chapter 4**) and *Leptospira* (**Chapter 5**) in rodents from Bangladesh, it can be concluded that presence of these pest rodents do not only cause damage to stored produce, it is also a risk for human health as these rodents contaminate the food and environment of the people with their droppings and urine. It was found that daily rodent removal trapping proved to be most effective to diminish stored produce loss. The effectiveness of predators (cats) was limited. Losses in households were up to 1051.3 grams per month. The attitude of farmers in Asia is contradictory to the attitude of farmers in Europe. Losses experienced during rodent outbreaks in Europe are hardly accepted by farmers, especially given the multitude of associated problems that cause additional costs (purchase of substitute fodder, reseeding, weeding, ploughing of degraded soil)(Jacob & Tkadlec, 2010). In Asia, farmers have a different attitude, they feel that losses due to rodents are not that large and ‘part of the job’ (Brown, P. R. *et al.*, 2008; John, 2014; Singleton, Grant R, 2003). By conducting measurements on the actual size of the losses, farmers knowledge can be upgraded in order to gain a different attitude towards (pest) rodents and the need for management.

7.3. Rodent management

With rodent pest species around, the need for management arises. In literature it is stated that removing rat populations by reactive culling is often ineffective (Colvin & Jackson, 1999; Cowan, D. P., Quy, & Lambert, 2003; Meyer, 2003). A recent study on *Leptospira* (Lee *et al.*, 2018) in urban rats even found that lethal, urban rat control was associated with a significant increase in the odds that surviving rats carry *Leptospira*. These results suggest that human interventions have the potential to affect and even increase the prevalence of zoonotic pathogens within rat populations. Further research to demonstrate a direct link between the killing and increased pathogen transmission form rats to humans are difficult due to both practical and ethical considerations. Removing animal reservoirs of human pathogens might have unintended consequences on the disease risks. Again, I underline the importance of understanding the ecology of the targeted animal reservoir to design effective control programs. In **Chapter 7** the use of the *Landscape of Fear* as a rodent management strategy is described. With the current reactive pest management methods having serious drawbacks, rodent control needs to be based on pest-species ecology and ethology. Gaining insight in the behaviour of specific pest-species is a key aspect ecologically-based rodent management (EBRM). The *Landscape of Fear* (LOF) is a mapping of the spatial variation in the foraging cost arising from the risk of predation and reflects levels of fear a prey species perceives at different locations within its home range. In practice, the LOF is a mapping of habitat use as a result of perceived fear, which shows where bait or traps are most likely to be encountered and used by rodents. An opportunity for rodent control strategies lies in the integration of the LOF of rodents in EBRM methodologies. Rodent management could be more efficient and effective by concentrating on those areas where rodents perceive the least levels of predation risk. The LOF could be used for every pest species, in every climate.

In conclusion, as answer to the research question: “What is the efficacy of rodent management and monitoring methods on post-harvest losses by rodents in Bangladesh” it can be concluded that presence of pest rodents do not only cause damage to stored produce, it is also a risk for human health as these rodents contaminate the food and environment of the people with their droppings and urine. Therefore rodent management should be applied.

7.4. Rodent fear

In this thesis the use of preventive measures for managing rodent pests are promoted, such as the LOF. In **Chapter 7** several types of fear are discussed, as well as how they could be used in management. A natural response of plants, vertebrates and invertebrates to predation threat is the production of alarm signals (Verheggen, Haubruge, & Mescher, 2010). Rats and mice are known to produce alarm pheromones, which are chemical cues to warn others for potential danger (Kiyokawa, Shimozuru, Kikusui, Takeuchi, & Mori, 2006; Zalaquett & Thiessen, 1991). Laboratory experiments showed that rodents are even able to detect fear via alarm pheromones in conspecifics (a second-hand fear cue). In 2018, Haapakoski *et al*. published their research on the effect of alarm pheromones of wild bank voles to predator-exposed conspecifics. Wild bank voles were exposed to bedding material used by predator-exposed voles, and the control group was given bedding use by voles with no predator experience. It was found that litter size of the voles exposed to the bedding material of predator-exposed voles increased with about 50%. This shows that indirect predation risk can affect population levels, by increasing the amount of individuals. This is not what was expected when discussed in the chapter on the LOF that increasing the perceived risk of predation as management strategy could be a key point for rodent management. Although concluding in the chapter on LOF (**Chapter 7**) with the remark that no reliable scientific evidence is published on the assumption that biocontrol via predation minimizes rodent population size, I did not expect that the effect would be contradictory. However, I think that the statement made in **Chapter 7** is still valid, as one could use the rodents fear not to be able to frighten them, but by concentrate trapping methods on the areas where the pest animal perceives the least amount of fear.

The final research question was on rodent fear “Can the landscape of fear of pest species be used within rodent pest management strategies” and should be answered with an indisputable “yes” as an opportunity for rodent control strategies lies in the integration of the LOF of rodents in EBRM methodologies. Rodent management could be more efficient and effective by concentrating on those areas where rodents perceive the least levels of predation risk.

7.5. Other findings

Besides finding answers to the research questions, there were other interesting aspects which I came across or had to deal with, which I would like to point out below.

Laboratory rodents versus wild rodents

Laboratory rodents are formed by the human by intense selection and are highly inbred (Boonstra, 2013) and thus results from research on laboratory rodents cannot directly be translated to wildlife (Barnett, 1958). However, Berdoy (2002) shows in his documentary on lab rats, that after releasing 75 lab rats in an Oxfordshire farmyard, the rats still possess their natural wild behaviours and are able to survive outdoors.

Due to the importance of rats and mice in medical and experimental research, knowledge of rodent biology is heavily overrepresented by an overwhelming emphasis on commensal rodents. In literature there remains a significant skew towards laboratory studies. Although the number of studies on wild rodents is increasing, from 2008 to 2010 the citation index of rats was over 99,000 and still only 10% of which were studies conducted on rodents living in the wild (Buckle & Smith, 2015; Macdonald, D. *et al.*, 1994).

Besides the difference in breeding strategy (lab vs wild) the stressors laboratory rodents are subjected to are artificial and cannot be compared to the real 'outside' stressor to which their wild colleagues are exposed to (Koolhaas, Meerlo, De Boer, Strubbe, & Bohus, 1997). In the wild, animals are able to adapt as solution to ecological problems. The main knowledge on the effects of stress in wild animals is influenced by laboratory research, however it should be underpinned by the ecological and evolutionary setting within which the rodents actually live. Boonstra (2013) argues that when stressors such as high predation risk or food limitation occur, the animal responses in an adaptive manner in order to promote population fitness, even when the animal is chronically stressed. This statement of Boonstra (2013) is in line with the findings of Haapakoski *et al.* (2018), and contradictory to our expectations as we argue in favour of increasing the predation risk as part of using LOF as pest-rodent management tool. This directly leads to a question for further research: will increasing the perceived risk of predation as management strategy be an effective strategy for rodent management on short, but also on long term?

Pest animals and ethics

Animal welfare is high on the public agenda since the last decade. Recently, a report was published about the relation between humans and animals in the Netherlands

(Schukken, van Trijp, van Aplhen, & Hopster, 2019). This Dutch report discusses human interpretations and opinions on animal welfare, for different animal types. It can be concluded that different feelings come along when an animal is considered as pest-animal than when one considers an animal as a pet.

For laboratory animals strict regulations are set, based on criteria known as the 'Three Rs' -refinement; Replacement, Reduction, and Refinement. This means that methods are used only if the harms are outweighed by the benefits; that harms are reduced by replacement, reduction and refinement and that there is personal responsibility. Thus the first question to ask yourself before designing an animal experiment is: are there alternatives? Followed by: what is the smallest number of animals I need for this research? To be able to conduct animal experiments in the Netherlands, one needs to acquire an institutional permit from The Netherlands food and consumer product safety authority (NVWA). Furthermore, for every project with laboratory animals a licence needs to be requested at the central committee animal experiments (CCD). These instances and their licences ensure animal welfare. Most countries have animal welfare regulations, however protection levels for laboratory animals vary per country (Meerburg, B. G. *et al.*, 2008).

However, for pest-animals, inhumane control methods are still allowed, and an approach of 'the end justify the means' is used. There are many different techniques to control rodents (e.g. rodenticides, snap-traps, gluebords, chemosterilants), but they are not evenly humane. Gluebords for example are considered to be inhumane because the trapped animal has a slow death (multiple hours)(Mason & Littin, 2003; Meehan, 1984; Randall, 1999), and before it dies the animal can hurt itself through torn skin, broken limbs, and forceful removal of hair in their attempt to escape (Frantz & Padula, 1983). In the Netherlands, gluebords are forbidden as rodent control method. However, in many countries this method is still allowed (even western countries such as the USA and Canada)(Meerburg, B. G. *et al.*, 2008; Studer & Jones, 2014). There are even countries that have problems with living up to minimum standards of animal ethics, for example Bangladesh (Farhana, 2016). In this example, animal ethics are often not considered for production and domestic animals, let alone for pest animals. In an ideal situation there would be a clear framework for every animal species per country, including for pest species.

Research and pest animals

In this thesis, pest rodents from Bangladesh and the Netherlands were used. At the start of the thesis there were no specific licences that needed to be acquired, as I worked with pest animals. However, when writing the manuscripts and submitting them to journals, I had major difficulties with their submissions due to the lack

of ethical approval. Some journals would not even send the manuscripts to their reviewers when there was no code of the ethical approval present. Journals that did read our manuscripts, often gave us a general animal-welfare check-list with questions set to indicate welfare of laboratory animals. There questionnaires were difficult to apply to pest animals. This would mean that future research into pest animals that are not protected by ethical regulations, will become more and more difficult to share and publish as the journals will reject the manuscripts without even reading them. Editors should re-think the strict animal research procedures, especially in cases like ours: when research is done on already dead pest animals such as rodents trapped by professional pest-managers. In the third world, like in Bangladesh, many people suffer from undernourishment, are homeless and lack substantial rights, struggle to live and even die on the streets (Harriss-White, 2005; Rahman & Hakim, 2016; Uddin, Walters, Gaillard, Hridi, & McSherry, 2015). It feels contradictory then when an editor is not willing to consider a manuscript on pest-animals related to human health, only because of the lack of an ethics approval code. Therefore I suggest that journals should make a distinction between laboratory animals and pest animals, especially because the wild animals give insight in risks for humans (e.g. diseases) and without publishing results, risks may be underestimated or even unknown. It would even be better if there would be global regulations which apply for everyone, in order to prevent that each journal creates its own guidelines on animal welfare and ethics.

7.6. Concluding remarks

The main aim of this thesis was to gain knowledge on rodent-borne health risks in farming systems. I can conclude that there are serious rodent-borne health risks in farming systems in both Europe and Asia.

In this thesis, rodent presence is demonstrated in both studied countries, as well of the presence of zoonotic pathogens in these animals. The results of this thesis may help to improve the preparedness for potential disease outbreaks. Of course it is unable to totally prevent outbreaks of rodent-borne diseases. However, one can be prepared to take appropriate measures at both animal and human level when necessary.

It is essential to gain a more thorough understanding of the ecology of rodent-borne pathogens in rodents and humans in order to determine the public health risks associated with commensal rodents. Furthermore, this knowledge is of the essence in order to develop strategies to monitor and mitigate the risks (Himsworth, C.G. *et al.*, 2013). Even though the ecology of rodent-associated zoonoses is complex, shared elements of human disease can still be identified by studying the manifold ways in which rodents, pathogens, vectors, humans, and the environment possibly will interact. This will help to reduce the impact of disease outbreaks on animal and human health. This thought is endorsed by results of a recent study of Morand et al. (2019), which indicates that the ever decreasing biodiversity due to humans, will lead to an intensification of disease risks as result of more contact between humans, domesticated animals, and wildlife. Furthermore, transmission mechanisms and methods of pathogen spill-over should be studied in more depth in order to explore the role of the rodent as vector for zoonotic pathogens (Bordes, Frédéric *et al.*, 2015).

Gaining detailed knowledge about the rodent pest-species, its behaviour ecology, and on knowledge of the pathogen and its hosts, as well as their interactions is essential to estimate human health risks. Current and new technical solutions possibilities should be implemented to enhance pest species knowledge. It is crucial to integrate knowledge from several different and distinct fields to prevent future (large) disease outbreaks. The need for a multidisciplinary approach to deal with rodent-borne zoonoses is mainly due to the complexity of the diseases, the interactions between intermediate and final hosts, species specific host behaviour and ecology, economic importance, changing climate, and the multifaceted management of control and prevention (IPM) (Costello *et al.*, 2009; Davis, S., Calvet, & Leirs, 2005). Although strategies to prevent rodent-borne disease outbreaks are

limited, it is essential to rapidly respond to rodent-borne zoonoses in order to reduce the impact of emerging rodent-borne zoonoses in the coming era.

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Summary

Rodents represent the largest order of mammals (>40%) and consist of over 2000 species. However, only a small portion (<10%) of all rodent species can be referred to as pest species. From a human perspective, rodents have always been connected with disease. There are numerous pathogens that can be transferred from rodents to humans, called zoonoses. Currently, there are over 60 rodent-borne zoonoses known. Little research has been conducted on current rodent-borne zoonoses in regions of Asia, which raises the need to determine pathogen prevalence. Furthermore, impaired knowledge on zoonotic pathogens in rodents and insectivores limits opportunities for preventive measures and complicates risk-assessments for zoonotic transmission to humans. Besides being able to transmit diseases, rodents are also known for causing damage and losses to stored food. Asia has the highest undernourishment rate with an estimated number of over 275 million people suffering from hunger. In Bangladesh the proportion of undernourished in 2017 was almost 25 million people on a population of 164.7 million. A factor contributing to food insecurity is the presence of rodents. On yearly basis, rodents cause 5-10% loss to rice production in Asia, which leads to a worldwide estimated loss of 11 kg of food per person per year. There is a knowledge gap on the biology and habitat specialisations and distribution of many rodent species in Asia and in Europe, which is essential for the species-specific management of pest rodents. The main aim of this thesis was to obtain more knowledge about rodent-borne health risks in farming systems in both Europe and Asia. The chapters of this thesis describe several studies into rodents from the Netherlands and Bangladesh in order to find an answer to the main research question of this thesis.

In Chapter 2, presence of the zoonotic enteropathogen *Clostridium difficile* in wild rodents and insectivores is studied. Because *C. difficile*, an opportunistic anaerobic bacteria, is distributed globally and can be carried by both animals and humans, it is important to gain more knowledge whether and to what extent this zoonotic pathogen is present in Dutch wild rodents and insectivores. It is known that there is genetic overlap between human and animal sources of *C. difficile*. In our study, the aim was to assess the presence of *C. difficile* in rodents and insectivores trapped on and around pig and cattle farms in the Netherlands. In total 347 rodents and insectivores (10 different species) were trapped and 39.2% tested positive for presence of *C. difficile*. For all positive samples the ribotype (RT) was determined, and in total there were 13 different RTs found (in descending order of frequency: 057, 010, 029, 005, 073, 078, 015, 035, 454, 014, 058, 062, 087). Six of the RTs isolated from rodents and insectivores are known to be associated with human *C. difficile* infection; RT005, RT010, RT014, RT015, RT078 and RT087. The presence of rodents and insectivores in and around food production buildings (e.g. farms) could contribute to the spread of *C. difficile* in the human environment. In order to enable

on-farm management for pathogen control, it is essential to comprehend the role of wild rodents and insectivores that could potentially affect the ecology of pathogens on farms.

The aim of Chapter 3 was to assess the presence of two other pathogens in wild rodents and insectivores from the Netherlands; *Leptospira* spp. and *Toxoplasma gondii.* These two zoonotic pathogens are present on a list of prioritized emerging pathogens in the Netherlands and were therefore the focus of this chapter. Both pathogens have the ability to survive under moist environmental conditions. In total, a group of 379 small mammals (rodents & insectivores) were tested on pathogenic *Leptospira* spp, and 312 on *Toxoplasma gondii*. Rodents and insectivores were trapped at various sites, but mostly on pig and dairy farms throughout the country. Over five percent of the animals (5.3%, n=379) tested positive for *Leptospira* DNA, and five of the animals (1.6%, n=312) tested were positive for *Toxoplasma gondii* DNA. The animals positive for *T. gondii* were all brown rats and the ones for *Leptospira spp*. were various species. Our results show that insectivores and rodents might be used as an indicator for the environmental contamination and/or the contamination in wildlife for *Leptospira* spp.

In Chapter 4 the study location changed to a totally different environment: the study described was conducted in Bangladesh. As there is limited scientific knowledge available about the incidence and prevalence of *T. gondii* in commensal rodents in many Asian countries, we tested rodents from a commercial rice mill and eight local villages in Bangladesh for the presence of *T. gondii* DNA using rodent brain material preserved in ethanol. Rodents contribute to the life cycle of the protozoan parasite Toxoplasma gondii as an intermediate host and key prey animal of cats, the definitive host. Overall, 10 of 296 (3.4%) rodent samples tested positive for *Toxoplasma* DNA. Our results indicate that rodents present in food production and food storage facilities may carry *T. gondii*.

The aim of Chapter 5 was to assess the prevalence of pathogenic *Leptospira* species in rodents from Bangladesh. Worldwide, *Leptospira* infection poses an increasing public health problem. In 2008, leptospirosis was recognised as a re-emerging zoonosis of global importance with South-East Asia being one of the most significant centres of the disease. Because Bangladesh offers a suitable humid climate for the survival of these pathogenic bacteria, the presence of rodents could be a serious risk for human infection, especially in peri-urban areas or locations where food is stored. Rodents are thought to be the most important host for a variety of *Leptospira* serovars. Real-time Polymerase Chain Reaction (qPCR) and sequencing showed that 13.1% (61/465) of the trapped rodents were infected with

pathogenic *Leptospira*. Sequencing of the qPCR products identified the presence of three species: *Leptospira interrogans*, *Leptospira borgpetersenii*, and *Leptospira kirschneri*. Rodents of the genus, *Bandicota*, were significantly more likely to be positive than those of the genus, *Rattus* and *Mus*. Our results confirm the importance of rodents as hosts of pathogenic *Leptospira* and indicate that human exposure to pathogenic *Leptospira* may be considerable, also in places where food (rice) is stored for longer times. This chapter also emphasizes the need to improve rodent management at such locations and to further quantify the public health impacts of this neglected emerging zoonosis in Bangladesh.

Then in Chapter 6 the efficacy of rodent management and monitoring methods on post-harvest losses by rodents in Bangladesh was assessed. The presence of pest rodents around food production and storage sites is one of many underlying problems contributing to food contamination and loss, particularly influencing food and nutrition security in low-income countries. By reducing both pre- and post-harvest losses by rodents, millions of food-insecure people would benefit. Studies on the impact of rodents is particularly lacking in post-harvest systems. As there is limited quantitative data on post-harvest rice losses due to rodents in Asia, we assessed stored rice-losses in local households from eight rural communities and two rice milling factories in Bangladesh in order to monitor the effect of different rodent control strategies. Four treatments were applied, of which three rodent management methods: (i) control (ii) use of domestic cats, (iii) use of rodenticides, (iv) use of snap-traps. In total, over a two year period 210 rodents were captured from inside people's homes, with *Rattus rattus* trapped most often (n= 91), followed by *Mus musculus* (n=75) and *Bandicota bengalensis* (n=26). In the milling stations, 68 rodents were trapped, of which 21 *M. musculus*, 19 *R. rattus*, 17 *B. bengalensis*, 8 *Rattus exulans*, and 3 *Mus terricolor*. In 2016, losses from rice-baskets within households were between 13.6-16.7%. In 2017, the losses were lower, ranging from 0.6-2.2%. Daily rodent removal trapping proved to be most effective to diminish stored produce loss. The effectiveness of domestic cats was limited.

The aim of Chapter 7 was to obtain knowledge to be able to optimize IPM (prevention and control) for the local situation in Bangladesh to reduce the actual post-harvest losses. Current reactive pest management methods have serious drawbacks such as the heavy reliance on chemicals, emerging genetic rodenticide resistance and high secondary exposure risks. Rodent control needs to be based on pest species ecology and ethology to facilitate the development of ecologically based rodent management (EBRM). An important aspect of EBRM is a strong understanding of rodent pest species ecology, behaviour and spatiotemporal factors. Gaining insight into the behaviour of pest species is a key aspect of EBRM. The landscape of fear

(LOF) is a mapping of the spatial variation in the foraging cost arising from the risk of predation, and reflects the levels of fear a prey species perceives at different locations within its home range. In practice, the LOF maps habitat use as a result of perceived fear, which shows where bait or traps are most likely to be encountered and used by rodents. Several studies have linked perceived predation risk of foraging animals with quitting-harvest rates or giving-up densities (GUDs). GUDs have been used to reflect foraging behaviour strategies of predator avoidance, but to our knowledge very few papers have directly used GUDs in relation to pest management strategies. An opportunity for rodent control strategies lies in the integration of the LOF of rodents in EBRM methodologies. Rodent management could be more efficient and effective by concentrating on those areas where rodents perceive the least levels of predation risk.

We can conclude that there are serious rodent-borne health risks in farming systems in both in the Netherlands and in Bangladesh. In this thesis, for both countries rodent presence is demonstrated, as well of the presence of zoonotic pathogens in these animals. The results of this thesis may help to improve the preparedness for potential disease outbreaks. Of course we cannot prevent the outbreaks of rodent-borne diseases. However, we can be prepared to take appropriate measures when necessary.

It is essential to gain a more thorough understanding of the ecology of rodent-borne pathogens in rodents and humans in order to determine the public health risks associated with commensal rodents. Even though the ecology of rodent-associated zoonoses is complex, shared elements of human disease can still be identified by studying the manifold ways in which rodents, pathogens, vectors, humans, and the environment possibly will interact. This will help to reduce the impact of disease outbreaks on animal and human health. Furthermore, transmission mechanisms and methods of pathogen spill-over should be studied in more depth in order to explore the role of the rodent as vector for zoonotic pathogens. It is crucial to integrate knowledge from several different and distinct fields to prevent future (large) disease outbreaks. The need for a multidisciplinary approach to deal with rodent-borne zoonoses is mainly due to the complexity of the diseases, the interactions between intermediate and final hosts, species specific host behaviour and ecology, economic importance, changing climate, and the multifaceted management of control and prevention (Integrated Pest Management, IPM). Although strategies to prevent rodent-borne disease outbreaks are limited, it is essential to rapidly respond to rodent-borne zoonoses in order to reduce the impact of emerging rodent-borne zoonoses in the coming era.

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thank you. It was a memorable visit. It was impressive to see, feel, hear and live a total different culture. Thank you for your support and help with setting up the field work, and thank you for your efforts to get the samples shipped to the Netherlands.

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Curriculum Vitae

Inge Milou Krijger was born on 18 August 1989 in Valkenburg, Zuid Holland, The Netherlands and grew up in Bleskensgraaf. After obtaining her propedeuse diploma in Industrial Design at the Technical University in Delft in 2008, she decided that animals were more her cup of tea. She switched to the University of Applied Sciences in Delft to study Animal Husbandry. In her final year, Inge decided to do her thesis project in Wageningen. Her bachelor's thesis research was on moles to determine whether moles could serve as an indicator species for *Toxoplasma gondii* infections in livestock. This research project triggered her to come to Wageningen in 2013 to do a Master's degree. She decided to finish her degree with two MSc theses projects. One was on the presence and species distribution of biting midges on horses to help assess the role of midges in causing insect bite hypersensitivity in horses. For her second project, Inge investigated the role of rodents and ticks in the presence of the bacteria *Borrelia burgdorferi* s.l. in a natural ecosystem. After graduating with the degree of Master in Animal Sciences in 2015, she started her PhD research work at the Farm Technology Group of Wageningen University & Research, under the supervision of Peter W.G. Groot Koerkamp and Bastiaan G. Meerburg. The main aim of the study was to gain insight in rodent-borne health risks in farming systems. The results of this PhD work are presented in this thesis.

List of publications

Refereed scientific papers

Krijger, I. M., Ahmed, A. A., Goris, M. G., Groot Koerkamp, P. W., & Meerburg, B. G. (2019). Prevalence of Leptospira Infection in Rodents from Bangladesh. *International Journal of Environmental Research and Public Health*, 16(12), 2113.

Krijger, I. M., Belmain, S. R., Singleton, G. R., Groot Koerkamp, P. W., & Meerburg, B. G. (2017). The need to implement the landscape of fear within rodent pest management strategies. *Pest Management Science*, 73(12), 2397-2402.

Krijger, I. M., Cornelissen, J. B., Belmain, S. R., Shafali, R. B., & Meerburg, B. G. (2019). Evidence of Toxoplasma gondii in Rodents from Bangladesh. *Vector-Borne and Zoonotic Diseases*.

Krijger, I. M., Cornelissen, J. B., Wisselink, H. J., & Meerburg, B. G. (2014). Prevalence of Toxoplasma gondii in common moles (Talpa europaea). *Acta Veterinaria Scandinavica*, 56(1), 48.

Krijger, I. M., Meerburg, B. G., Harmanus, C., & Burt, S. A. (2019). Clostridium difficile in wild rodents and insectivores in the Netherlands. *Letters in Applied Microbiology*, 69(1), 35-40.

Submitted papers

Krijger, I.M., Gort, G., Belmain, S.R., Groot Koerkamp, P.W., Shafali, R.B., Meerburg, B.G. Efficacy of rodent management and monitoring methods on post-harvest losses by rodents in Bangladesh. Submitted. (Chapter 6 in this thesis)
Krijger, I.M., Ahmed, A. A., Goris, M. G., Cornelissen, J. B., Groot Koerkamp, P. W., & Meerburg, B. G.

Wild rodents and insectivores as carriers of pathogenic *Leptospira* spp. and *Toxoplasma gondii* in The Netherlands. Submitted. (Chapter 2 in this thesis).

Conference contributions.

Krijger, I. M., Belmain, S. R., Singleton, G. R., Groot Koerkamp, P. W., & Meerburg, B. G.. Integration of the landscape of fear of rodents in EBRM methodologies. 6th International Conference of Rodent Biology and Management & 16th Rodens et Spatium, 2018, Potsdam.

Krijger, I. M., Cornelissen, J. B., Belmain, S. R., Shafali, R. B., & Meerburg, B. G. Rodents from a rice milling station in Bangladesh infected with *Toxoplasma gondii.*

6th International Conference of Rodent Biology and Management & 16th Rodens et Spatium, 2018, Potsdam.

Krijger, I.M., Ahmed, A. A., Goris, M. G., Cornelissen, J. B., Groot Koerkamp, P. W., & Meerburg, B. G.. Leptospirosis and toxoplasmosis in wild rodents in The Netherlands. 6th International Conference of Rodent Biology and Management & 16th Rodens et Spatium, 2018, Potsdam.

Krijger, I. M., Ahmed, A. A., Goris, M. G., Groot Koerkamp, P. W., & Meerburg, B. G.. Leptospirosis in rodents in peri-urban Bangladesh. 6th International Conference of Rodent Biology and Management & 16th Rodens et Spatium, 2018, Potsdam

Other publications

Krijger, Inge. Mollen als indicatorsoort voor Toxoplasma? Dierplagen Informatie, jaargang 16 nr. 3 -2013

Krijger, Inge. Darmbacterie *Clostridium Difficile* in knaagdieren? Dierplagen Informatie, jaargang 22 nr. 4 – 2019

Meerburg, Bastiaan, Krijger, Inge, Wildcamera welkome aanvulling bij plaagdierbeheersing, Dierplagen Informatie, jaargang 22 nr. 1 – 2019

Training and Education statement

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (5 ECTS)

- Assessment of transmission of zoonotic pathogens by rodents in granaries, rodenticide use and rodenticide resistance in Bangladesh in order to reduce post-harvest losses

Writing of project proposal (1 ECTS)

- Assessment of transmission of zoonotic pathogens by rodents in granaries, rodenticide use and rodenticide resistance in Bangladesh in order to reduce post-harvest losses

Post-graduate courses (3.3 ECTS)

- Opleiding Bestrijdingstechnicus; KAD (2019)

Laboratory training and working visits (1.5 ECTS)

- Leptospirosis laboratory training: KIT Biomedical research (NL) (2015)

Invited review of (unpublished) journal manuscript (3 ECTS)

- Veterinary parasitology: Toxoplasmosis in pork (2017)
- Journal of Veterinary Medicine and Research: Sero-prevalence of toxoplasmosis in Boranabreed cattle in three selected district of borena zone, Oromia regional state, southern Ethiopia (2018)
- Frontiers Ecology And Evolution: Parameters that affect fear responses in rodents and how to use them for management (2019)

Deficiency, refresh, brush-up courses (0.9 ECTS)

- Competence assessment; WGS (2015)
- Carrier orientation; WGS (2019)

Competence strengthening / skills courses (5.1 ECTS)

- College geven; Educational Staff Development WUR (2015)
- LAS Course (Laboratory Animal Science) (2016)
- Supervising thesis students; Educational Staff Development WUR (2018)
- Infographics and iconography; WUR Library (2018)
- Brain training; WGS (2019)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)

- PE&RC First years weekend (2015)
- PE&RC Day (2016)

Discussion groups / local seminars / other scientific meetings (4.8 ECTS)

- WEES (2016)
- Livestock technology (2016)
- Wetenschappelijk onderzoek brengt het plaagdiermanagement naar een hoger PLA..N" (PLA..N=brancheorganisatie platform plaagdierbestrijding Nederland); oral presentation (2016)
- Plaagdierbeheersing; oral presentation; gemeente Leudal (2017)
- Presentation at meeting plaagdierbeheersing zuid NL (2017)
- Scientific meeting: vector meeting; WUR (2017)
- Technisch overleg KAD (2017-2019)
- WIAS Science day (2018)

International symposia, workshops and conferences (9.7 ECTS)

- AgEng Conference (2018)
- 6th International Conference of Rodent Biology and Management
- 16th Rodens et Spatium

Lecturing / supervision of practicals / tutorials (19.5 ECTS)

- FTE Engineering design (2015-2019)

Supervision of MSc students (6 ECTS)

- Designing a system to evaluate a rodent control method
- Analysis of current rice storage systems in Bangladesh and the begin of a design for a new storage system
- Leptospirosis in rats from Bangladesh

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