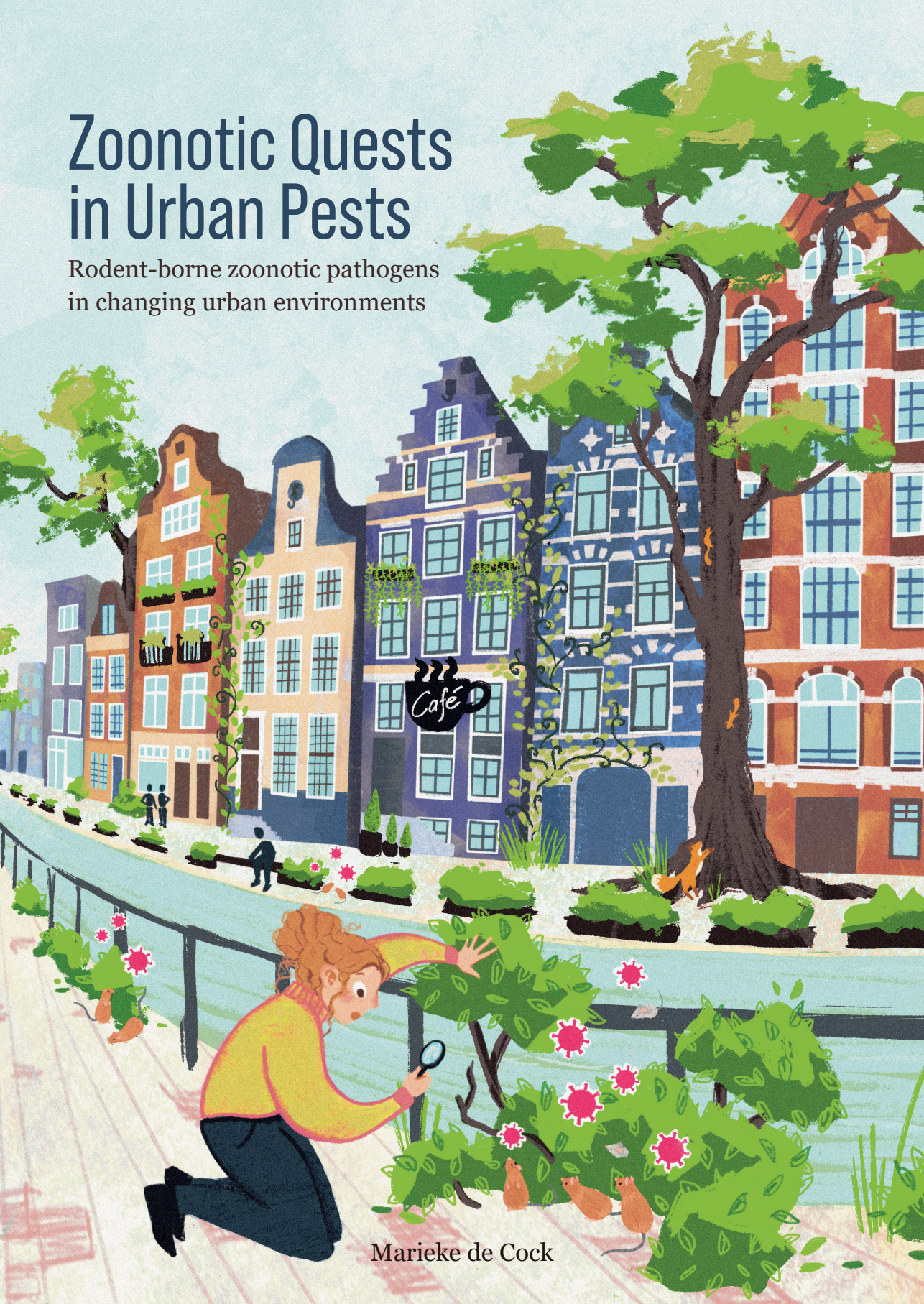
Zoonotic Quests in Urban Pests

Rodent-borne zoonotic pathogens in changing urban environments



Marieke de Cock

Propositions

1. Urban greening will result in more wildlife-borne zoonoses.
   (this thesis)

2. Keeping domestic cats indoors will increase rodent nuisance in residential areas.
   (this thesis)

3. PhD students' mental health should get more attention to prevent them from developing a psychiatric disorder (Levecque et al., 2017).

4. Being able to communicate research to others is as important as the research itself.

5. Controlling rats based on nuisance is like taking a paracetamol when you do not feel well, it addresses the symptoms but not its cause.

6. All animal are equal, but some animals, even within the same species, are more equal than others – adapted from George Orwell.

Propositions belonging to the thesis, entitled

Zoonotic quests in urban pests
Rodent-borne zoonotic pathogens in changing urban environments

Marieke de Cock
Wageningen, 15 March 2024

Zoonotic quests in urban pests

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Zoonotic quests in urban pests

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Marieke P. de Cock

Thesis

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Restaurant

Chapter 1

General introduction

Zoonoses

Zoonoses are infectious diseases caused by pathogens transmitted from animals to humans. These pathogens can be bacteria, viruses, parasites, helminths or fungi. They can be transmitted either directly, via contact with the animal or with its secreta or excreta, indirectly, via contact with contaminated food or environment (e.g., water or soil), or by vectors, via biting or mechanical transfer by arthropods such as ticks and fleas [1]. Most of the infections with zoonotic pathogens run an asymptomatic course [2]. This, in combination with often generic or broad-spectrum infection symptoms, such as fever, muscle ache and malaise, leads to misidentification and underreporting of infection with zoonotic diseases in humans [3]. Depending on the type of zoonosis, infections in humans can lead to severe health issues. For instance, each year more than 10,000 cases of human infections with orthohantaviruses are diagnosed in Europe, of which rodents are the main reservoir host [4]. Infection with orthohantaviruses can result in renal disease and cardiac, pulmonary, ocular and hormonal disorders. Dobrava-Belgrade orthohantavirus has a high case fatality rate, in contrast to Puumala orthohantavirus (PUUV). Still, PUUV infection can also lead to complications and long-term hormonal, renal and cardiovascular disorders [4]. Another example of a zoonosis, transmitted by rodents, is Weil's disease, caused by *Leptospira* bacteria, resulting in more than 1,000 human cases per year in Europe [5]. Although most *Leptospira* cases are asymptomatic or have only flu-like symptoms, infection can progress to renal failure and even multi-organ failure [6].

Emerging zoonoses

When a zoonosis has recently evolved, is recently recognized, or increases in incidence or geographical distribution, it is called an emerging zoonosis [7]. It is estimated that more than 60 % of all emerging infectious diseases are zoonotic, of which about 75 % originates from wildlife [8, 9]. Occasionally, emerging zoonoses can have major impact on human and/or animal health, and can manifest as an epidemic and sporadically even lead to a pandemic. Examples are the recent SARS-CoV-2 (Covid-19) pandemic which started in 2019 [10], and the Avian influenza virus outbreaks in wild bird populations throughout the world since 2020 [11]. The increase in the frequency of emergence of zoonotic pathogens is linked to climate change, contact rates between humans and (wild) animals, human behavior, the expansion of human populations, globalization of trade and travel (including movement of animals and animal products), changes in agricultural practices, and modification and fragmentation of natural habitats that alter the distribution of wild hosts and vectors. These factors (or a combination of them) could influence contact rates amongst animals and between animals and humans, facilitating the transmission and spillover of zoonotic pathogens between (wild) animals and humans [12-14].

Rodents are competent hosts for zoonotic pathogens

In contrast to many other (larger) wildlife mammals, rodents can host a very high diversity of zoonotic pathogens, especially rodents with a fast life history (e.g., an early and frequent reproduction) [15, 16]. Therefore, when rodents would get infected with a new emerging pathogen, this could result in a rapid increase in human disease hazard. The competence of rodents to host a high diversity of zoonotic pathogens is related to various factors.

One of them is the high abundance and diversity of rodent species [16]; rodents (order *Rodentia*) consist of more than 2,500 distinct species [14]. In particular house mice (*Mus musculus*), brown rats (*Rattus norvegicus*) and black rats (*Rattus rattus*), species that are living close to humans, form the highest risk for pathogen transmission to humans and animals, which is likely due to higher exposure rates [17]. Wild brown and black rats are able to host dozens of zoonotic pathogens, including *Yersinia pestis* bacteria (the causative agents of plague), *Leptospira* bacteria and orthohantaviruses [18]. The disease hazard and risk posed by rodents is likely larger than by other wildlife species.

Another factor contributing to the competence of rodents to host a high diversity of pathogens is the modulation of the rodent's immune response upon infection, resulting in a low level of infection or the ability of the rodent to tolerate replication of the pathogen, such that the pathogen may persist [19]. Many pathogens may infect and persist (lifelong) in rodents without causing clinical disease, which promotes pathogen transmission in the population [19, 20]. However, even in the absence of clinical disease symptoms, the host can still have pathologic signs of infection, such as signs of mild inflammation [21]. For example, rats are often asymptomatically infected with *Leptospira* bacteria, even though they show pathological signs of infection [22], and they can continue to shed infectious *Leptospira* bacteria in their urine during their entire lifetime [6]. Some pathogens do cause disease or come at a fitness cost for the rodent host, such as PUUV in bank vole (*Myodes glareolus* syn. *Clethrionomys glareolus*) populations [23].

Certain behavioral and demographic characteristics of rodents may also contribute to their capacity to host a high diversity of pathogens, such as the trade-offs between reproduction and life span. Rodents are characterized by an early sexual maturity and large litter sizes [15], which makes them highly dependent on the current availability of food resources. The changing availability of food resources (e.g., per season) affects rodents' reproductive activity, thereby causing fluctuations in rodent populations [24]. This, in turn, may affect the prevalence and dynamics of pathogens present in these rodent populations, for example by influencing the number of infected and susceptible animals. Mast seedings in European forests, which provide ample food resources for rodents, can lead to a huge increase in the population of mice and voles the next year, which in case of bank voles correlates with human PUUV outbreaks [25], and in case of mice correlates with densities of immature tick stages and thereby with the prevalence of Lyme disease [26]. Adverse conditions could force rodents to move to another area, which could be closer to humans, leading to increased transmission risk of zoonotic pathogens to humans [27].

Ecology and population dynamics of urban rodents

The anthropophilic nature of some rodent species (especially brown rats, black rats and house mice), and their high adaptive capacity to adjust to new environments has made them some of the most successful and abundant mammal species in urban areas [28]. Rodents mainly select their habitat based on the availability of food, shelter and water resources [29, 30]. Living close to humans provides them with abundant food and shelter resources that are not affected by seasonal fluctuations. Examples of such food resources are household waste, pet and bird food, chicken coops and compost heaps [29, 31-34].

Rats are omnivores that can eat a large variety of food resources, in contrast to voles (e.g., bank voles and field voles) and mice (e.g., house mice and wood mice), whose diets mostly consists of more natural food resources such as plants, or plants and invertebrates, respectively [35-37]. Water resources are especially important for rats, which require daily access to fresh water for their survival [30]. In contrast, house mice often do not require access to water, depending on the water content of their diet [38]. Rodent shelter can vary from natural soil for making burrows to woodpiles, old or poorly maintained buildings or sheds, or even the sewers in case of brown rats [29, 32-34, 39].

Depending on resource availability, rodents may reproduce year round in urban environments. However, there seems to be some variation in reproductive activity linked to season, with peaks in spring and fall [40-42]. Rats have on average five litters per year with four up to eight young per litter, and mice may have a new litter every 20-30 days with about 6 young per litter [40-43]. The offspring of rats and mice can already reach reproductive maturity after three months and 40 days, respectively [42, 44], which can result in exponential growth of rodent populations when the conditions are favorable.

Most rodents do not live longer than one or a few years, which is likely caused by a combination of resource limitation and interspecific competition rather than predation [28, 40, 44-46]. However, other research suggests that populations of mice, voles and shrews are affected more by top-down control (e.g., by predators), whereas populations of rats are controlled bottom-up (e.g., by limitation of food resources) [47]. While cats, foxes, martens and bird of prey may prey on rodents [48, 49], they do not seem to be able to sufficiently reduce population numbers for effective biological control [44] except for cats, which are able to reduce the abundance of smaller rodents such as mice and voles [50-52]. Still, the extent of rodent predation by natural predators remains unclear, partly because carnivores in urban areas tend to shift their diet to a wider range of food resources. For example, more than half of the stomach contents of red foxes in Switzerland was from anthropogenic origin [53]. The ample and easy accessible food resources in urban areas do not require these animals to show their natural hunting behavior [54], which may limit predation on rodents.

Potential effects of urban greening on rodents and rodent-borne zoonotic pathogens

Cities are constantly changing environments, driven by human adaptations. The increased urbanization in the last century was accompanied by negative side-effects, such as air pollution, water pollution and heat-island effects [55], for which countermeasures are increasingly being implemented. Urban greening is one of those measures that improves air and water quality, makes cities more resilient to climate change, and improves mental and physical health [56-58]. Urban greening is the process of creating more greenspace in cities, for example by planting more or replacing paved areas with trees and shrubs.

Besides the aforementioned beneficial effects, urban greening also helps to increase urban biodiversity by creating more suitable habitat for wildlife [59-62]. If this positive effect holds true for all urban wildlife species, urban greening will also increase the abundance of unwanted or pest species, such as wild rats or house mice. While a few studies hint at

a positive relationship between the presence of urban green space and the abundance of rodents, specifically wild rats [32, 39], the extent of the relationship between urban greening and the abundance of rodents has not been thoroughly investigated yet.

In addition, little is known about the deleterious effects urban greening might consequently have on wildlife-borne zoonotic pathogens. An increase in the abundance of rodents might increase the transmission of zoonotic pathogens via density-dependent transmission between rodents. This may result in higher numbers of infected rodents and thus a higher zoonotic disease hazard [63, 64]. However, pathogen transmission may vary depending on the location or pathogen considered [65, 66]. For instance, not all pathogens are transmitted in a density-dependent manner. Transmission could also be frequency-dependent, meaning that pathogen transmission increases with the proportion of infected animals in the population [67]. When the rodent population mainly consists of young individuals, this may result in a lower pathogen prevalence because those individuals have had less time to be exposed to pathogens, may be protected by maternal antibodies, or may express less behavior related to increased risk of infection, such as mating or fighting, compared to older individuals [68-71].

Potential effects of urban greening on urban wildlife, vectors and disease dynamics

If urban greening increases the total wildlife biodiversity within cities, this may result in a higher diversity of potential reservoir hosts for pathogens, which are not necessarily competent reservoir hosts (i.e., hosts that participate significantly in the circulation of the pathogen in nature) [72]. This may lead to the so-called "dilution effect", in which the presence of incompetent reservoir hosts dilutes the effect of competent reservoir hosts, resulting in an overall lower pathogen prevalence and diversity and thereby reducing the disease risk [73-75]. On the other hand, an increase in the abundance and diversity of wildlife species in urban areas and their overlap in space use with other (wild and domestic) animals and humans, might lead to an increased risk of pathogen spillover [14, 76, 77]. In addition, a higher wildlife biodiversity might alter pathogen transmission cycles in urban areas by using different hosts (e.g., domestic animals) compared to natural settings [78-80].

Urban greening might also attract more natural predators of rodents, which might reduce rodent populations and thus the zoonotic disease hazard [48, 49]. However, the extent of rodent predation by wild predators in urban areas remains unclear [54]. In addition, predator species can also host a wide range of zoonotic pathogens, which would not necessarily reduce the overall zoonotic disease risk, and which could even increase the risk of introduction of new zoonotic pathogens into urban areas [81-83].

Additionally, urban greening may increase the survival and abundance of vectors, such as ticks and fleas, by providing appropriate microclimatic conditions (e.g., temperature and humidity), which enhances their survival [84-87]. This, in turn, might increase the infection prevalence of tick-borne pathogens in rodents. However, some tick-borne pathogens require the presence of a larger wildlife mammal to complete their lifecycle [88-90], of which the abundance might still be too low in urban areas [91, 92]. Urban greening

may also increase the survival of pathogens in the environment by mediating extreme temperatures and increasing humidity and moisture levels. For example, *Leptospira* bacteria can persist in contaminated water or soil for a few hours to days or even weeks if conditions are favorable [93].

Rodent-borne zoonotic disease surveillance

Surveillance is the key tool of governmental organizations, like the Dutch National institute for Public Health and the Environment (RIVM), to give direction for measures that prevent and control infectious diseases. According to ECDC, "*Surveillance of health and disease includes ongoing data collection, analysis to convert this data into statistics, interpretation of this analysis to produce information and dissemination of this information to those who can take appropriate action*." Countries that perform disease surveillance are more likely to detect the presence of infectious diseases, better understand the underlying disease dynamics, are better prepared, and can rapidly take appropriate measures [94]. For many infections, such as measles, sexually transmitted diseases, certain antibiotic resistant bacteria, and tick-borne diseases, surveillance systems are in place in the Netherlands [95, 96].

For rodents and rodent-borne diseases, the Dutch surveillance system is currently very basic. Monitoring of rodents and rodent-borne zoonotic pathogens is mostly performed on an ad hoc basis (e.g., reactive sampling when human cases arise or sampling in high risk areas) or by using convenience sampling (e.g., testing rodents that are captured for other research aims or captured as by-catch). Some municipalities have a monitoring system for rats and house mice in which they monitor citizen nuisance complaints about rats and house mice. However, this is not 100 % representative of the actual situation due to, for example, biases in filing complaints [97, 98]. The current way of monitoring results in a patchy and incomplete overview.

Surveillance often starts with monitoring activities, in which monitoring refers to the collection of data over a defined period of time. Based on the perceived threat resulting from these monitoring activities, the designated public health authority will make a prioritization and decide whether to take action and in what form (e.g., take control measures, make contingency plans, change legislation or intensify monitoring; Figure 1) [99]. The implemented actions or interventions will subsequently be monitored. This monitoring can focus on different parts, depending on the focus of the action or intervention. In light of the One Health approach, monitoring should focus on humans, animals and the influence of the environment (Figure 1). Based on the outcomes of these monitoring activities, new prioritizations will be made, and actions or interventions will be adjusted accordingly, which will again be monitored, and so on.

Outline of this thesis

To prevent increased disease transmission to humans in urban areas, it is important to get more insight in rodent-borne zoonotic disease risks, and how this may be affected by urban greening. In this thesis, the overall main question is: "**What are the public health risks of rodent-borne zoonoses in urban environments?"**. To assess these risks, both pathogen exposure and disease hazard need to be investigated. In this thesis, we only

focused on disease hazard, by investigating the hosts (i.e., rodent populations), zoonotic pathogens, in relation to the urban environment (Figure 1 and 2). We aim to answer the following sub questions: "**Which zoonotic pathogens can be found in urban rodents?**" and "**How does urban greening influences rodent-borne zoonotic disease hazard?**". We specifically focused on brown rats, but we also included data about other rodents to get a broader overview. These results will be used to answer the sub question: **"How can rodent-borne disease surveillance be improved?"**.

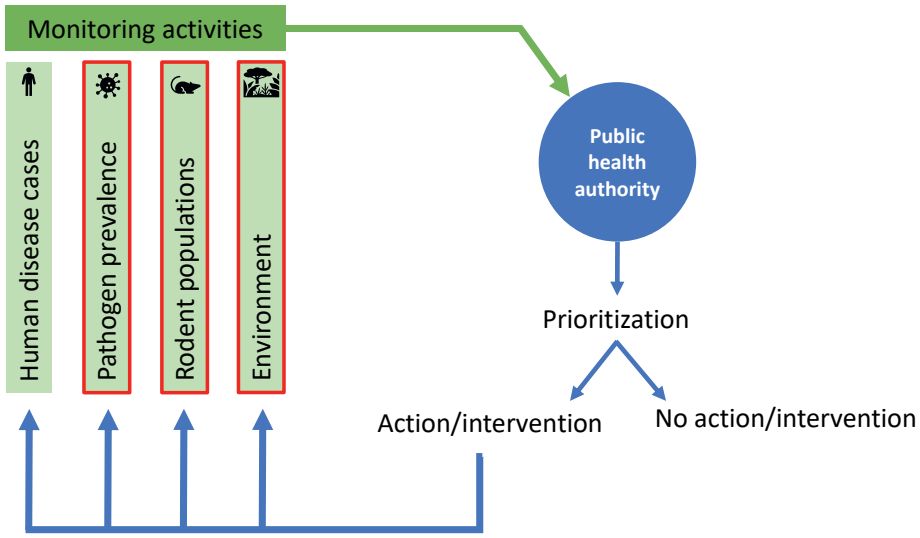

Figure 1. *Simplified scheme for rat-borne zoonotic disease surveillance system, adapted from Braks et al., 2011. The monitoring activities outlined in red are the ones this thesis focuses on.*

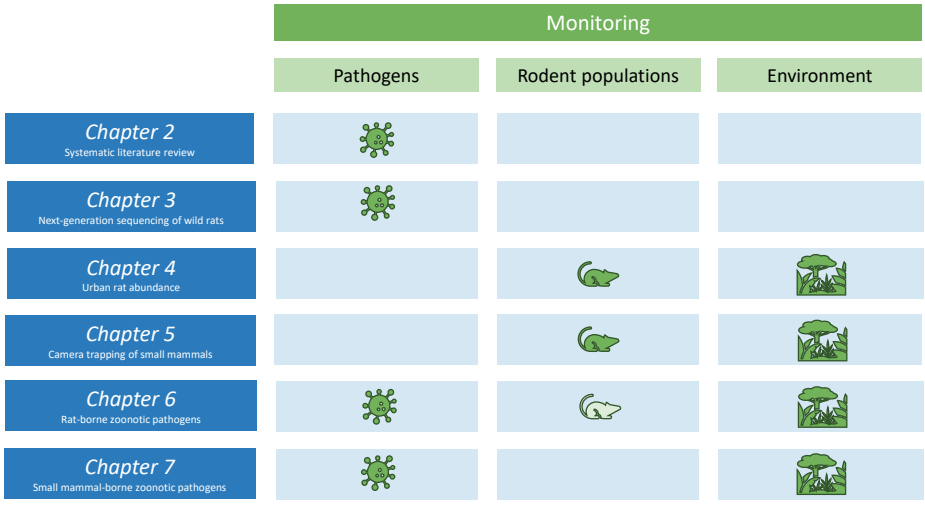

Figure 2. *Overview of the outline of this thesis.*

To assess the public health risks and to improve the surveillance of rodent-borne diseases in urban areas, we need to know what the current situation is. While urban greening increases wildlife biodiversity in cities, which could increase the risk of zoonotic disease transmission to humans, there is relatively little research performed on zoonotic pathogens in wildlife from urban areas. We therefore performed a systematic literature review on zoonotic pathogens studied in 10 common urban wildlife mammals, including rodents such as brown rats, house mice, wood mice and common voles, in **Chapter 2**. We aimed to identify knowledge gaps that can be used to better assess the public health risks and to improve both rodent-borne and wildlife-borne zoonotic disease surveillance.

Before testing wild rodents for zoonotic pathogens, we assessed how pathogen detection can be best performed. Therefore, in **Chapter 3** we investigated the added value of using next-generation sequencing (NGS) to the more traditional molecular methods that are currently mostly used. One of the advantages of these methods is that they can detect almost any pathogen present, whereas more conventional methods, such as PCR, can only detect those pathogens that you are specifically looking for. The use of NGS could facilitate the detection of unexpected or emerging pathogens.

Since changes in the urban environment could affect disease hazard by altering rodent densities and pathogen transmission, we investigated the relationships between urban greenness and both rodent abundance and the prevalence and diversity of rodent-borne zoonotic pathogens. While a few studies hint at a positive relationship between the presence of urban green space and the abundance of wild rats [32, 39], the extent of this relationship has not been thoroughly investigated yet. In **Chapter 4**, we conducted a field study in the cities of Amsterdam, Rotterdam and Eindhoven, in which we systematically trapped wild rats using snap traps in locations with varying degrees of urban greenness. We modelled the relationships between the relative abundance of wild rats and the degree of urban greenness and various other environmental and socio-economic explanatory variables.

Since the use of snap traps for estimating relative rat abundance has some disadvantages, we also investigated the relationship between urban greenness and small mammals in a different way to verify our results from Chapter 4. We looked at the relationships between the degree of urban greenness and the presence and occupancy of small mammals (i.e., rats, mice, voles and shrews) in private gardens in the Netherlands using camera trapping data in **Chapter 5**. In this study, we also included small mammal predators (i.e., domestic cats, dogs, mustelids and red foxes) as predictor variables in the models, in addition to various environmental and socio-economic variables.

Although wild rats are known to host a wide variety of zoonotic pathogens (Chapter 2), we have only little information about the current infection status of wild rats in urban areas in the Netherlands, and whether this could increase by urban greening. In **Chapter 6**, we studied the effect of urban greenness on the prevalence and diversity of rat-borne zoonotic pathogens. In this study, we also combined the rat-borne zoonotic pathogen prevalence data with the data on rat abundance from Chapter 4, which we used to calculate the rat-borne zoonotic disease hazard in relation to urban greenness.

To broaden our view on the effects of urban greening on zoonotic pathogens beyond wild rats, and to look at potential common patterns and pathogen spillover events between wildlife species, we also investigated the effects of urban greening on zoonotic pathogens transmitted by other rodent species (i.e., mice and voles) in **Chapter 7**. Additionally, we also examined differences in the prevalence and diversity of zoonotic pathogens between mice and voles from urban and nature areas. An overview of the outline of this thesis and the connection between chapters can be found in Figure 2.

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Café

Chapter 2

WILDbase: towards a common database to improve wildlife disease surveillance in Europe

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Abstract

Background: To be better prepared for emerging wildlife-borne zoonoses, we need to strengthen wildlife disease surveillance.

Aim: To create a topical overview of zoonotic pathogens in wildlife species to identify knowledge gaps and opportunities for improvement of wildlife disease surveillance.

Methods: We created a database, which is based on a systematic literature review in Embase focused on zoonotic pathogens in ten common urban wildlife mammals in Europe, namely brown rats, house mice, wood mice, common voles, red squirrels, European rabbits, European hedgehogs, European moles, stone martens, and red foxes. In total, we screened 7,403 articles of which 1,047 articles were included.

Results: In total, 186 zoonotic pathogen species were described, including 88 bacteria, 42 helminths, 19 protozoa, 22 viruses, and 15 fungi. Most of these pathogens were only studied in one single animal species. Even taking into account that some pathogens are relatively species-specific, many European countries have no (accessible) data on zoonotic pathogens in these relevant animal species. We used the Netherlands as an example to show how this database can be used by other countries to identify wildlife disease surveillance gaps on a national level. Only four percent of all potential host-pathogen combinations have been studied in the Netherlands.

Conclusions: This database comprises a comprehensive overview that can guide future research on wildlife-borne zoonotic diseases both on a European and national scale. Sharing and expanding this database provides a solid starting point for future European-wide collaborations to improve wildlife disease surveillance.

Introduction

Over 60 % of all emerging infectious diseases are zoonotic, and of those, over 70 % originate from wildlife [1]. Emerging wildlife diseases are a result of spill-over events between wildlife and humans [2], and they seem to become more prominent, often due to increased contact rates between humans and wild animals, in part driven by habitat loss and/or modification, increased urbanization, changes in agricultural practices and the globalization of trade and travel [3-5]. The health, economic and societal consequences of zoonotic spill-over events from wildlife can be far-reaching, as exemplified by the recent COVID-19 pandemic [6-8].

Urban areas can be considered artificial ecosystems that provide ample opportunity for zoonotic pathogen spill-over between wildlife, domestic animals and humans, and they can therefore form hotspots of increased zoonotic disease risk [9, 10]. This is due to the close contact between humans and wildlife, and the altered wildlife composition in urban areas compared to nature areas, which could result in new spill-over events between wildlife species [2, 5, 11]. This in turn could lead to different pathogen transmission cycles and disease dynamics in urban areas and consequently to higher exposure rates to zoonotic pathogens [12-14]. Moreover, changes to the urban environment such as urban greening, that promote biodiversity [15-18], could further alter disease dynamics in urban areas. These complex interactions underline the importance of broadening wildlife disease surveillance by shifting the focus from one to multiple animal species and pathogens to increase our understanding of pathogen transmission cycles in urban areas, and to identify potential pathogen spill-over between wildlife species.

The different disease dynamics and increased risk of zoonotic disease transmission in urban areas compared to nature areas require an improvement of wildlife disease surveillance [2]. However, surveillance of wildlife diseases in urban areas can be challenging and most wildlife studies are performed outside of urban areas [19-21]. To facilitate wildlife surveillance in urban areas, having a comprehensive overview of zoonotic pathogens studied in urban wildlife species to determine how to set-up this surveillance will help to prioritize monitoring efforts.

While there are many studies investigating zoonotic pathogens in wildlife species, there is no common database where this information is systematically collected and stored. This study aims to improve the surveillance and cross-country collaboration of wildlife-borne zoonoses, especially for, but not limited to, urban areas, by creating such a comprehensive and easily accessible database. To create this database, we performed a systematic literature review of zoonotic pathogens studied in ten common urban wildlife mammals. We present and summarize knowledge gaps in the monitoring of wildlife-borne zoonotic pathogens in Europe per pathogen, animal species, country and habitat type. Also, we used the Netherlands as an example to demonstrate how this database can be used by other countries to identify their current knowledge gaps in wildlife disease surveillance. These knowledge gaps deserve more attention because they could indicate potential pathogen spill-over between species or the presence of multiple competent host species. The complete database underlying this study, named "WILDbase", is publicly available at www.wildbase.org and in Appendix 1.

Materials & Methods

In this review we considered zoonotic pathogens comprising bacteria, viruses, helminths (including Annelida, Platyhelminthes, Nematoda and Acanthocephala), unicellular eukaryotes (hereafter called protozoa, and including Apicomplexan parasites) and fungi. Ectoparasites were excluded. This review was not registered beforehand.

Selection of wildlife species

We selected ten common urban wildlife mammal species, based on their presence in Dutch cities. The majority of these species also occurs in most other European countries (Figure S1). For this, we used data from the Dutch National database for Flora and Fauna [22] in combination with expert opinions from urban ecologists from six of the larger Dutch cities (i.e. Amsterdam, Utrecht, Nijmegen, Eindhoven, Maastricht and Enschede). This led to the following list of ten common urban mammal species (excluding flying mammals, i.e. bats), in random order: brown rat (*Rattus norvegicus*), house mouse (*Mus musculus*), wood mouse (*Apodemus sylvaticus*), common vole (*Microtus arvalis*), red squirrel *(Sciurus vulgaris*), European rabbit (*Oryctolagus cuniculus*), European hedgehog (*Erinaceus europaeus*), European mole (*Talpa europaea*), stone marten (*Martes foina*), and red fox (*Vulpes vulpes*).

Search strategy

We conducted a systematic literature search according to the PRISMA guidelines to identify articles using the Embase database [23, 24]. Search terms included keywords related to (1) the Latin species name and any equivalent English names, AND (2) terms for 'zoonosis', and also (1) AND (3) an elaborate list of 86 important emerging zoonotic pathogens compiled by order of the Dutch government [25, 26]. The search terms included "wildcards" to capture term variations (e.g., zoono*). The search terms used per animal species can be found in Table S1. We included all articles published before 01-01-2023, which resulted in a total of 7,403 articles for initial consideration.

Article screening and exclusion

First, we performed a title and abstract screening, which excluded 5,649 out of 7,403 articles, followed by a full-text screening, which excluded 707 out of 1,754 articles, resulting in a total of 1,047 articles that were used for data extraction (Figure 1). All articles were assessed by two independent reviewers, or three in case of disagreement between the first two reviewers. For studies to be included, they needed to research: a) one of the ten mammal species of interest, b) wild animals (i.e., not pet or laboratory animals), and c) zoonotic pathogens tested in those animals. A pathogen species was considered zoonotic when we could find at least one case of human infection with the pathogen in scientific literature. In case a pathogen was only identified to genus level, but not to species level, information on zoonotic potential could often not be determined. Therefore, a pathogen genus was considered 'zoonotic' when a zoonotic species belonging to that genus was also detected at least once in the same animal species in Europe. A pathogen genus was considered 'potentially zoonotic' when no specific zoonotic species belonging to that genus had been detected in the same animal species in Europe until 2023, although that genus can contain zoonotic species. Exclusion criteria included: a) research performed outside of Europe (a

list of included European countries can be found in Table S2), b) article does not contain original data (e.g., a review or modelling paper using published data), c) species-specific pathogen data could not be extracted from the article (e.g., different rodent species were grouped together and only the total prevalence of that group was reported), d) article was not peer-reviewed (e.g., a preprint or conference abstract), e) full article is written in a language other than English, f) duplicated article, and g) no full text available of the article (mainly the case for older articles). An overview of the article screening process is visualized in Figure 1.

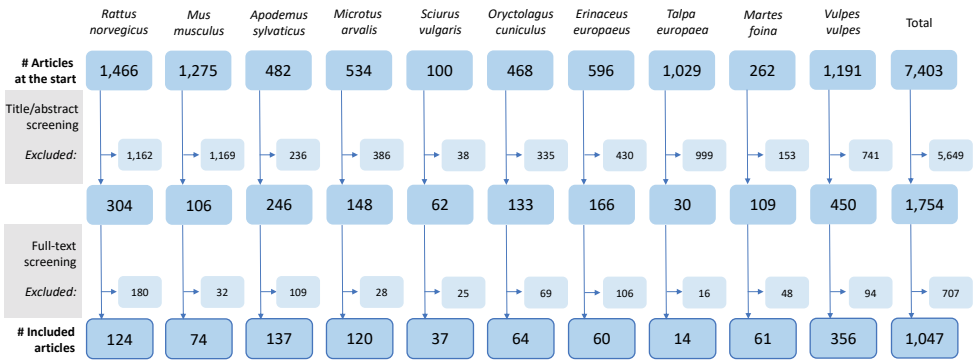

Figure 1. *Overview of the article screening process: initial number of articles after literature search in Embase, number of articles excluded after title/abstract screening, number of articles subjected to full-text screening, number of articles excluded after full-text screening, and the total number of included articles per animal species.*

Data extraction and processing

We extracted the following data from the articles: pathogen species (or genus, in case the pathogen was not determined to species level), tissue(s) sampled, laboratory methods used, mammal species studied, total number of animals tested, number of animals testing positive, country, publication year, and habitat type of trapping. Habitat type was classified into urban (including peri- and suburban areas), rural, agriculture (including animal farms, crop farms and fields), nature, or other (e.g., wild animals in an animal rescue center). This classification was made based on the habitat information provided in the articles. When animals were trapped in multiple habitat types, but the results were not reported per habitat type separately, we classified the habitat type as 'mixed'. In case no or too few details were given about the habitat type to be classified with certainty into one of the categories, we classified the habitat type as 'unspecified'. Some older articles contained obsolete country names (e.g., Yugoslavia), which we changed to current country names by matching them with the exact trapping locations.

To determine the completeness of wildlife disease surveillance on a European or national level, we calculated the 'coverage' by dividing the number of studied host-pathogen combinations by the total number of possible host-pathogen combinations, multiplied by 100. The distribution of animals in Europe was retrieved from the International Union for Conservation of Nature's Red List of Threatened Species website [27]. We used R version 4.3.1 (21-09-2023) for the creation of distribution maps [28].

Results and discussion

Data overview

To identify gaps and opportunities to improve wildlife disease surveillance, we created a database of zoonotic pathogens in ten common urban wildlife mammals in Europe ("WILDbase", accessible at www.wildbase.org and in Appendix 1), based on a systematic review of the literature. In total, 1,047 articles were included, providing data on 102 zoonotic pathogen genera, 21 potentially zoonotic pathogen genera, and 186 zoonotic pathogen species (Table 1 and Table S3). Table S3 gives an overview of all studied zoonotic pathogens in the selected ten wildlife species and visualizes all current knowledge gaps per pathogen group, genus and species, and per animal species. As an example, we provide a shortened version of this table focusing only on viruses in Table 1. Most studied pathogens belonged to the group of bacteria (44 genera, 88 species, and 459 studies),

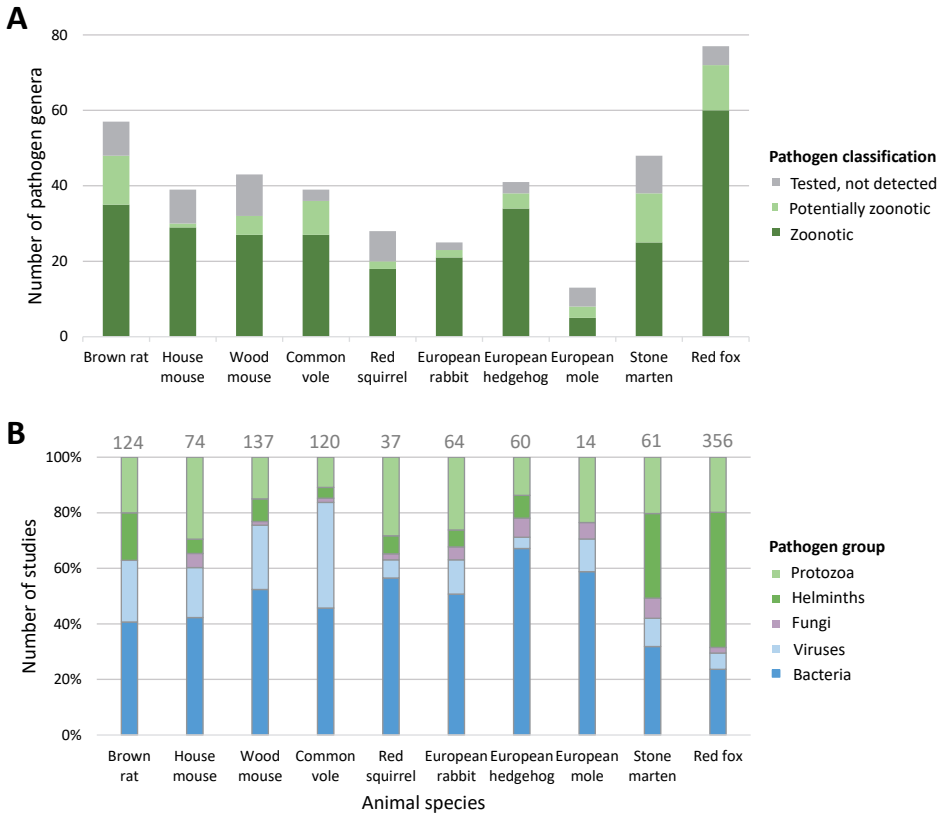

Figure 2. *A. Number of pathogen genera detected per animal species. Pathogen genera are classified as zoonotic, potentially zoonotic, or tested and (potentially) zoonotic but not detected. All zoonotic and potentially zoonotic genera were also detected in the specific animal species. B. Relative number of studies performed on each pathogen group (e.g., bacteria, viruses, helminths, protozoa and fungi) per animal species. The number above each bar shows the total number of studies per animal species.*

followed by helminths (37 genera, 42 species, and 270 studies), protozoa (11 genera, 19 species, and 223 studies), viruses (20 genera, 22 species, and 174 studies), and lastly fungi (11 genera, 15 species, and 31 studies; Figure 1-2, Table 1 and Table S3). This resembles the order in which these pathogen groups cause zoonoses globally, except for viruses, which are in second place globally versus fourth place in this study [29]. This suggests that there is an underrepresentation of studies focusing on viruses in these wildlife species in Europe.

Coverage of the database

The total coverage for all host-pathogen combinations in this database was 26 %, meaning that only a quarter of all potential host-pathogen combinations from Table S3 have been studied in Europe so far (Table S4). The coverage ranged from 19 % for helminths and fungi, up to 37 % for protozoa (Table S4). Per animal species, coverage ranged from 7 % for European moles to 57 % for red foxes (Table S4). This shows that there is a large surveillance bias regarding both animal species and pathogens, which hinders comparisons of relative zoonotic disease risk posed by these different animal species [29]. Therefore, these understudied pathogens and animal species need more attention.

Host specificity or surveillance specificity?

Most zoonotic pathogens were studied in only a single animal species (54 %), and only 11 % of all pathogens (mostly bacteria and protozoa) were studied in five or more different animal species (Figure S2). For some of those host-pathogen combinations the absence of research might be related to host specificity [30], or the current absence of the pathogen or its vector in a specific area due to environmental or climatic conditions [31-33]. However, since the majority of pathogens can infect more than one host species [30], most of these pathogens likely have not been tested in multiple animal species yet, while they could infect multiple hosts. *Toxoplasma gondii* was detected in all ten animal species (Figure S2 and Table S3), which shows that multiple animal species can serve as (accidental) reservoir host. Likewise, *Leishmania infantum*, *Francisella tularensis* and *Anaplasma phagocytophilum* have been detected in eight different animal species (Figure S2 and Table S3). In contrast, for example *Corynebacterium ulcerans*, *Proteus mirabilis*, *Streptococcus suis* and *Sindbis virus* have only been studied in one animal species. While some pathogens or host-pathogen combinations have been studied extensively, others have just been studied once. Pathogen detection is sensitive to research effort, which may cause some poorly studied species to be misclassified as non-hosts. This highlights the importance of, and opportunities for, monitoring a broader spectrum of wildlife species to detect pathogen spill-over and potentially new, unexpected reservoir hosts.

Differences between environments

Pathogen transmission can differ per environment, for example due to the availability of suitable hosts for efficient transmission [13, 34]. Urban and nature environments are very different in terms of animal species composition, abundance, and richness [35], which may affect contact rates between animal species and pathogen transmission cycles. We observed that in > 60 % of the studies the type of environment (e.g., urban, rural,

2

Table 1. *Overview of studied (potentially) zoonotic viruses in different wildlife species, showing the number of studies in which the pathogen was detected/the total number of studies performed. Where no studies have been performed for a specific host-pathogen combination (i.e., a knowledge gap), the cell is empty. Detected pathogens are highlighted in green. Table S3 shows the results for bacteria, protozoa, helminths and fungi. An orange dot represents host-pathogen combinations not studied in the Netherlands. An asterisk indicates host-pathogen combinations studied in the Netherlands but without any positive results.*

			Animals			
	Order		*Rodentia*			
	Family		*Muridae*			*Cricetidae*
	Genus	**Species**	**Brown rat**	**House mouse**	**Wood mouse**	**Common vole**
Viral pathogens	*Alphainfluenza-virus*	*Influenza A virus*	0/1 •			
	Alphavirus	*Sindbis virus*				
	Aphthovirus	*Foot and mouth disease virus*				
	Betacoronavirus	*SARS-CoV-2*	0/1 •	0/1 •	0/2 •	0/1 •
	Cardiovirus	*Encephalo-myocarditis virus*	0/1 •			
	Coltivirus	*Eyach coltivirus*			0/1	
	Flavivirus	spp	0/1 •		0/1 •	
		Tick-borne encephalitis virus		0/1 •	2/4*	4/4
		Usutu virus	0/1 •			
		West Nile virus	0/1 •		0/1 •	
	Kobuvirus	spp	1/1 •			
	Lyssavirus	*European bat lyssavirus*				
		Lyssavirus rabies				1/2 •
	Mammarena-virus	*Lymphocytic choriomeningitis mammarenavirus*		4/4 •	5/6 •	3/3 •
	Norovirus	spp	4/4 •			
	Orthobornavirus	*Borna disease virus*				
	Orthohantavirus	spp	4/5 •	2/2 •	3/6 •	5/7 •
		Dobrava-Belgrade orthohantavirus		0/1 •	2/8 •	2/2 •
		Puumala orthohantavirus		2/3 •	3/10*	1/3 •

	Lago-morpha	*Eulipotyphla*		*Carnivora*		
Sciuridae	*Leporidae*	*Erinaceidae*	*Talpidae*	*Mustelidae*	*Canidae*	Detected in # animal species
Red squirrel	**European rabbit**	**European hedgehog**	**European mole**	**Stone marten**	**Red fox**	
		0/1 •		1/2 •	3/4	2
		1/1 •				1 •
		1/1 •				1 •
				1/2	0/2 •	1
						0 •
	0/1 •					0
						0 •
0/1 •		1/1 •	2/2 •	0/1 •	1/2 •	5
						0 •
		1/1 •			1/2 •	2 •
					1/1 •	2 •
				1/1 •		1 •
				1/2 •	3/8 •	3 •
					1/1 •	4 •
						1 •
				0/1 •	2/4 •	1 •
					2/2 •	5 •
						2 •
					1/1 •	4

Table 1. *continued*

	Genus	Species	Brown rat	House mouse	Wood mouse	Common vole
Viral pathogens	*Orthohantavirus*	*Seoul orthohantavirus*	9/10	0/1 •		
		Tula orthohantavirus		0/2 •	2/6*	25/26
	Orthohepevirus	spp	1/1 •			2/2 •
		Hepatitis E virus	10/12•	0/1 •	0/1 •	
	Orthonairovirus	*Crimean-congo haemorrhagic fever virus*			0/1 •	
	Orthopoxvirus	spp	0/1 •	1/1 •	4/7 •	2/2 •
		Cowpoxvirus	3/4	1/1 •	3/3 •	3/3 •
	Parechovirus	*Ljungan virus*		1/1 •	1/1 •	
	Phlebovirus	spp				
	Rotavirus	spp	2/2 •			
	Varicellovirus	*Pseudorabies virus*				
	# Viral pathogens detected		8	6 •	9	10

agriculture and nature) was not specified (Figure S3), which hampers (risk) comparisons between environments. Despite the presumed increased zoonotic disease risks in urban areas due to increased contact between humans and animals [9, 29, 36], only few studies (< 10 %) have been performed in urban areas, and of those, almost half were conducted on brown rats (Figure S3). For example, *Cryptosporidium parvum*, *Taenia martis* and *Enterocytozoon bieneusi* have not been studied in urban areas. This highlights the need for studies to better specify where tested animals are from, and to increase the testing of animals from urban areas.

Differences between European countries; the Netherlands as an example

There are large differences in surveillance effort between European countries, both per pathogen group and per animal species (Figure 3 and Figure S1). The total surveillance effort per country ranges from zero studies per country (for Albania, Kosovo and Moldova) to > 100 (for Spain, the United Kingdom and Germany; Figure 3). These differences are likely due to country-specific variation in resource allocation for disease-related research [37], and to different perceived zoonotic risks from wildlife to humans. To demonstrate how this database could be used by other countries to identify their current knowledge gaps in wildlife disease surveillance, we highlighted the gaps for the Netherlands in Table 1 and Table S3 using orange circles (•). An orange circle indicates that while that specific pathogen has been studied in a specific animal species in Europe, none of those studies were performed in the Netherlands. As a result, even more knowledge gaps arise on a national level, and the coverage decreases from 26 % on European level to

Red squirrel	European rabbit	European hedgehog	European mole	Stone marten	Red fox	Detected in # animal species
						1
						2
						2 •
	5/6				2/4 •	3
	0/1 •					0 •
						3 •
						4
1/1 •						3 •
					1/1 •	1 •
0/1 •					2/2 •	2 •
					1/1 •	1 •
1 •	1	4 •	1 •	4	13	

only 4 % for the Netherlands. It is important to increase these percentages to identify potential additional host reservoir species to better understand disease dynamics and to prevent human infections. An example is tick-borne encephalitis virus (TBEV), which is an emerging zoonotic virus in the Netherlands [38]. Our overview shows that TBEV has already been detected in five different animal species, but we only tested for TBEV in two of those species in the Netherlands (Table 1). In this way, TBEV could infect humans via other animal species than we might expect, which shows the importance of increasing the coverage of knowledge gaps by testing multiple animal species.

Stepwise approach to start filling knowledge gaps

Deciding where to start with filling the knowledge gaps (e.g., focusing on a specific pathogen or animal species) can be difficult and varies per country. We suggest to first focus surveillance on host-pathogen combinations that have been detected in other countries. Secondly, the focus could be broadened to host-pathogen combinations that have been previously tested, yet not detected, but for which the presence of a pathogen cannot be ruled out based on the number of studies or tested animals, or the lack of current knowledge of host-pathogen potential. Thirdly, the focus could be on the remaining host-pathogen combinations that have not been studied yet, by first focusing on those pathogens that are likely to infect multiple animal species. However, another option is including a risk assessment, as some pathogens pose a greater risk to human health than others, which can give additional directions for wildlife disease surveillance programs. A risk assessment often includes parameters based on the (perceived) threat

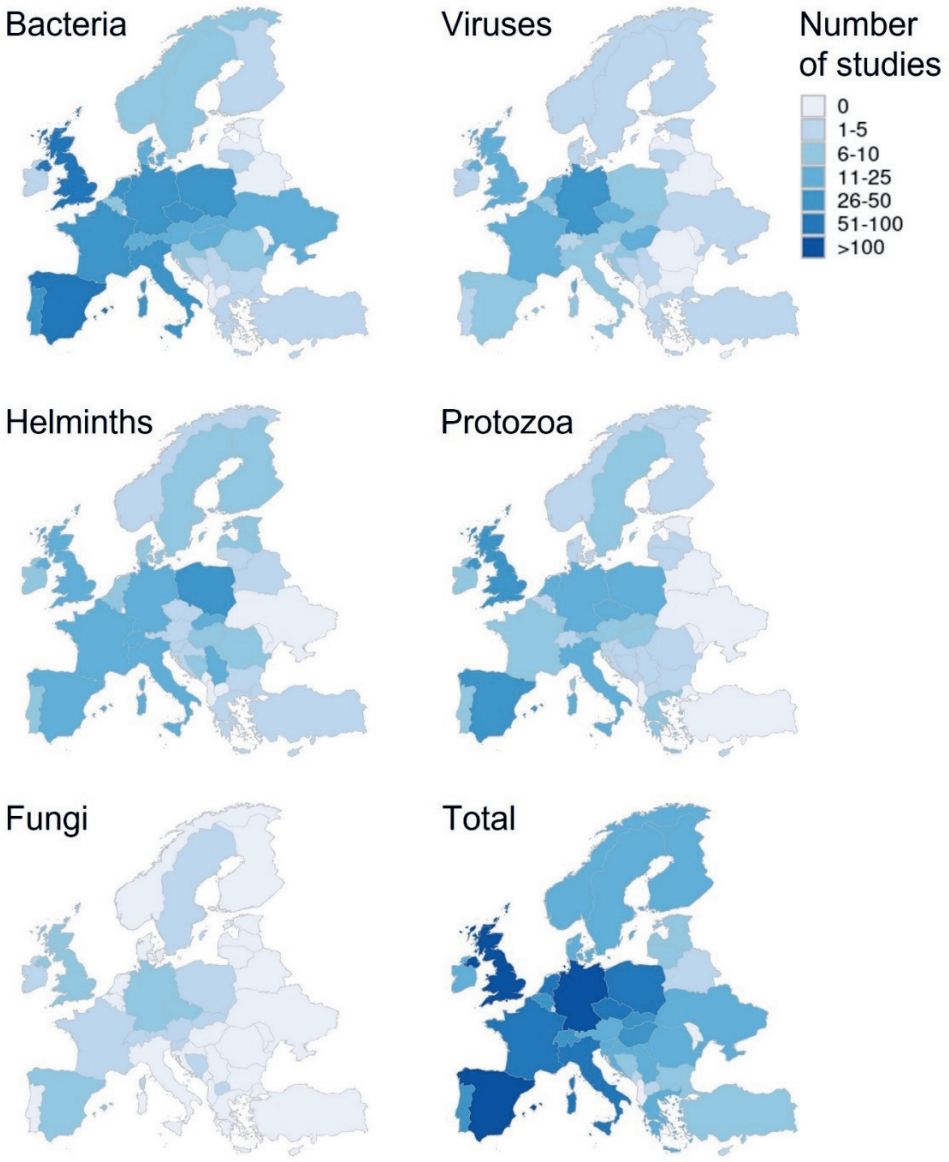

Figure 3. *Number of studies conducted per pathogen group per country (bacteria, viruses, helminths, protozoa, fungi, and total).*

posed by a pathogen to human and/or animal health, which, amongst other things, depends on pathogen prevalence in the animal population, the abundance of the animals, contact rates between humans and animals, pathogen transmission, infection prevalence in humans, and disease symptoms in humans [25]. However, these parameters may be difficult to assess. For example, there is a lack of reliable data on animal abundance. Having

a European database for wildlife abundance data, like VectorNet for the abundance and distribution of vectors in Europe [39], would be very useful. Besides that, the prevalence may vary depending on the test method used (e.g., PCR versus serology) and the type of tissue(s) tested (e.g., blood, organs or feces), which may differ not only per study, but also per pathogen. This complicates comparisons of relative zoonotic disease risk between pathogens.

In addition, the reliability of the observed prevalence is subject to the number of animals tested. We noticed that the average number of animals tested per study varies a lot; from 21 animals tested per study for European moles, to an average number of 465 for red foxes (Figure S4). This is partly related to the type of pathogens tested and the abundance of the animal species. For example, non-invasive fecal samples can be easier to collect and therefore result in higher sample sizes compared to organ or blood samples, which also often require ethical approval [40]. Furthermore, especially for endangered or protected animal species, samples sizes may be very low, which makes it even more important to make efficient use of the available animal samples. From the ten animal species in this study, the European rabbit is the only one listed as 'near threatened' [27], while the average sample size of studies performed on European rabbits is higher than those performed on wood mice, brown rats, house mice, European hedgehogs, red squirrels, stone martens and European moles. A higher sample size not only makes the resulting prevalence more reliable, it also increases the chance of detecting pathogens that occur with low prevalence.

Database limitations

This systematic literature review resulted in a comprehensive, yet incomplete database of zoonotic pathogen research conducted in European wildlife species. One limitation is that we could have missed some relevant studies because our search was limited to the Embase literature database, or because of the search terms we used. For example, our search did not result in any studies about *Streptobacillus moniliformis* in wild brown rats in Europe, although brown rats are one of the main reservoir hosts [41]. All studies about *S. moniliformis* in brown rats were either performed outside Europe or performed on pet or laboratory rats. An additional search did result in one relevant study that was not included in our search because 'brown rat', '*Rattus norvegicus*' or an equivalent name from our search terms was not present in the title, abstract or keywords of that study [42]. A second limitation is that only few studies reported negative results, likely caused by publication bias of positive results [43]. However, the publication of negative results is very valuable, and might significantly enhance the coverage and accuracy of this database. Therefore, it is important to extend this database with multiple literature databases, and to have country-specific experts to validate and enhance the accuracy of this database.

The value of a common database

While many studies have investigated zoonotic pathogens in wildlife species, this database shows that there is still a large surveillance bias regarding both wildlife species and zoonotic pathogens, which results in numerous knowledge gaps on a European level, and even more gaps on a national level. This emphasizes the need for a European-

wide collaboration to improve zoonotic wildlife disease surveillance. A common topical database of published literature, for example an extension of "WILDbase" as presented here, preferably in combination with an online biobank for sharing animal samples, could help to localize and fill current knowledge gaps both on national and European level, improve wildlife disease surveillance, and justify or give direction to surveillance funding. As such, WILDbase would complement existing European networks such as EVD-Net (for emerging and vector-borne diseases) and EARS-Net (for antimicrobial resistance) [44]. In addition, such a database and biobank could result in more efficient use of animal samples (e.g., testing one sample for many pathogens), especially with regard to ethical requirements related to invasive research on wildlife [40], the limited number of samples from protected or endangered species, and the time needed to collect and analyze the samples.

Increasing our knowledge about potential reservoir hosts for zoonotic pathogens can not only help us to better understand pathogen transmission cycles and spill-over, but will raise awareness amongst health care professionals, which may result in better identification of human disease cases [45]. We encourage the usage and sharing of WILDbase by a broad range of European research institutes and professionals, as well as lay audiences. Expanding this comprehensive database (e.g., by consulting multiple literature databases, or by including more animal species) and keeping it up to date, can provide a solid starting point for future European-wide collaborations to improve wildlife disease surveillance.

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Supplementary data

Table S1. *Search terms per animal species. Per species, a combination of search terms (1) and (2), and of search terms (1) and (3) was used.*

(1) Species name	**Brown rat**	'rattus norvegicus*':ti,ab,kw OR 'brown rat*':ti,ab,kw OR 'norway rat*':ti,ab,kw OR 'norwegian rat*':ti,ab,kw OR 'common rat*':ti,ab,kw OR 'street rat*':ti,ab,kw OR 'sewer rat*':ti,ab,kw OR 'hannover rat*':ti,ab,kw OR 'wharf rat*':ti,ab,kw OR 'tunnel rat*':ti,ab,kw OR 'gutter rat*':ti,ab,kw OR 'rattus norvegicus'/exp OR 'brown rat'/exp OR 'norway rat'/exp OR 'norwegian rat'/exp OR 'common rat' OR 'street rat' OR 'sewer rat' OR 'hannover rat' OR 'wharf rat' OR 'tunnel rat' OR 'gutter rat' OR 'urban rat*':ti,ab,kw OR 'urban rat'
	House mouse	'house mouse*':ti,ab,kw OR 'house mice*':ti,ab,kw OR 'mus musculus*':ti,ab,kw OR 'house mouse'/exp OR 'house mice' OR 'mus musculus'/exp OR 'mus domesticus*':ti,ab,kw OR 'mus domesticus'/exp
	Wood mouse	'wood mouse*':ti,ab,kw OR 'apodemus sylvaticus*':ti,ab,kw OR 'field mouse*':ti,ab,kw OR 'wood mouse'/exp OR 'apodemus sylvaticus'/exp OR 'field mouse' OR 'wood mice':ti,ab,kw OR 'field mice':ti,ab,kw OR 'wood mice' OR 'field mice'
	Common vole	'common vole*':ti,ab,kw OR 'microtus arvalis*':ti,ab,kw OR 'field mouse*':ti,ab,kw OR 'field mice*':ti,ab,kw OR 'common vole' OR 'microtus arvalis'/exp OR 'field mouse' OR 'field mice' OR 'field vole'
	Red squirrel	'red squirrel*':ti,ab,kw OR 'sciurus vulgaris*':ti,ab,kw OR 'red squirrel' OR 'sciurus vulgaris'/exp
	European rabbit	'oryctolagus cuniculus*':ti,ab,kw OR 'european rabbit*':ti,ab,kw OR 'oryctolagus cuniculus'/exp OR 'european rabbit'/exp OR 'wild rabbit*':ti,ab,kw OR 'wild rabbit'
	European hedgehog	'hedgehog*':ab,ti,kw OR 'erinaceus europaeus*':ab,ti,kw OR 'hedgehog'/exp OR 'erinaceus europaeus'/exp
	European mole	'mole':ti,ab,kw OR 'talpa europaea*':ti,ab,kw OR 'moles':ti,ab,kw OR 'mole'/exp OR 'talpa europaea'/exp OR 'moles'/exp
	Stone marten	'marten*':ti,ab,kw OR 'beech marten*':ti,ab,kw OR 'martes foina*':ti,ab,kw OR 'stone marten*':ti,ab,kw OR 'house marten*':ti,ab,kw OR 'white breasted marten*':ti,ab,kw OR 'marten'/exp OR 'beech marten'/exp OR 'martes foina'/exp OR 'stone marten'/exp OR 'house marten'/exp OR 'white breasted marten'
	Red fox	'red fox*':ti,ab,kw OR 'vulpes vulpes*':ti,ab,kw OR 'red fox'/exp OR 'vulpes vulpes'/exp
(2) Emtree terms for 'zoonosis'	'zoono*':ti,ab,kw OR 'zoonosis'/exp OR 'zoonotic'	

Table S1. *continued*

(3) Emzoo list	'ehrlichi*':ti,ab,kw OR 'ehrlichia'/exp OR 'bartonell*':ti,ab,kw OR 'bartonella'/exp OR 'brucell*':ti,ab,kw OR 'brucella'/exp OR 'burkholderia*':ti,ab,kw OR 'burkholderia'/exp OR 'chlamydophil*':ti,ab,kw OR 'chlamydophila'/exp OR 'clostridi*':ti,ab,kw OR 'clostridium'/exp OR 'coxiell*':ti,ab,kw OR 'coxiella burnetii'/exp OR 'anaplasm*':ti,ab,kw OR 'anaplasma'/exp OR 'escherichia coli':ti,ab,kw OR 'escherichia coli'/exp OR 'salmonell*':ti,ab,kw OR 'salmonella'/exp OR 'yersin*':ti,ab,kw OR 'yersinia'/exp OR 'erysipelothrix':ti,ab,kw OR 'erysipelothrix'/exp OR 'capnocytophag*':ti,ab,kw OR 'capnocytophaga'/exp OR 'francisella':ti,ab,kw OR 'francisella'/exp OR 'tularem*':ti,ab,kw OR 'tularemia'/exp OR 'leptospir*':ti,ab,kw OR 'leptospira'/exp OR 'weil s disease':ti,ab,kw OR 'weil s disease'/exp OR 'mycobacteri*':ti,ab,kw OR 'mycobacterium'/exp OR 'pasteurell*':ti,ab,kw OR 'pasteurella'/exp OR 'rickettsi*':ti,ab,kw OR 'rickettsia'/exp OR 'borreli*':ti,ab,kw OR 'borrelia'/exp OR 'staphylococc*':ti,ab,kw OR 'staphylococcus'/exp OR 'streptococc*':ti,ab,kw OR 'streptococcus'/exp OR 'campylobacter*':ti,ab,kw OR 'campylobacter'/exp OR 'cryptococc*':ti,ab,kw OR 'cryptococcus'/exp OR 'ascari*':ti,ab,kw OR 'ascaris'/exp OR 'baylisascari*':ti,ab,kw OR 'baylisascaris'/exp OR 'toxocar*':ti,ab,kw OR 'toxocara'/exp OR 'fasciol*':ti,ab,kw OR 'fasciola'/exp OR 'dirofilaria*':ti,ab,kw OR 'dirofilaria'/exp OR 'echinococc*':ti,ab,kw OR 'echinococcus'/exp OR 'taenia*':ti,ab,kw OR 'taenia'/exp OR 'trichin*':ti,ab,kw OR 'trichinella'/exp OR 'bovine spongiform encephalopathy':ti,ab,kw OR 'bovine spongiform encephalopathy'/exp OR 'babesi*':ti,ab,kw OR 'babesia'/exp OR 'cryptosporidi*':ti,ab,kw OR 'cryptosporidium'/exp OR 'giardi*':ti,ab,kw OR 'giardia'/exp OR 'toxoplasm*':ti,ab,kw OR 'toxoplasma'/exp OR 'leishmani*':ti,ab,kw OR 'leishmania'/exp OR 'lymphocytic choriomeningitis':ti,ab,kw OR 'lymphocytic choriomeningitis virus'/exp OR 'batai virus':ti,ab,kw OR 'batai virus'/exp OR 'bhanja virus':ti,ab,kw OR 'bhanja virus'/exp OR 'california encephalitis':ti,ab,kw OR 'california encephalitis'/exp OR 'crimean-congo hemorrhagic fever':ti,ab,kw OR 'crimean-congo hemorrhagic fever virus'/exp OR 'dobrava-belgrade':ti,ab,kw OR 'dobrava':ti,ab,kw OR 'dobrava-belgrade virus' OR 'erve virus':ti,ab,kw OR 'erve virus' OR 'puumala':ti,ab,kw OR 'puumala virus'/exp OR 'rift valley fever':ti,ab,kw OR 'rift valley fever virus'/exp OR 'seoul virus':ti,ab,kw OR 'seoul virus'/exp OR 'hanta*':ti,ab,kw OR 'hantavirus'/exp OR 'tahyna virus':ti,ab,kw OR 'tahyna virus' OR 'encephalitis':ti,ab,kw OR 'encephalitis virus'/exp OR 'louping ill':ti,ab,kw OR 'louping ill virus'/exp OR 'rocio':ti,ab,kw OR 'rocio virus'/exp OR 'wesselsbron':ti,ab,kw OR 'wesselsbron virus' OR 'west nile':ti,ab,kw OR 'west nile virus'/exp OR 'hepatitis e':ti,ab,kw OR 'hepatitis e virus'/exp OR 'dhori virus':ti,ab,kw OR 'dhori virus' OR 'batken virus':ti,ab,kw OR 'batken virus' OR 'influenza':ti,ab,kw OR 'influenza'/exp OR 'thogoto virus':ti,ab,kw OR 'thogoto virus'/exp OR 'thogotovirus':ti,ab,kw OR 'thogotovirus'/exp OR 'ljungan virus':ti,ab,kw OR 'ljungan virus'/exp OR 'cowpox':ti,ab,kw OR 'cowpox virus'/exp OR 'monkeypox':ti,ab,kw OR 'monkeypox virus'/exp OR 'orf virus':ti,ab,kw OR 'orf virus'/exp OR 'colorado tick fever':ti,ab,kw OR 'colorado tick fever virus'/exp OR 'eyach virus':ti,ab,kw OR 'eyach virus' OR 'tribec':ti,ab,kw OR 'tribec virus' OR 'lyssavirus':ti,ab,kw OR 'lyssavirus'/exp OR 'rabies':ti,ab,kw OR 'rabies virus'/exp OR 'barmah forest':ti,ab,kw OR 'barmah forest virus'/exp OR 'ross river':ti,ab,kw OR 'ross river virus'/exp OR 'sindbis':ti,ab,kw OR 'sindbis virus'/exp OR 'anisakis simplex*':ti,ab,kw OR 'anisakis simplex'/exp OR 'plague':ti,ab,kw OR 'plague'/exp OR 'typhus':ti,ab,kw OR 'typhus'/exp OR 'rat bite fever':ti,ab,kw OR 'rat bite fever'/exp OR 'rat-bite fever':ti,ab,kw OR 'rat-bite fever'/exp OR 'haverhill fever':ti,ab,kw OR 'haverhill fever'/exp OR 'anthrax':ti,ab,kw OR 'anthrax'/exp OR 'bacillus anthracis':ti,ab,kw OR 'bacillus anthracis'/exp OR 'botulism':ti,ab,kw OR 'botulism'/exp OR 'listeri*':ti,ab,kw OR 'listeria'/exp OR 'tuberculos*':ti,ab,kw OR 'tuberculosis'/exp OR 'q fever':ti,ab,kw OR 'q fever'/exp

Table S2. *European countries (n = 39) included in the literature search. Islands located far from mainland Europe (i.e., The Azores, Canary Islands, Faroe Islands and Madeira) were excluded from this review.*

Albania	Estonia	Lithuania	Slovakia
Austria	Finland	Luxembourg	Slovenia
Belarus	France	Macedonia	Spain
Belgium	Germany	Moldova	Sweden
Bosnia and Herzegovina	Greece	Montenegro	Switzerland
Bulgaria	Hungary	Norway	The Netherlands
Croatia	Ireland	Poland	Turkey
Cyprus	Italy	Portugal	Ukraine
Czech Republic	Kosovo	Romania	United Kingdom
Denmark	Latvia	Serbia	

Table S3. *Overview of all studied (potentially) zoonotic pathogens (bacteria, viruses, helminths, protozoa and fungi) in different wildlife species, showing the number of studies in which the pathogen was detected/the total number of studies performed. Where no studies have been performed for a specific host-pathogen combination (i.e., a knowledge gap), the cell is empty. Detected pathogens are highlighted in green. An orange dot represents host-pathogen combinations not studied in the Netherlands. An asterisk indicates host-pathogen combinations studied in the Netherlands but without any positive results.*

		Animals			
	Order	*Rodentia*			
	Family	*Muridae*			*Cricetidae*
Genus	**Species**	**Brown rat**	**House mouse**	**Wood mouse**	**Common vole**
BACTERIA					
Aerococcus	*viridans*				
Anaplasma	spp	0/1 •		0/1 •	0/1 •
	phagocytophilum	1/2 •	1/4 •	7/11	2/3 •
Arcanobacterium	*haemolyticum*				
Bacillus	spp	1/1			
	cereus				
Bacteroides	*pyogenes*				
Bartonella	spp	6/7 •	0/1 •	7/11 •	7/8 •
	alsatica				
	clarridgeiae				
	grahamii		1/1 •	3/3	
	henselae	1/1 •		1/1 •	
	melophagi				
	rochalimae				
	tribocorum	3/3			
	vinsonii			1/1 •	
	washoensis				
Bordetella	*bronchiseptica*				
Borrelia	spp	0/2 •	1/1 •	2/6 •	2/3 •
	afzelii		1/2 •	5/6	2/2 •
	bavariensis				
	bissettiae				
	burgdorferi s.l.	2/3 •	3/3 •	13/16	7/7 •
	burgdorferi s.s.		1/2 •	1/1 •	1/1 •
	garinii		0/2 •		2/2 •
	lusitaniae		0/1 •	1/1 •	
	miyamotoi			2/3	0/1 •
	spielmanii				
Brucella	spp	0/1			1/1 •
Campylobacter	spp	2/2	1/2	0/1 •	
	coli		1/1		

		Lagomorpha	*Eulipotyphla*		*Carnivora*		
	Sciuridae	*Leporidae*	*Erinaceidae*	*Talpidae*	*Mustelidae*	*Canidae*	
	Red squirrel	**European rabbit**	**European hedgehog**	**European mole**	**Stone marten**	**Red fox**	Detected in # animal species
	0/1 •						0 •
					0/1 •	1/4 •	1 •
	3/4 •		4/4 •	0/3 •	2/3 •	14/18	8
			1/1 •				1 •
	1/1 •		1/1 •				3
	1/1 •						1 •
			1/1 •				1 •
	2/2 •	1/1	2/2 •	1/1 •	0/2 •	0/4 •	7
		4/4					1
					1/1 •		1 •
	1/1 •						3
							2 •
			1/1 •				1 •
						5/5 •	1 •
							1
							1 •
	3/3		1/1 •				2
		1/1 •	1/1 •				2 •
			2/2 •	0/1 •		2/4 •	5 •
	5/5 •		4/4 •		2/2 •	3/4 •	7
			2/2 •				1 •
						1/1 •	1 •
	4/4 •		2/2 •	0/2 •	0/1 •	5/8 •	7
	4/4 •		1/1 •				5 •
	3/3 •		2/2 •			4/4 •	4 •
						2/2 •	2 •
	1/1 •		0/1 •	0/1 •	0/1 •	0/1 •	2
			3/3 •			1/1 •	2 •
						1/2 •	2
						0/1 •	2
							1

Table S3. *continued*

Genus	Species	Brown rat	House mouse	Wood mouse	Common vole
Campylobacter	*hyointestinalis*		1/1		
	jejuni	1/1 •	1/1		
Chlamydia	spp	1/1			
Chlamydophila	spp				
	psittaci				1/1 •
Citrobacter	*freundii*				
Clostridium	*difficile*	1/1	4/4	2/2	1/1
Coccobacillus	spp				
Corynebacterium	spp	1/1			
	confusum				
	diphtheriae				
	ulcerans				
Coxiella	*burnetii*	6/6	2/2 •	5/8 •	1/4 •
Dermatophilus	*congolensis*				
Ehrlichia	spp			0/2 •	0/1 •
	canis				
Enterococcus	spp			1/1 •	
	spp AMR			1/1 •	
	avium				
	faecalis				
	faecium				
	hirae				
Escherichia	spp	1/2	0/1 •		
(/Shigella)	*coli*		0/1 •		1/1 •
	coli AMR	5/5 •	1/1 •	2/2 •	
Francisella	*tularensis*	1/3 •	1/1 •	2/5 •	6/10*
Helicobacter	spp				
Klebsiella	spp				
	pneumoniae				
Lactococcus	*garvieae*				
Leptospira	spp	13/14 •	4/4 •	9/12*	14/14 •
	borgpetersenii	1/1 •	2/2 •	1/1 •	
	interrogans	13/13	4/5 •	4/5 •	3/3
	kirschneri	0/1	1/2 •		
Listeria	spp	1/1 •			
	ivanovii				
	monocytogenes				
Morganella	*morganii*				
Mycobacterium	spp	1/1	1/1 •		1/1 •
	avium		1/1 •	1/1 •	
	bovis	1/1 •		1/1 •	

Red squirrel	European rabbit	European hedgehog	European mole	Stone marten	Red fox	Detected in # animal species
						1
1/1 •	1/1 •	1/1				5
						1
					0/1 •	0 •
						1 •
		2/2 •				1 •
						4
1/1 •						1 •
						1
		1/1 •				1 •
					1/1 •	1 •
		3/3 •				1 •
	4/5 •		0/2 •	0/2 •	4/8 •	6
1/1 •						1 •
					2/2	1
				0/1 •	6/16 •	1 •
					2/3 •	2 •
						1 •
		2/2 •				1 •
1/1 •	1/1 •	1/1 •			2/2 •	4 •
1/1 •	1/1 •				2/2 •	3 •
	1/1 •	1/1 •			2/2 •	3 •
				1/1 •	0/1 •	2
1/1 •	2/2 •	4/4 •			2/2 •	5 •
	3/4 •	1/1		1/1 •	7/8 •	7
1/2 •	3/3 •		0/1 •	2/2 •	4/7 •	8
					2/2 •	1 •
		1/1 •		1/1 •		2 •
		2/2 •				1 •
		1/1 •				1 •
		3/3	0/3 •		2/2 •	6
		4/4				4
		7/7		2/2 •	9/10 •	7
						1
					1/1 •	2 •
					1/1 •	1 •
		1/1 •		1/3 •	2/4 •	3 •
		2/2 •				1 •
1/2 •		1/1 •	1/1 •	1/1 •	1/2 •	8
	8/8 •	1/1 •	1/1 •	2/3 •	2/3 •	7 •
	0/1 •	0/1 •		1/1 •	9/9 •	4 •

Table S3. *continued*

Genus	Species	Brown rat	House mouse	Wood mouse	Common vole
Mycobacterium	*caprae*				
	leprae				
	lepromatosis				
Mycoplasma	spp				1/1 •
	haemofelis	1/1 •	1/1 •		
	pulmonis	1/1 •			
Neoehrlichia	spp				
	mikurensis	1/1 •	0/1 •	2/2	1/2 •
Pantoea	*agglomerans*				
Pasteurella	spp	1/1 •			
	canis				
	multocida	0/1 •			
	pneumotropica	1/1 •			
Proteus	spp	1/1			
	mirabilis				
Pseudomonas	spp	1/1 •			
Rickettsia	spp	2/2 •	0/1 •	1/5*	4/8 •
	conorii	1/1 •			
	helvetica	0/1 •	1/1 •		
	massiliae				
	prowazekii				
	slovaca				
	typhi	4/4 •	1/1 •	1/1 •	
Salmonella	spp	2/5*	0/4	0/1	0/1 •
	enterica		2/2		
Spiroplasma	*ixodetes*			0/1	
Staphylococcus	spp	2/2			
	aureus	1/1		0/1 •	1/1 •
	aureus (MRSA)	1/2*	1/2*	1/2*	
	lentus				
	delphini				
	equorum				
	hycus				
	pseudintermedius				
	sciuri				
	simulans				
Streptobacillus	*moniliformis*				
Streptococcus	spp	1/1			
	canis				
	gallinaceus				
	pyogenes				

Red squirrel	European rabbit	European hedgehog	European mole	Stone marten	Red fox	Detected in # animal species
					2/3 •	1 •
2/4*						1
1/3*						1
						1 •
						2 •
						1 •
					4/4 •	1 •
0/1 •				1/1 •	0/4 •	4
1/1 •						1 •
				1/1 •		2 •
					1/1 •	1 •
		1/1 •				1 •
						1 •
						1
		2/2 •				1 •
						1 •
		1/2 •	1/2 •	0/1 •	0/10 •	5
	1/1 •				1/1 •	3 •
			0/1 •	1/1 •		2 •
					1/1 •	1 •
	0/1 •					0 •
	1/1 •				1/1 •	2 •
	0/1 •				1/1 •	4 •
		1/1			1/3 •	3
	1/1 •	7/7		1/1 •	8/8 •	5
						0
				1/1 •	1/1 •	3
5/5 •		1/1 •		1/1 •	1/1 •	6
	2/2 •	8/8 •			0/2 •	5
		1/1 •			1/1 •	2 •
				1/1 •	1/1 •	2 •
					1/1 •	1 •
0/1 •						0 •
				1/1 •	1/1 •	2 •
1/1 •		1/1 •			1/1 •	3 •
		1/1 •				1 •
			1/1 •			1 •
				1/1 •		2
		1/1 •			2/2 •	2 •
		1/1 •				1 •
		2/2 •				1 •

Table S3. *continued*

Genus	Species	Brown rat	House mouse	Wood mouse	Common vole
Streptococcus	*suis*				
	thoraltensis				
Trueperella	*pyogenes*				
Vagococcus	*fluvialis*				
	lutrae				
Yersinia	spp	2/2 •			1/1 •
	enterocolitica	5/5 •	2/3 •	3/4 •	2/2 •
	pseudotuberculosis	1/1 •	2/2 •		
# Bacterial pathogens detected		**39**	**27**	**27**	**22**
VIRUSES					
Alphainfluenzavirus	*Influenza A virus*	0/1 •			
Alphavirus	*Sindbis virus*				
Aphthovirus	*Foot and mouth disease virus*				
Betacoronavirus	*SARS-CoV-2*	0/1 •	0/1 •	0/2 •	0/1 •
Cardiovirus	*Encephalo-myocarditis virus*	0/1 •			
Coltivirus	*Eyach coltivirus*			0/1	
Flavivirus	spp	0/1 •		0/1 •	
	Tick-borne encephalitis virus		0/1 •	2/4*	4/4
	Usutu virus	0/1 •			
	West nile virus	0/1 •		0/1 •	
Kobuvirus	spp	1/1 •			
Lyssavirus	*European bat lyssavirus*				
	Lyssavirus rabies				1/2 •
Mammarenavirus	*Lymphocytic choriomeningitis mammarenavirus*		4/4 •	5/6 •	3/3 •
Norovirus	spp	4/4 •			
Orthobornavirus	*Borna disease virus*				
Orthohantavirus	spp	4/5 •	2/2 •	3/6 •	5/7 •
	Dobrava-Belgrade orthohantavirus		0/1 •	2/8 •	2/2 •
	Puumala orthohantavirus		2/3 •	3/10*	1/3 •
	Seoul orthohantavirus	9/10	0/1 •		
	Tula orthohantavirus		0/2 •	2/6*	25/26
Orthohepevirus	spp	1/1 •			2/2 •
	Hepatitis E virus	10/12 •	0/1 •	0/1 •	

Red squirrel	European rabbit	European hedgehog	European mole	Stone marten	Red fox	Detected in # animal species
	1/1 •					1 •
		1/1 •				1 •
		1/1 •				1 •
		1/1 •				1 •
		1/1 •				1 •
					1/1 •	3 •
				3/3 •	6/7 •	6 •
				1/1 •	1/1 •	4 •
24	**17**	**51**	**5** •	**23** •	**51**	
		0/1 •		1/2 •	3/4	2
		1/1 •				1 •
		1/1 •				1 •
				1/2	0/2 •	1
						0 •
	0/1 •					0
						0 •
0/1 •		1/1 •	2/2 •	0/1 •	1/2 •	5
						0 •
		1/1 •			1/2 •	2 •
					1/1 •	2 •
				1/1 •		1 •
				1/2 •	3/8 •	3 •
					1/1 •	4 •
						1 •
				0/1 •	2/4 •	1 •
					2/2 •	5 •
						2 •
					1/1 •	4
						1
						2
						2 •
	5/6				2/4 •	3

Table S3. *continued*

Genus	Species	Brown rat	House mouse	Wood mouse	Common vole
Orthonairovirus	*Crimean-congo haemorrhagic fever virus*			0/1 •	
Orthopoxvirus	spp	0/1 •	1/1 •	4/7 •	2/2 •
	Cowpoxvirus	3/4	1/1 •	3/3 •	3/3 •
Parechovirus	*Ljungan virus*		1/1 •	1/1 •	
Phlebovirus	spp				
Rotavirus	spp	2/2 •			
Varicellovirus	*Pseudorabies virus*				
	# Viral pathogens detected	**8**	**6** •	**9**	**10**
HELMINTHS					
Alaria	spp				
	alata	1/1 •			
Ancylostoma	spp				
	caninum				
Angiostrongylus	spp				
	cantonensis	1/1 •			
Brachylaima	spp			3/3 •	
Capillaria	spp	2/2 •			
	aerophila				
	hepatica	7/7 •	0/1 •	2/2 •	1/1 •
Dicrocoelium	*dendriticum*				
Dioctophyma	*renale*				
Dipetalonema	spp				
Diphyllobothrium	spp				
Dipylidium	spp				
	caninum				
Dirofilaria	spp				
	immitis				
	repens				
Echinochasmus	*perfoliatus*				
Echinococcus	spp	0/1 •			
	granulosus				
	multilocularis	1/1 •	1/1 •	0/1 •	3/3 •
Fasciola	*hepatica*	0/1 •			
Heterophyes	*heterophyes*				
Hymenolepis	spp			3/3 •	
	diminuta	7/7	1/1 •	0/1	
	microstoma	1/1 •			
	nana	7/7	0/1 •		
Mesocestoides	spp			3/3 •	

Red squirrel	European rabbit	European hedgehog	European mole	Stone marten	Red fox	Detected in # animal species
	0/1 •					0 •
						3 •
						4
1/1 •						3 •
					1/1 •	1 •
0/1 •					2/2 •	2 •
					1/1 •	1 •
1 •	**1**	**4 •**	**1 •**	**4**	**13**	
				1/1 •	1/1 •	2 •
					21/21	2
					1/1 •	1 •
					7/7 •	1 •
				1/2 •		1 •
						1 •
						1 •
		5/5 •		2/3 •	8/8	4
					26/26	1
2/2 •					1/1 •	5 •
					1/1 •	1 •
					1/1 •	1 •
					0/1 •	0 •
					3/3 •	1 •
					2/2 •	1 •
					18/18 •	1 •
					1/2 •	1 •
				0/1 •	13/16 •	1 •
				1/1 •	4/6 •	2 •
					3/3 •	1 •
					1/3*	1
					2/6 •	1 •
				0/4 •	70/75	4
	3/3					1
					1/1 •	1 •
					2/2	2
					1/1 •	3
						1 •
					1/1 •	2
				1/1 •	22/23*	3

Table S3. *continued*

Genus	Species	Brown rat	House mouse	Wood mouse	Common vole
Mesocestoides	*lineatus*				
Metagonimus	*yokogawai*				
Metorchis	*bilis*				
Moniliformis	*moniliformis*	1/1 •			
Onchocerca	spp				
Opisthorchis	*felineus*				
Pelodera	*strongyloides*			1/1 •	
Physaloptera	spp				
Plagiorchis	*muris*			1/1 •	
Pseudamphistomum	*truncatum*				
Schistosoma	spp		0/1 •		
Spirometra	spp				
	erinacei				
Strongyloides	spp	1/1			
Taenia	spp				
	crassiceps				1/1 •
	martis			4/4 •	
	multiceps				
	serialis				
	taeniaeformis	4/4 •	0/1 •	6/6 •	2/2 •
Thelazia	*callipaeda*				
Toxascaris	*leonina*				
Toxocara	spp	0/1 •	1/1 •		
	canis				
	cati	1/1 •			
Trichinella	spp	4/7*			
	britovi	0/1 •			
	nativa	0/1 •			
	pseudospiralis	1/2 •			
	spiralis	3/4 •			
Trichostrongylus	spp				
Trichuris	spp				
	vulpis				
Uncinaria	spp				
	stenocephala				
# Helminth pathogens detected		**15**	**3** •	**8**	**4** •
PROTOZOA					
Babesia	spp	0/3 •	0/1 •	2/3	0/1 •
	microti		1/1 •	2/4	6/6 •
Blastocystis	spp	1/2 •			
Cryptosporidium	spp	2/5 •	1/1 •	0/2 •	2/2 •

Red squirrel	European rabbit	European hedgehog	European mole	Stone marten	Red fox	Detected in # animal species
					3/3 •	1 •
					1/1 •	1 •
					4/4 •	1 •
						1 •
					0/1 •	0 •
					4/4 •	1 •
						1 •
					2/2 •	1 •
						1 •
					6/6 •	1 •
						0 •
					1/1 •	1 •
					1/1 •	1 •
				1/1 •	4/4	3
				1/1 •	20/20	2
				1/1 •	14/14	3
1/1 •				1/1 •	1/1 •	4 •
					3/3 •	1 •
					2/2 •	1 •
					6/6 •	4 •
	1/1 •			3/3 •	5/6 •	3 •
				1/3 •	27/28	2
				1/2 •	5/5 •	3 •
				0/1 •	42/42	1
				0/2 •	2/4 •	2 •
		0/1 •		4/5 •	32/39	3
				3/3 •	31/31 •	2 •
					11/12 •	1 •
					7/10 •	2 •
					27/28 •	2 •
					1/1 •	1 •
				0/1 •	0/1 •	0 •
					16/16	1
				1/1 •		1 •
				0/1 •	32/32	1
2 •	**2**	**1 •**	**0 •**	**15 •**	**52**	
0/1 •		0/1 •	1/1 •	0/2 •	9/11	3
					0/1 •	3
1/2 •	1/1 •					3 •
1/3 •		2/2		0/2 •	6/7 •	6

Table S3. *continued*

Genus	Species	Brown rat	House mouse	Wood mouse	Common vole
Cryptosporidium	*andersoni*	1/1 •			
	canis				
	chipmunk				
	cuniculus				
	felis				
	hominis				
	muris	1/1 •	1/1 •	2/2 •	
	occultus	1/1 •			
	parvum	2/2 •	1/1 •	2/2 •	1/1 •
	suis				
	tyzzeri				
	ubiquitum				
Dientamoeba	*fragilis*	1/1 •			
Entamoeba	spp	0/1 •			
Giardia	spp	0/1 •			2/2 •
	duodenalis	1/1 •			
Leishmania	spp	3/3 •	2/2 •	0/1 •	
	infantum	5/5 •	3/3 •	2/3 •	
Neospora	*caninum*	2/3*	3/6		2/2
Sarcocystis	spp	0/1 •	0/1 •	0/1 •	
Toxoplasma	spp	1/1 •			
	gondii	8/9	11/13	5/9	3/4
Trypanosoma	spp	1/1 •	0/3	2/2 •	1/1 •
	lewisi	3/3 •	0/1		
# Protozoal pathogens detected		**15**	**8**	**7**	**7**
FUNGI					
Aspergillus	spp				
	fumigatus				1/1 •
Cryptococcus	*neoformans*				
Emmonsia	*crescens*				1/1 •
Encephalitozoon	*cuniculi*		2/3 •	2/2 •	1/1 •
	hellem		1/1 •		
	intestinalis		1/1 •		
Enterocytozoon	*bieneusi*		2/2 •		
Histoplasma	*capsulatum*				
Microsporum	*canis*				
Nannizzia	*fulva*				
	gypsea				
	nana				
Penicillium	spp				1/1 •
Scopulariopsis	*brevicaulis*				1/1 •

Red squirrel	European rabbit	European hedgehog	European mole	Stone marten	Red fox	Detected in # animal species
	1/1				1/1	3
					2/2	1
2/2						1
	1/1					1
					1/1	1
					1/1	1
		0/1				3
						1
1/1		3/3			3/3	7
					1/1	1
					1/1	1
					2/2	1
						1
0/1						0
0/1		1/1		1/2	3/4	4
0/1	1/1				4/4	3
0/1	1/1			1/2	1/5	5
1/1	9/9	3/4		4/4	13/15	8
				0/2	11/17	4
				1/1	2/2	2
	1/1					2
5/5	5/5	1/1	3/3	4/5	28/32	10
	1/1					4
						1
6	**9**	**5**	**2**	**5**	**17**	
				1/1		1
						1
					1/1	1
			1/1			2
	0/2			1/2	1/4	5
	0/1					1
	2/2				1/1	3
0/1	1/2			2/2	2/3	4
		1/1		0/1		1
		1/1				1
		1/1				1
		1/1				1
		1/1				1
						1
						1

Table S3. *continued*

Genus	Species	Brown rat	House mouse	Wood mouse	Common vole
Trichophyton	*benhamiae*				
	mentagrophytes		1/1 •		
	# Fungal pathogens detected	**0** •	**5** •	**1** •	**5** •

•: Host-pathogen combinations not studied in the Netherlands.
*: Host-pathogen combinations studied in the Netherlands but without any positive results.

Table S4. *Coverage percentages per pathogen group, animal species , and in total. The coverage percentage was calculated by dividing the number of studied host-pathogen combinations by the total number of host-pathogen combinations, multiplied by 100. HPCs = host-pathogen combinations. Coverage percentages > 50 % are highlighted in bold.*

		Brown rat	House mouse	Wood mouse	Common vole	Red squirrel	European rabbit	European hedgehog	European mole	Stone marten	Red fox	Total
Bacteria	# studied HPCs	45	35	33	26	27	20	53	13	30	59	341/1210
	Coverage %	37 %	29 %	27 %	21 %	22 %	17 %	44 %	11 %	25 %	49 %	28 %
Viruses	# studied HPCs	15	12	15	11	3	3	5	1	6	14	85/300
	Coverage %	**50 %**	40 %	**50 %**	37 %	10 %	10 %	17 %	3 %	20 %	47 %	28 %
Helminths	# studied HPCs	20	7	10	4	2	2	2	0	21	55	123/650
	Coverage %	31 %	11 %	15 %	6 %	3 %	3 %	3 %	0 %	32 %	**85 %**	19 %
Protozoa	# studied HPCs	19	12	10	8	11	9	7	2	8	18	104/280
	Coverage %	**68 %**	43 %	36 %	29 %	39 %	32 %	25 %	7 %	29 %	**64 %**	37 %
Fungi	# studied HPCs	0	5	1	5	1	4	7	1	4	4	32/170
	Coverage %	0 %	29 %	6 %	29 %	6 %	24 %	41 %	6 %	24 %	24 %	19 %
Total	# studied HPCs	99	71	69	54	44	38	74	17	69	150	685/2610
	Coverage %	38 %	27 %	26 %	21 %	17 %	15 %	28 %	7 %	26 %	**57 %**	26 %

Red squirrel	European rabbit	European hedgehog	European mole	Stone marten	Red fox	Detected in # animal species
		1/1 •				1 •
		4/4 •				2 •
0 •	2 •	7 •	1 •	3 •	4 •	

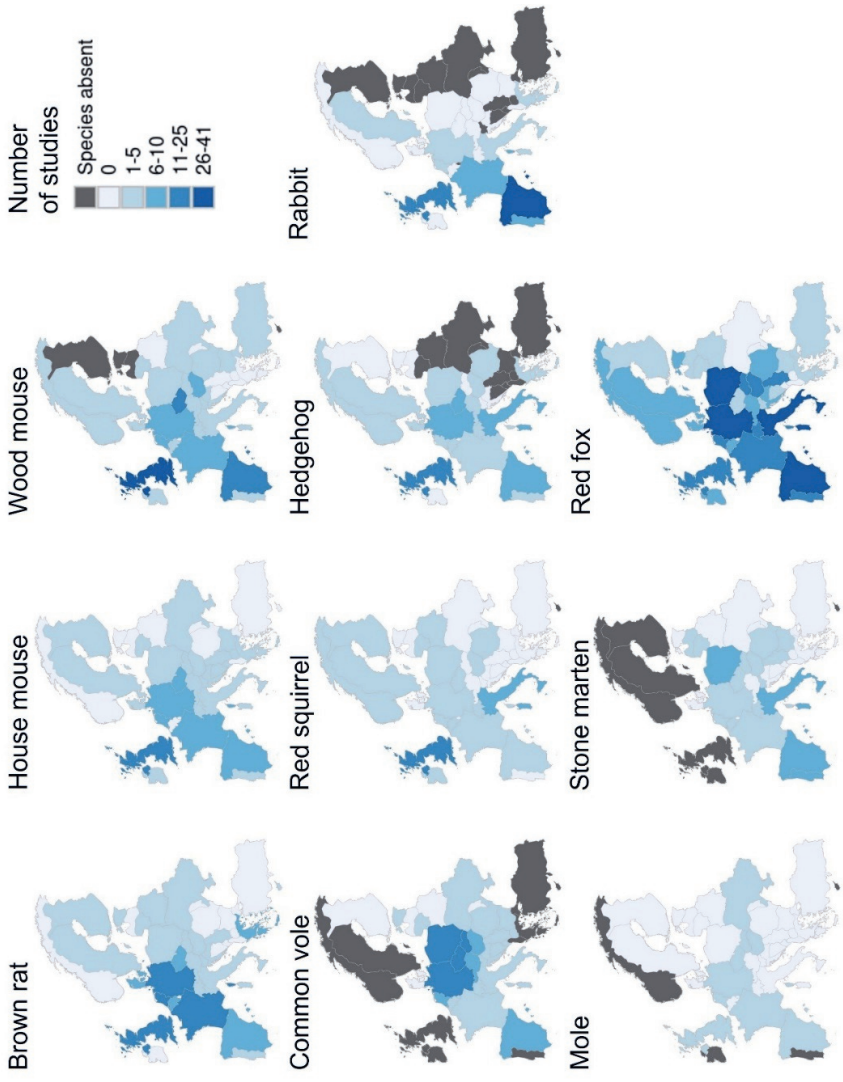

Figure S1. *Number of studies conducted per animal species per country. 'Species absent' indicates when an animal species does not occur in a country. Animal distributions are taken from the IUCN Red List Version 2022-2 (IUCN, 2023).*

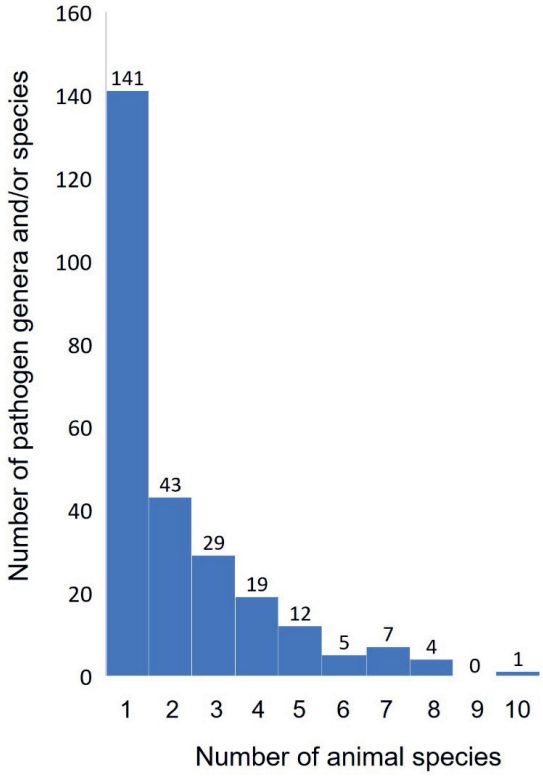

Figure S2. *Number of pathogens (genera and/or species) detected in one or multiple animal species.*

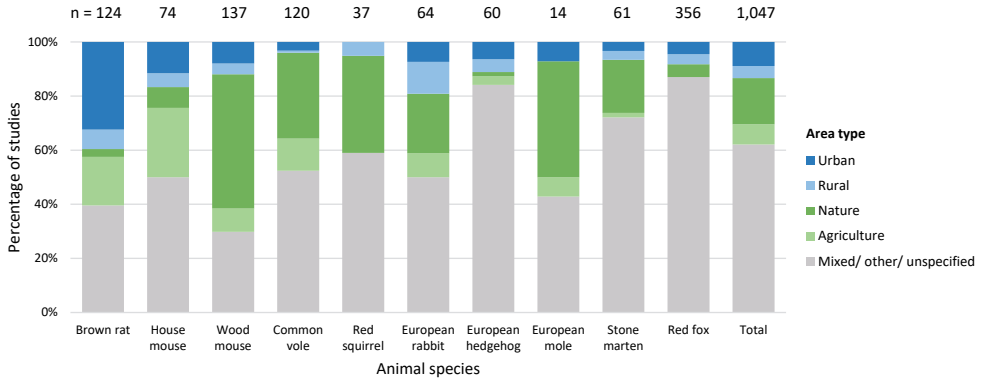

Figure S3. *Relative percentage of studies performed in a specific habitat type (e.g., urban, rural, nature, agriculture or mixed/other/unspecified) per animal species and in total. Mixed habitats included > 1 habitat type but were not specified separately. Above the bars, the total number of studies per animal species is shown.*

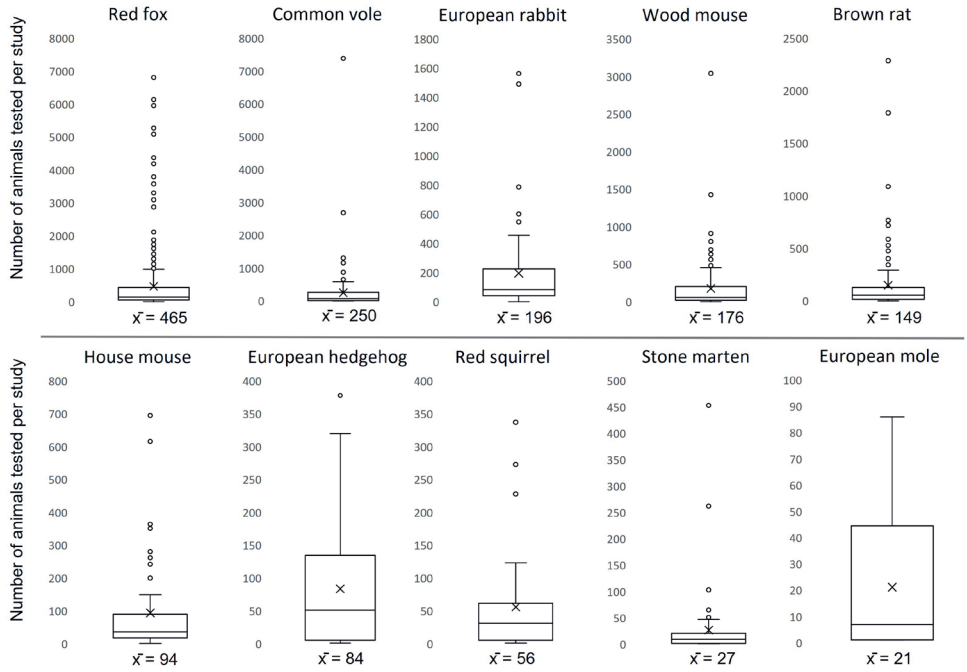

Figure S4. *Number of animals tested per study, per animal species. x shows the mean number of animals tested per study. The y-axes have different scales.*

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Restaurant

Chapter 3

Screen the unforeseen: microbiome-profiling for detection of zoonotic pathogens in wild rats

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Abstract

Wild rats can host various zoonotic pathogens. Detection of these pathogens is commonly performed using molecular techniques targeting one or a few specific pathogens. However, this specific way of surveillance could lead to (emerging) zoonotic pathogens staying unnoticed. This problem may be overcome by using broader microbiome-profiling techniques, which enable broad screening of a sample's bacterial or viral composition. In this study, we investigated if 16S rRNA gene amplicon sequencing would be a suitable tool for the detection of zoonotic bacteria in wild rats. Moreover, we used virome-enriched (VirCapSeq) sequencing to detect zoonotic viruses. DNA from kidney samples of 147 wild brown rats (*Rattus norvegicus*) and 42 black rats (*Rattus rattus*) was used for 16S rRNA gene amplicon sequencing of the V3–V4 hypervariable region. Blocking primers were developed to reduce the amplification of rat host DNA. The kidney bacterial composition was studied using alpha and beta-diversity metrics and statistically assessed using PERMANOVA and SIMPER analyses. From the sequencing data, 14 potentially zoonotic bacterial genera were identified from which the presence of zoonotic *Leptospira* spp. and *Bartonella tribocorum* was confirmed by (q)PCR or Sanger sequencing. In addition, more than 65 % of all samples were dominated (> 50 % reads) by one of three bacterial taxa: *Streptococcus* (n = 59), *Mycoplasma* (n = 39) and *Leptospira* (n = 25). These taxa also showed the highest contribution to the observed differences in beta diversity. VirCapSeq sequencing in rat liver samples detected the potentially zoonotic rat *hepatitis E virus* in three rats. Although 16S rRNA gene amplicon sequencing was limited in its capacity for species level identifications and can be more difficult to interpret due to the influence of contaminating sequences in these low microbial biomass samples, we believe it has potential to be a suitable pre-screening method in the future to get a better overview of potentially zoonotic bacteria that are circulating in wildlife.

Introduction

Wild brown rats (*Rattus norvegicus*) and black rats (*Rattus rattus*) can host various zoonotic pathogens including *Seoul orthohantavirus*, *Leptospira* spp. and vector-borne bacteria [1, 2]. Their synanthropic lifestyle increases the risk of pathogen transmission from rats to humans via (in-)direct contact. Screening of zoonotic pathogens of wild rats gives insight in the potential public health risks. Early detection and identification of these pathogens is crucial to respond faster and more adequate to emerging infectious diseases.

Currently, diagnostic techniques such as (q)PCR and PCR-based reverse line blot hybridization assays are often used for detection of zoonotic pathogens [3]. Though they are designed to be highly sensitive and specific [4], they have the disadvantage that they can detect only a limited number of specific pathogens per test. As a consequence, certain pathogens can remain undetected, because they are not expected to be found in a particular species and therefore are not targeted [5]. Therefore, it is important to keep optimizing detection methods and to test potential new techniques. In the last decades, huge advances have been made in developing new pathogen identification tools such as (metagenomic) deep sequencing [4, 6]. The advantage of such techniques is the ability to broadly screen a sample's bacteriome or virome composition without exact sequence knowledge about the presence or absence of specific bacteria or viruses. This feature could make metagenomic sequencing a suitable tool to screen for zoonotic pathogens in wildlife and it could facilitate the detection of unexpected or emerging pathogens. Although numerous microbiome-profiling studies have been performed on humans, such studies performed on wildlife are limited [5, 7-10].

In this study, we investigated if 16S rRNA gene amplicon sequencing would be a suitable tool for detection of zoonotic pathogens in wild rats using kidney samples. These results were compared with qPCR results. In addition, we used VirCapSeq sequencing on liver samples to detect zoonotic viruses. Furthermore, we examined the kidney bacterial composition in more detail and investigated if there were internal (microbial diversity, species, bodyweight/age and sex) or external (trapping location type) factors correlated with zoonotic pathogen carriage in these wild rats. With this information we aim to improve targeted screening and surveillance of zoonotic pathogens in wild rats to enhance early detection.

Materials and Methods

Sample collection

From 2013 to 2018, pest control agencies captured brown and black rats in different municipalities across the Netherlands using live traps and snap traps for various surveillance studies [11]. From all trapped rats, we included 189 rats in this study based on species and trapping location type (Table S1). Trapping locations were divided into the following categories: urban, rural, agriculture and industry. Locations with < 1000 addresses/km2 were defined as rural and locations with > 1000 addresses/km2 were defined as urban. When rats were captured on farms, their location was defined as

agriculture, and when they were captured in industrial areas, their location was defined as industry. The live trapped rats were anaesthetized using isoflurane and euthanized by cervical dislocation or an isoflurane overdose after which they were dissected. The rats captured using snap traps were stored at −20°C until dissection. During dissection, we collected data on species, sex and bodyweight, as well as kidney and liver samples. Samples were stored at −80°C until DNA extraction. Kidney and liver samples were used for bacteriome and virome analyses, respectively. Bodyweight was used to divide rats into age classes. For males we used: juvenile (< 100 g), subadult (101–200 g), and adult (> 200 g), and for females we used: juvenile (< 100 g), sub-adult (101–175 g) and adult (> 175 g) [12].

Kidney bacterial composition

DNA extraction and 16S rRNA gene quantification

A small cross section of each kidney was cut and weighed. We adjusted the volume of lysis buffer according to weight and subsequently used equal volumes per sample for DNA extraction. We extracted DNA using the DNeasy Blood and Tissue Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's protocol, including extraction controls to monitor potential contamination during DNA extraction. We performed a 16S rDNA gene qPCR as previously described [13] to quantify the amount of 16S rRNA gene copies present in the samples.

Development of a rat mitochondrial 16S rRNA gene targeted blocking primer

During an initial pilot study where 16S rRNA gene amplicons of the hypervariable V3–V4 region were sequenced, a substantial amount of sequences derived from rat host DNA were obtained. This was probably due to the low microbial biomass of kidney samples and the non- specific amplification of 16S rat mitochondrial rRNA. To prevent the amplification of the untargeted rat-derived DNA, peptide nucleic acid (PNA)-based blocking primers were designed, in collaboration with BaseClear (Leiden, the Netherlands). In short, unique amplicon-derived sequences obtained during the pilot were aligned against mitochondrial 16S rRNA amplicon sequences from other mammals obtained from DNA databases (Table S2). The sequence alignment was manually inspected for regions of identity. One region was identified from which 15–25-nt sequences were extracted as candidate blocking primers. Candidate blocking primers were aligned to 16S rRNA gene databases, and primers that showed matches in the database were excluded from further analysis. Ultimately, a single remaining candidate sequence was obtained that matched the rat mitochondrial sequences as well as those from other eukaryotic host species, such as *Vulpes vulpes*, *Meles meles*, *Martes martes*, *Mustela nivalis*, *Cervus elaphus, Dama dama, Sciurus vulgaris, Talpa europaea, Crocidura russula* and *Lepus europaeus*. The resulting PNA blocking primer (5'-TGGTAAATTTCGTGCCAGCCA-3') was synthesized by Eurogentec (Maastricht, the Netherlands) and used at a final concentration of 800 nM. The blocking primer reduced the amount of host DNA sequenced with approximately 15 % – 45 % per sample (Figure S1). Blocking primers were included in the final sequencing PCR to gain more bacteria-derived sequences from the microorganisms in the samples.

16S rRNA gene amplification and sequencing

The extracted DNA was used as template for 16S rRNA gene amplification followed by sequencing of the amplicons by BaseClear (Leiden, the Netherlands) using the Illumina MiSeq platform. In short, amplicons of V3–V4 hypervariable regions of 16S rRNA genes were generated by PCR using a limited number of cycles using the forward primer 341F (5'-CCTACGGGNGGCWGCAG-3'), reverse primer 785R (5'- GACTACHVGGGTATCTAATCC-3' [14] and the PNA blocking primer described above, followed by a second PCR to incorporate the Illumina sequencing adaptors. PCR products were purified using a magnetic bead–based protocol, and DNA concentration was measured by fluorometric analysis (Qubit, Thermo Fisher Scientific). Subsequently, PCR amplicons were equimolarly pooled, and samples or controls with negligible amplicon DNA concentrations were added at the maximum allowed volume in the library tagging procedure. Pooled amplicons were sequenced on an Illumina MiSeq run with the paired- end 300 cycles protocol. The sequencing data was demultiplexed with the Illumina CASAVA pipeline (v1.8.3) based on sample-specific barcodes. The raw sequencing data was processed by removing the sequence reads of too low quality (only "passing filter" reads were selected) and discarding reads containing adaptor sequences or PhiX control with an in-house filtering protocol. A quality assessment on the remaining reads was performed using the FastQC quality control tool version 0.10.0.

Data preparation

The data preparation and all analyses were performed in R 4.1.1 (R Core Team, 2021) and RStudio (RStudio Team, 2021). To go from raw reads to community analyses, we mostly followed a predefined workflow [15]. The Illumina demultiplexed paired-end sequence reads were processed using the *DADA2* R package. Paired- end reads were merged, primers were removed (trimLeft (27,31)), sequences were trimmed to 280 bp (forward reads) and 240 bp (reverse reads) and a maximum of two ambiguous nucleotides was used. This was followed by error correction, data pooling, merging of sequences, chimaera filtering and clustering the reads into amplicon sequence variants (ASVs) using the reference database SILVA 16S version 138.1 [16]. Then we combined the taxonomy counts into a phyloseq R object [17] containing the ASV counts, taxonomy data and sample metadata. We applied taxonomic filtering to remove non-bacterial ASVs (almost 70 % were eukaryotic ASVs and a single archaeal ASV) and a few ASVs that were unidentifiable at Phylum level. We subjected the 16S rRNA amplicon sequences of all potentially zoonotic genera also to NCBI BLAST against the nr/nt database (date accessed: 10-Nov-2021) to identify identical matching species.

Diversity analyses

Alpha diversity was calculated using the Shannon diversity index. Because we wanted to compare only alpha diversity versus number of reads per sample, we did not use rarefied data as this would force us to remove a substantial number of samples with very low read counts (e.g. when rarefied to 1000 reads, almost 50 % of samples would be removed). Prior to performing beta-diversity analysis, we removed ASV singletons and doubletons in each sample to reduce the influence of contamination, and we performed a Hellinger

transformation on the data. Beta-diversity was assessed by principal coordinate analyses (PCoAs) on relative abundance data using Bray–Curtis dis- similarities using the *vegan* R package [18]. To identify significant differences between community structures, PERMANOVA was performed using *adonis (vegan*; $p = 0.05$, 999 permutations and set. seed(100)) and dispersions were tested for homogeneity using *betadisper* (*vegan*). To assess the contribution of individual taxa to the observed differences in beta diversity between groups we used SIMPER analysis (*simper_pretty* function; [19]). The SIMPER-identified taxa that contributed most to the observed group differences were tested for significance with the Kruskal-Wallis test (*R_krusk* function, [19]). Taxa were considered significant if fdr-adjusted *p*-values < 0.05.

Liver virome

DNA and RNA extraction followed by enriched-metagenome sequencing

A total of 189 frozen liver samples were sliced. Per 0.5-g liver, we added 1.75-ml cold PBS with 1 % complete EDTA-free protease inhibitor cocktail (Roche, ref: 11873580001), followed by a 4-h benzonase treatment at 37°C to remove free (non-encapsulated) nucleic acids. Samples were pooled in batches of 3–5 samples per pool based on rat species and trapping location. Subsequent DNA purification was performed using the Qiagen QIAamp MinElute Virus Spin Kit (Qiagen, Venlo, the Netherlands) according to the manual prescription. For the RNA fraction extraction, the Zymo Research Direct-zol kit (BaseClear, Leiden, the Netherlands) was used following the kit instructions with an internal DNase treatment. To increase the amount of RNA and DNA, a pre-enrichment was performed with a random SISPA amplification [20]. Shotgun sequence libraries were created from the extracted DNA and reverse transcribed RNA following enrichment using VirCapSeq (according to Roche) and subsequently sequenced using Illumina short-read sequencing (MiSeq). When one of the pools was found positive for a relevant virus, the individual samples were processed separately.

Data analysis

For downstream analysis, the Illumina raw sequencing data was demultiplexed using the Illumina software (bcl2fastq v2.20.0.422, Illumina Inc) and subsequently polished (trimmed for artefacts and QC (BBMap – Bushnell B. – sourceforge.net/projects/bbmap/). To determine the presence of viruses in the samples, the polished reads were mapped to the present viruses of the NCBI database. The output table was manually inspected and the top scoring accession numbers were downloaded and used in further analyses.

The NCBI database was used to in silico enrich the viral reads per sample. The selected polished reads were used as input for de novo assembly using default settings in spades (SPAdes v3.13.0) [21]. Blast analysis was performed on the output files of the assembly.

Zoonotic pathogen identification and confirmation

Pathogen confirmation analyses were performed for *Leptospira* spp., *Bartonella* spp., *Mycoplasma* spp. and *Brucella* spp. For the first three pathogens, we extracted DNA from kidney samples using the DNeasy Blood and Tissue Kit (Qiagen, Venlo, the Netherlands)

according to the manufacturer's protocol. For *Brucella*, we isolated DNA from *Brucella*-suspected colonies by suspending the colony in 200-µl nuclease-free water (Sigma-Aldrich, https://www.sigmaaldrich.com) and subsequent boiling at 100°C for 8 min, followed by centrifugation for 2 min at 20,000 × g.

For ***Leptospira* spp**. identification, we used a previously described qPCR specifically targeting pathogenic *Leptospira* species with forward primer LipgrF2 5'-CGC-TGA-AAT-GGG-AGT-TCG-TAT-GAT-TTC-C-3', reverse primer LipgrR2 5'-GGC-ATT-GAT-TTT-TCT-TCY-GGG-GTW- GCC-3' and probe LipgrP1 5'-Fam-AGG-CGA-AAT-CGG-KGA-RCC- AGG-CGA-YGG-BHQ1-3' [22]. The *Leptospira interrogans kantorow* strain was used as positive control. Samples with sigmoid melting curves and Ct values < 45 were considered positive.

For ***Bartonella* spp**. identification, we first performed a qPCR on genus level with forward primer ssrA-F 5'- GCTATGGTAATAAATGGACAATGAAATAA-3', reverse primer ssrA-R 5'-GCTTCTGTTGCCAGGTG-3' and probe 5'-atto520- ACCCCGCTTAAACCTGCGACG-3'-BHQ1 [23]. Subsequently, we performed conventional PCRs for Sanger sequencing on the samples detected positive by qPCR. These PCRs targeted two different genes: gltA and rpoB. We first used gltA primers: *gltA-2 (*Bhcs.781p fwd: 5'-GGGGACCAGCTCATGGTGG-3' & Bhcs.1137n rev: 5'-ATTGCAAAAAGAACAGTAAACA-3') [24]. In case results were negative, we also used rpoB primers: *rpoB* (1400F: 5'-CGCATTGGCTTACTTCGTATG-3' & 2300R 5-GTAGACTGATTAGAACGCTG-3') [25]. The strain of *B. henselae* ATCC 49882 was used as positive control. PCR products were sequenced by BaseClear (Leiden, the Netherlands).

For ***Mycoplasma* spp.** identification we performed a *M. pulmonis* specific PCR and a *Mycoplasma* genus PCR. For the *M. pulmonis* specific PCR we used forward primer MP1 5'- AGC-GTT-TGC-TTC-ACT-TTG-AA-3' and reverse primer MP2 5'-GGG-CAT-TTC-CTC-CCT-AAG-CT-3' [26]. For the *Mycoplasma* genus PCR we targeted the 16S rRNA gene using forward primer HemMyco16S-41s 5'-GYATGCMTAAYACATGCAAGTCGARCG-3' and reverse primer HemMyco16S-938as 5'-CTCCACCACTTGTTCAGGTCCCCGTCGTC-3' [27]. The obtained PCR products were sequenced by BaseClear (Leiden, The Netherlands).

For ***Brucella* spp.** identification we cultured and isolated *Brucella spp*. using the Castañeda method and selective media according to the OIE protocol [28]. Suspected colonies were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry on the Bruker MALDI Biotyper (Bruker, https://www.bruker.com) by using an extended in-house *Brucella* spp. database [29] and PCR. On the isolated DNA we performed qPCR targeting the IS711 sequences of *Brucella* spp. [30] using forward primer IS711F 5'-GACCAAGCTGCATGCTGTTG-3', reverse primer IS711R 5'-GCCGGGTGTTGGCTTTATT-3' and probe IS711P FAM-CGATGCTATCGGCCTACCGCTGCG-BHQ1. Colonies and tissue samples were considered positive after qPCR if the results showed a cycle threshold (Ct) value of < 36 (with sigmoid curve), inconclusive if Ct value was > 36 but < 40 (with inconclusive sigmoid curve), and negative if Ct value was > 40 or if there was no Ct detected.

Statistical analyses

In addition to the diversity analyses, we performed statistical analyses to investigate the correlation between the presence of zoonotic pathogens detected in these rats and

specific rat characteristics (species, weight/age, location type and sex). The prevalence of zoonotic pathogens was tested for both qPCR and 16S rRNA amplicon sequencing results separately. The primary outcome variable was infection status (positive versus negative). We included the following explanatory variables: location type (urban, rural, agriculture or industry), species (*R. norvegicus or R. rattus*), sex (male or female), age class (juvenile, sub-adult or adult) and bodyweight (g). Trapping area (municipality where the rats were trapped) was included as a random factor. We used generalized linear mixed models (GLMM) with binomial logit link function to examine the relationship between infection status and the explanatory variables using the *glmer* function (*lme4*). Variables that were significantly associated with infection status ($p < .05$) were included in the final model. Rats with missing data for one or more variables were excluded. All statistical analyses were performed in R 4.1.1 and RStudio [31]. Confidence intervals were computed using the 95 % Wald confidence interval. Variables were considered significant when $p < 0.05$.

Results

16S rRNA gene amplicon sequencing of rat kidney microbial DNA

Sequencing resulted in 3,416,038 16S rRNA amplicon read pairs, of which 3,330,609 were derived from Bacteria (97.5 %) and 85,429 were derived from Eukarya (rats) (2.5 %; Figures S1 and S4). The data showed large variation in the final number of reads per sample, ranging from 12 to 57,798 with a mean of 17,622 and median of 5388 reads per sample (Figure S2). Rarefaction curves indicate that sufficient reads were obtained to capture the variation present in the samples (Figure S3). After filtering the sequence data, we identified a total of 854 unique ASVs of which 504 could be classified to genus level and 229 to species level, resulting in 233 distinct bacterial genera and 173 distinct bacterial species. The most prevalent and abundant genera were *Streptococcus* (93 %), *Mycoplasma* (81 %) and *Leptospira* (54 %; Figure 1). These three genera already comprised almost 90 % of taxonomy counts. Many samples were dominated (> 50 % reads) by one of these three

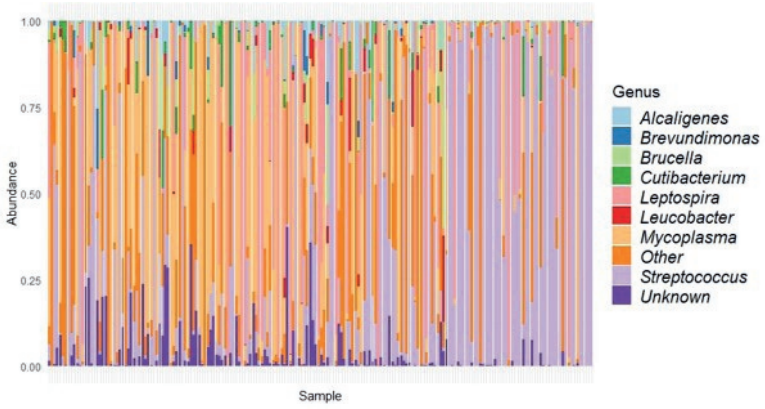

Figure 1. *Bar plot displaying the relative abundance of bacteria (y-axis) per sample (x-axis) at genus level. Samples are in order of 16S rRNA amplicon concentration from low (left) to high (right).*

genera, most often by *Streptococcus* (n = 59), followed by *Mycoplasma* (n = 39) and *Leptospira* (n = 25; Figure 1).

Potentially zoonotic bacteria detected using 16S rRNA gene amplicon sequencing

We identified 14 out of 233 bacterial genera as potentially zoonotic (Table 1). The prevalence and total number of reads of these potentially zoonotic bacterial genera varied considerably (Table 1). *Streptococcus, Mycoplasma* and *Leptospira* were detected with high prevalence and abundance, whereas other potentially zoonotic genera (*Bacillus, Mycobacterium, Chlamydia* and *Campylobacter*) and members of the Erysipelotrichaceae family were detected in very low quantities (< 0.1 % of total reads), which makes the presence of these genera in the samples uncertain. Most of these potentially zoonotic genera could also be identified to species level, resulting in seven potentially zoonotic bacterial species: *Leptospira interrogans, Brucella melitensis, Bartonella vinsonii, Staphylococcus aureus, Escherichia coli, Proteus mirabilis* and *Bacillus anthracis* (Table 1). From the NCBI BLAST, we identified (almost) identical matching zoonotic species for the same seven genera that were identified using the SILVA v138.1 species reference database. For some potentially zoonotic genera, more than one identical matching species was found, belonging to different genera. This was the case for *Brucella, E. coli, Bacillus* and Erysipelotrichaceae.

(Potentially) zoonotic bacteria: species identification and prevalence comparison based on 16S rRNA gene amplicon sequencing and qPCR results

From the 14 potentially zoonotic genera identified earlier, four genera (*Leptospira, Bartonella, Mycoplasma* and *Brucella*) were selected to compare the results from 16S rRNA gene amplicon sequencing with those from quantifications using PCR-based assays and to further identify them to species level. The *Leptospira* prevalence based on 16S rRNA gene amplicon sequencing reads (with > 2 reads per ASV per sample considered positive) was 55 % (103/189), and the prevalence based on qPCR (targeting pathogenic Leptospira species) was 46 % (86/189; 95 %). These two prevalences are not significantly different (binomial GLM; p = .100). In addition, we found a negative correlation between the 16S rRNA amplicon concentration and the qPCR Ct-values (Figure 2). In total, 133 samples (70 %) were positive in both assays, 36 samples (19 %) were positive in the 16S rRNA gene amplicon sequencing only and 20 samples (11 %) were positive in qPCR only. In a previous study, 22 of the rats used in this study were typed to serovar level and *L. interrogans* serovar Icterohaemorrhagiae (n = 8; 36 %) and serovar Copenhageni (n = 14; 64 %) belonging to serogroup Icterohaemorrhagiae were detected [11]. Both serovars are zoonotic. The identified species (*L. interrogans*) was the same as the species identified based on the SILVA database and BLAST result.

The *Bartonella* prevalence based on 16S rRNA gene amplicon sequencing reads was 18 % (33/189) and the prevalence based on qPCR (targeting a selection of 30+ *Bartonella* species) was 23 % (44/189). These two prevalences are not significantly different (binomial GLM; p = .161). We also found a negative correlation between the 16S rRNA amplicon concentration and the qPCR Ct-values for *Bartonella* (Figure 2). In total, 160 samples (85 %) were positive in

Table 1. *Potentially zoonotic bacterial genera detected in rat kidneys using 16S rRNA gene amplicon sequencing*

Potentially zoonotic genus	***Species according to 16S SILVA v138.1 reference database***	***Prevalence (%, n)[a]***	***Total nr of reads (%, n)***	***Nr of ASVs[b]***
Streptococcus	> 2 species (most prevalent one is *S. ruminantium)*	93 %, n = 176	59 %, n = 1,963,767	18
Mycoplasma	*M. coccoides* and *M. haemomacaque*	81 %, n = 153	7.9 %, n = 262,265	10
Leptospira	*L. interrogans*	54 %, n = 103	23 %, n = 750,323	2
Brucella	*B. melitensis*	38 %, n = 71	0.2 %, n = 6,175	1
Bartonella	*B. vinsonii*	17 %, n = 33	2.4 %, n = 81,158	2
Staphylococcus	> 2 species (most prevalent one is *S. aureus)*	17 %, n = 32	0.3 %, n = 8,911	10
Escherichia/ Shigella	*E. coli*	11 %, n = 20	0.9 %, n = 30,190	1
Corynebacterium	> 2 species (most prevalent one is *C. tuberculostearicum)*	10 %, n = 19	< 0.1 %, n = 2,066	14
Proteus	*P. mirabilis*	3 %, n = 5	0.2 %, n = 5,615	4
Bacillus	> 2 species (most prevalent one is *B. anthracis)*	2 %, n = 4	< 0.1 %, n = 306	4
Mycobacterium	N/A	1 %, n = 1	< 0.1 %, n = 70	1
Chlamydia	*C. muridarum*	1 %, n = 1	< 0.1 %, n = 32	1
Campylobacter	*C. sputorum*	1 %, n = 1	< 0.1 %, n = 5	1
Erysipelotrichaceae family	N/A	1 %, n = 1	< 0.1 %, n = 5	1

Abbreviation: ASV, amplicon sequence variant. [a]Prevalence: number of rats in which > 2 reads of a specific taxa was found with 16S rRNA gene amplicon sequencing, displayed in percentages and number of positive animals (=n). [b]Multiple ASVs, consisting of different DNA sequences, can be assigned to the same genus. [c]Shows matching species based on 16S DNA sequence. Identity percentage per BLAST result is shown, all sequences have 100 % query cover.

both assays, 9 samples (5 %) were positive in the 16S rRNA gene amplicon sequencing only, and 20 samples (11 %) were positive in qPCR only. DNA from 22 qPCR-positive rats was successfully isolated and sequenced. We observed high similarity (97.7 % – 100 %) of all sequences to *Bartonella tribocorum* (GenBank accession numbers MT741530 (gltA), MG027996 (gltA) and AF165996 (rpoB)). This species is potentially zoonotic [32]. The species *B. tribocorum* is different from the species identified based on the SILVA database (*B. vinsonii*).

For *Mycoplasma*, only a subset of 20 samples positive according to 16S rRNA gene amplicon sequencing were tested by PCR. From these, 19 out of 20 samples were positive in PCR and were further sequenced. From 14 samples we could obtain good sequences all with high similarity (99.8 % - 100 %) to Candidatus *Mycoplasma haemomuris* subspecies ratti (Genbank accession number AB758439), which is considered not zoonotic. This species is also different from the species identified based on the SILVA database (*M. coccoides*

BLAST result[c]	***BLAST identity %***	***Known zoonotic species***	***References for known zoonotic species***
> 2 *Streptococcus* species	> 97.05 %	*S. canis, S. iniae, S. suis* and *S. equi sub. zooepidemicus*	[33]
Mycoplasma spp.	> 98.95 %	*M. pulmonis* and *M. arginini*	[34, 35]
> 2 *Leptospira* species (including *L. interrogans)*	All 100 %	*L. interrogans*	[36]
> 2 *Brucella* species (including *B. suis* and *B. melitensis)* and *Ochrobactrum* spp.	All 100 %	*B. suis, B. melitensis, B. abortus* and *B. canis*	[37]
> 2 zoonotic *Bartonella* species	All 100 %	*> 14 different species*	[38]
> 2 *Staphylococcus* species (including *S. aureus)*	> 99.75 %	*Methicillin resistant S. aureus* and *S. pseudointermedius*	[39, 40]
E. coli, Escherichia spp. and *Shigella flexneri*	All 100 %	*ESBL/AmpC producing E. coli*	[41]
> 2 *Corynebacterium* species	> 99.48 %	*C. ulcerans, C. diphtheriae* and *C. pseudotuberculosis*	[42]
> 2 *Proteus* species (including *P. mirabilis)*	> 99.75 %	*P. mirabilis*	[43]
> 2 *Bacillus* species (including *B. anthracis), Neobacillus* spp., *Cytobacillus firmus* and *Priestia* spp.	All 100 %	*B. anthracis*	[44]
M. wolinskyi, Mycobacterium spp.	All 100 %	*M. bovis, M. avium*	[45]
C. muridarum	100 %	*C. trachomatis*	[46]
C. sputorum	100 %	*C. jejuni* and *C. coli*	[47]
Erysipelotrichaceae spp., *Erysipelatoclostridium* spp., *Clostridium* spp., *Longibaculum* spp. and *Faecalibacillus* spp.	All 100 %	*E. rhusiopathiae*	[48]

and *M. haemomacaque*). Similarly, a subset of 20 samples that were positive for *Brucella* according to 16S rRNA gene amplicon sequencing results were tested by culture and qPCR. The BLAST result of the 16S *Brucella* sequence resulted in high similarity with both *Brucella* (including species *B. suis* and *B. melitensis*) and *Ochrobactrum* (Table 1). However, the presence of *Brucella* spp. could not be confirmed by either qPCR or culture.

Influence of internal and external factors on carriage of zoonotic bacteria

We investigated the effect of species, sex, bodyweight and location type on *Leptospira* and *Bartonella* carriage in wild rats, using the results from both 16S rRNA gene amplicon sequencing and qPCR, using binomial GLMMs. From both 16S rRNA gene amplicon sequencing and qPCR results, we observed that bodyweight was positively correlated with *Leptospira* carriage ($p < 0.01$; Table S3). For *Bartonella*, we observed a significantly higher prevalence in brown rats compared to black rats from both 16S rRNA gene amplicon sequencing and qPCR results ($p < 0.05$; Table S3).

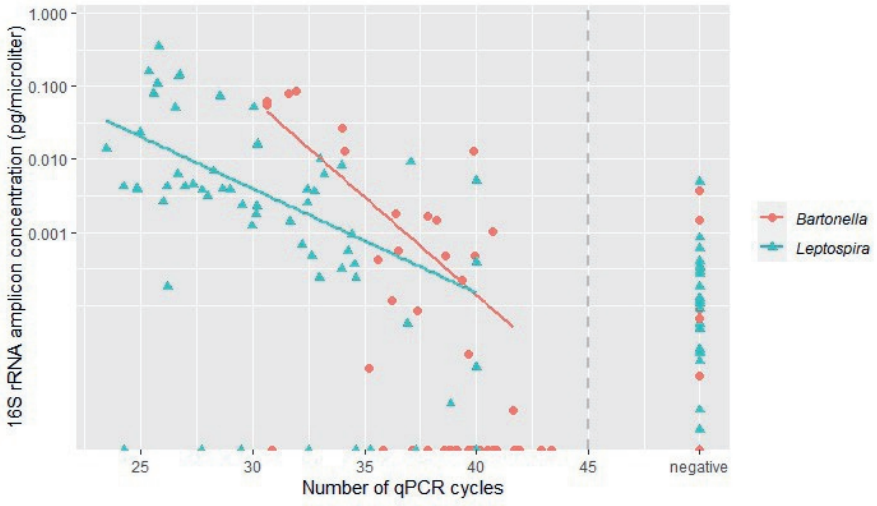

Figure 2. *Comparison between the number of qPCR cycles and the 16S rRNA amplicon concentration for both* Leptospira *and* Bartonella*. The qPCRs are pathogen-specific and shows at which cycle number the samples were found positive. The 16S rRNA amplicon concentration was calculated by multiplying the percentage of* Leptospira *or* Bartonella *reads per sample with the 16S rRNA gene amplicon concentration per sample. The y-axis is log-scaled. Samples with Ct values > 45 and/or non-sigmoidal curves are included in 'negative'. The qPCR cut-off value of 45 is represented by a grey dashed line. There is a negative correlation between the 16S rRNA amplicon concentration and the number of qPCR cycles for both* Leptospira *and* Bartonella*.*

Influence of in- and external factors on kidney bacterial composition

We visualized the overall differences in beta diversity in Bray–Curtis dissimilarity ordination (PCoA) plots for the factors species, location type, age and sex (Figure 3). Significant differences were observed for a multi-variable model, including species, location type and age (PERMANOVA, $p < 0.05$). The kidney bacterial composition of brown and black rats was also significantly different (PERMANOVA $p < 0.05$), whereas both dispersions were homogenous (betadisper $p > 0.05$; Figure 3a). This difference was attributed to significant differences in the abundance of *Streptococcus* (3 ASVs), *Leptospira* and *Mycoplasma* (SIMPER, Kruskal–Wallis $p < .05$; Table S4), where the abundance of all taxa was higher in brown rats compared to black rats, except for two out of three *Streptococcus* ASVs.

Significant differences were also observed for location type (urban, rural, agriculture and industry; PERMANOVA $p < 0.05$) while dispersions were not homogenous (betadisper $p < 0.05$), which makes it uncertain whether the observed differences are indeed significant differences in group means or caused by dispersion variation between samples (Figure 3b). Differences between location types were predominantly attributed to differences in the abundance of *Streptococcus, Leptospira* and *Mycoplasma* (SIMPER, Kruskal–Wallis $p < 0.05$; Table S4). A lower mean abundance of *Leptospira* in industry was observed compared to the other three location types (urban, rural and agriculture).

Significant differences between age classes were observed (PERMANOVA $p < 0.05$) while dispersions were homogenous (betadisper $p > 0.05$; Figure 3c). Differences between adult and sub-adult and between adult and juvenile were both attributed to a difference in the

abundance of *Leptospira*, which was higher in adults compared to sub-adults and juveniles (SIMPER, Kruskal–Wallis $p < 0.05$; Table S4). Because age class is based on bodyweight, this result coincides with our earlier finding that *Leptospira* carriage was positively correlated with bodyweight. For sex, no significant differences in beta diversity were observed (Figure 3d).

As might be expected, the genera mostly contributing to differences in beta diversity (*Streptococcus, Leptospira* and *Mycoplasma*) are also the main dominant genera identified earlier. The influence of these dominant genera on differences in beta diversity was visualized in a PCoA plot (Figure 4). Significant differences were observed between *Leptospira* and all other groups (*Mycoplasma, Streptococcus*, other and none) and between none and both *Streptococcus* and *Mycoplasma* (PERMANOVA $p < 0.05$). However, there was large variation in the dispersion of the data (betadisper $p < 0.05$).

Relation between sequencing depth, alpha diversity and contamination

We observed that the Shannon diversity index was negatively correlated with the total number of reads per sample (Spearman correlation $R = -0.7$; $p < 0$ .05; Figure 5), which implies that samples with higher numbers of reads are less diverse than samples with lower numbers of reads (Figure 5). This coincides with the domination patterns we observed before in these low microbial load samples (Figure 1). This high diversity in samples with low numbers of reads is probably related to contamination, which was detected in sequenced mock communities as well (Figure S1). Besides that, we observed that the percentage of host DNA blocked by the blocking primers varied per sample and that the percentage of eukaryotic reads in the sample were negatively correlated to the 16S rRNA concentration (Figures S1 and S4).

Zoonotic viruses detected in rat liver

Rat liver samples were used for the detection of DNA and RNA viruses. Besides very low levels of different virus sequences, we detected only two viruses at levels of infecting agents: *Minute virus of mice (Rodent protoparvovirus 1*) and rat *Hepatitis E virus* (rat HEV) (also referred to as Orthohepevirus C). Of these two viruses, only rat HEV is potentially zoonotic. In total, 3/189 rats (6.3 %) tested positive for rat HEV with both VirCapSeq sequencing and qPCR. The rat HEV sequence was detected in two black rats trapped on a pig farm in the southern province of Noord-Brabant (2016) and in one brown rat trapped on an industrial location close to the harbour in Amsterdam (2014). One positive sample was further analysed, and the genome assemblies demonstrated that an almost full genome could be regenerated (ON644869). The sequence had most identity (87.96 % 1–2517 bp and 87.43 % 2619–6942 bp) with sequence KM516906 from the United States, followed by sequence MW795566 from a wild brown rat from Hungary captured in 2010 (86.82 % 1–2517 bp and 87.01 % 2619– 6941 bp) [49], and sequence GU345043 (86.39 % 1–2516 bp and 86.88 % 2631–6945 bp) from a wild brown rat from Germany captured in 2009 [50]. When we aligned the previously mentioned sequences with our sequences we observed a gap of 66 bp from 2522 to 2587 bp.

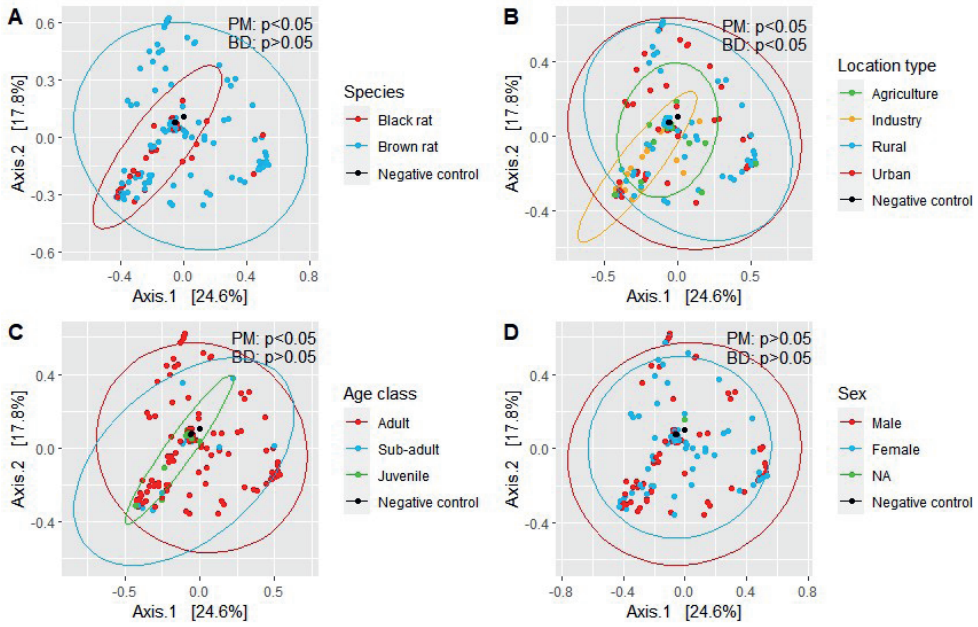

Figure 3. *Principal coordinate analysis (PCoA) of wild rats' kidney bacterial composition based on the Bray–Curtis dissimilarity, visualized per rat species (a), location type (b), age class (c) and sex (d). Ellipses are computed with 95 % coverage. BD, betadisper* p-*value; PM, PERMANOVA* p-*value*

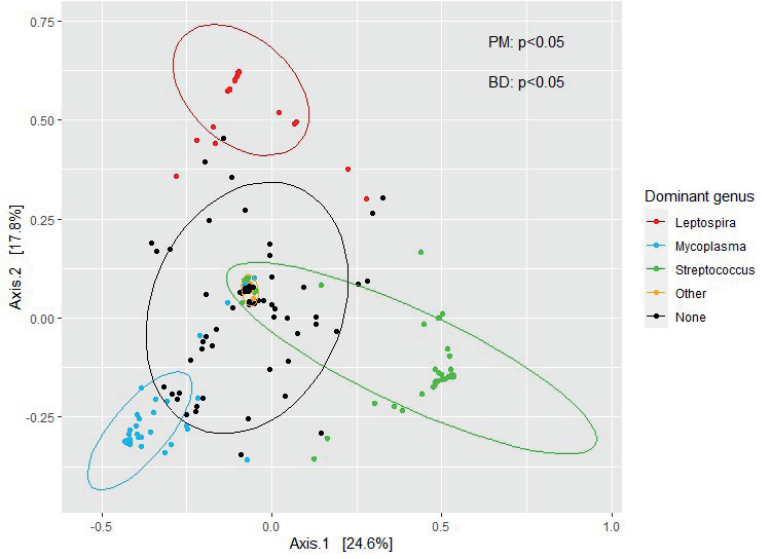

Figure 4. *Principal coordinate analysis (PCoA) of wild rats' kidney bacterial composition with Bray–Curtis distance. Visualized per dominating genus (> 50 % of total reads) per sample. Category 'none' consists of samples without a dominating taxa and 'other' consists of samples with a dominating taxa other than* Streptococcus, Leptospira *or* Mycoplasma. *BD, betadisper* p-*value; PM, PERMANOVA* p-*value*

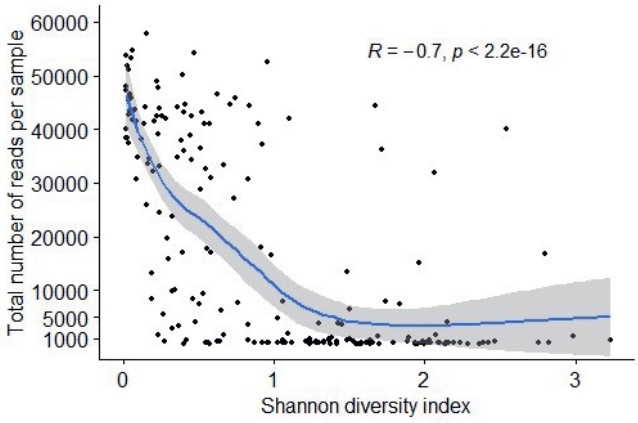

Figure 5. *Shannon diversity index per total number of reads per sample. Each dot represents a sample. Correlation was tested using Spearman correlation coefficients (R = –.7;* p < *2.2e*$^{-16}$*).*

Discussion

In this study, we investigated if 16S rRNA gene amplicon sequencing would be a suitable tool for the detection of zoonotic bacteria in wild rats. These results were compared with (q-)PCR results. In addition, we used VirCapSeq-enriched sequencing on liver samples to detect zoonotic viruses. We also examined if there were internal or external factors correlated with zoonotic pathogen carriage in these wild rats.

Zoonotic bacteria detected in rat kidney

Using 16S rRNA gene amplicon sequencing, we identified 14 potentially zoonotic bacterial genera and 7 potentially zoonotic bacterial species in rat kidneys. We selected four highly prevalent genera for confirmation of results and to be further tested to species level. The presence of zoonotic *Leptospira* species (*L. interrogans* serovar Icterohaemorrhagiae and Copenhageni [11]) and a potentially zoonotic *Bartonella* species (*B. tribocorum*) were confirmed. *B. tribocorum* has been detected previously in both *R. norvegicus* and *R. rattus* [1, 51]. The *Mycoplasma* sequence was identified as Candidatus *M. haemomuris* subspecies ratti, which is considered not zoonotic. However, the *Brucella* sequences identified by 16S rRNA gene amplicon sequencing could not be confirmed by either culture or qPCR. BLAST results from the *Brucella* 16S DNA sequence resulted in a match for both *Brucella* and *Ochrobactrum* species. Therefore, although it was taxonomically assigned to *Brucella*, the DNA sequence probably belonged to *Ochrobactrum*.

The identification to species level based on the 16S SILVA database and qPCR sequencing resulted in both similar (for *Leptospira*) and different species (for *Bartonella, Mycoplasma* and *Brucella*). This emphasizes the need to confirm 16S rRNA gene amplicon sequencing results by other established detection methods and to interpret these results with caution, especially in the case of zoonotic bacteria. Overall, the 16S rRNA gene amplicon sequencing results did not indicate the presence of emerging or unexpected zoonotic bacteria in

these wild rats. However, in this study, we tested only rat kidney tissue, likely leading to an underestimation of the number of zoonotic bacteria present in these rats. Therefore, the inclusion and comparison of multiple tissues per animal may improve future studies.

Comparing 16S rRNA gene amplicon sequencing with qPCR results

The results generated by 16S rRNA gene amplicon sequencing and qPCR showed 70 % correlation for *Leptospira* and 85 % for *Bartonella*. This resulted in non-significantly different pathogen prevalence estimates based on the threshold values used in this study (> 2 reads considered positive and pathogen-specific Ct-value cut-offs). We also observed a negative correlation between the number of qPCR cycles and the 16S rRNA amplicon concentration per zoonotic bacterium, which shows the resemblance of the results obtained with both methods, and which also has been observed previously [52]. In addition, both methods resulted in similar significant correlations between *Leptospira* carriage and weight, and between *Bartonella* carriage and rat species. Therefore, 16S rRNA gene amplicon sequencing could also be used to generate population prevalence estimates.

The observed differences in results from both detection methods can be caused by various factors, including the choice of threshold values, the primers used for amplicon sequencing, the specificity of the qPCRs and the influence of contamination in these low microbial biomass samples. Especially for samples that were negative in qPCR and positive in 16S rRNA gene amplicon sequencing, the species detection range of the qPCRs we used could have limited the detection of certain species. However, standardized experiments using dilutions of known samples in different bacterial combinations should be performed to correctly compare the sensitivity and specificity of these two methods and to potentially define positive infection thresholds for specific bacteria when using 16S rRNA gene amplicon sequencing [5].

Influence of in- and external factors on zoonotic pathogen carriage and kidney bacterial composition

We investigated the relation between internal (species, sex and bodyweight/age) and external factors (location type) and zoonotic pathogen carriage. We observed a significant positive correlation between *Leptospira* carriage and rat bodyweight, indicating that heavier rats, and thereby most likely also older rats, are more likely to carry *Leptospira*. This result is in-line with previous research [53, 54]. For *Bartonella*, we observed a significant difference between rat species, with a higher prevalence of *Bartonella* in brown rats (29.3 %) compared to black rats (2.4 %), which agrees with results reported in other studies [55-57]. Some studies explained this difference in prevalence by a similar difference in ectoparasite infestation levels [57], but that alone could not always fully explain the observed difference [58]. In light of risk surveillance, these results suggest the surveillance of zoonotic pathogens in wild rats should focus on adult brown rats.

We also investigated the influence of in- and external factors in a broader perspective, by looking at their correlation with the total bacterial diversity of a sample instead of with only the presence or absence of *Leptospira* and *Bartonella*. We found significant bacterial diversity differences associated with species and age, which could be mainly attributed to

differences in the abundance of *Streptococcus, Leptospira* and *Mycoplasma*, the three most dominant bacteria identified. Differences in bacterial diversity associated with species and age have also been observed in previous studies on rodent gut microbiome [59, 60]. *Streptococcus* was divided over three ASVs of which one ASV was more abundant in brown rats and the other two ASVs were more abundant in black rats. Therefore, we suspect there are multiple *Streptococcus* species present with different host specificities. *Mycoplasma* abundance was higher in brown rats, but the reason behind this is unclear.

Bacterial domination in rat kidneys

We observed that almost 70 % of all samples were dominated (> 50 % reads) by one genus, mainly *Streptococcus*, *Leptospira* or *Mycoplasma*. The presence of dominating taxa is in-line with previous studies looking at the bacterial composition of urine [61, 62]. In human female urine, these dominating taxa mostly consist of *Lactobacillus, Streptococcus, Corynebacterium, Bifidobacterium, Staphylococcus* and *Prevotella* [62, 63]. These bacteria were also detected in the rat kidney samples. In the past, this domination was linked to the presence of disease, but recent studies suggest more complex interactions where domination can be linked to both disease susceptible and protective effects [64]. For example, a protective effect could be that commensal bacteria outcompete pathogenic bacteria for nutrients, produce antimicrobial substances or stimulate the host immune system [65]. *Lactobacillus, Streptococcus* and *Bifidobacterium* have been identified as human commensals of the urine microbiome [66]. The following bacteria have been related to the kidney microbiome of healthy human subjects and also occur in rat kidneys from this study: *Microbacterium, Pelomonas, Staphylococcus, Streptococcus, Leuconostoc, Corynebacterium, Anaerococcus* and *Thermicanus* [67]. Therefore, these bacteria might be considered commensals of the rat kidney. *Streptococcus* was present in 93 % of all rat kidney samples and did not match with known zoonotic *Streptococcus* species. We, therefore, suspect *Streptococcus* to be a commensal of the rat kidney as well. This could also be the case for *Mycoplasma* (present in 81 % of all samples and identified as non-zoonotic), but more research is needed to identify the true bacterial community composition of the rat kidney.

Bacterial community or contamination?

In this study, rat kidney samples were analysed, which are typically low microbial biomass samples in which only few bacteria are expected to be found, unless the animals were heavily infected. There was large variation in the total number of reads per sample with almost 40 % of all samples having < 1000 reads in total, which is in-line with the low measured 16S rRNA amplicon concentration in the samples. Similar data was obtained in a study investigating the urine microbiome of cats, where more than 50 % of samples had < 500 reads [68]. We also observed a negative correlation between the total number of reads per sample and the Shannon diversity index, which indicates that samples with low numbers of reads (roughly < 1000 reads) show profiles that are indicative of contamination or that those bacteria are present in very low concentrations. This negative correlation is the opposite result of studies performed on the gut microbiome [69], but in-line with other studies performed on low microbial biomass samples [70, 71]. Although low numbers of reads are to be expected in low microbial biomass samples such as kidney, the low number of reads complicates distinguishing between DNA from bacteria truly present in

the sample (although in low numbers) and DNA from contaminants. Therefore, results with low numbers of reads should be interpreted with caution. In this study, we used only three control samples. Using a larger number of control and also mock samples in future studies will facilitate the identification of possible contaminants. This is especially important for epidemiological studies focusing on zoonotic bacterial genera that are also considered common contaminants [5].

Cross amplification of mitochondrial mammalian DNA during sequencing in low microbial biomass samples can also negatively affect the amount of bacterial DNA sequenced [65]. To reduce the interference of host DNA during sequencing, we designed blocking primers. Though the percentage of host DNA blocked varied per sample, it was negatively correlated with the 16S rRNA gene concentration, which implies that the blocking primers are less efficient in blocking host DNA in samples that contain only very few bacteria. To further reduce host DNA contamination, specific DNA isolation kits could be used that maximize the isolation of bacterial DNA [65]. Although low microbial biomass samples such as kidney bring new challenges regarding interpretation and accuracy, they also have an advantage compared to high microbial biomass samples: Low microbial biomass samples have less commensal/background bacteria, which makes it relatively easier to identify zoonotic bacteria.

Virus detection in rat liver

Only one potentially zoonotic virus was detected at levels of infecting agents: rat HEV. The whole genome could be sequenced except for the part between 2522 and 2587 bp, which could indicate a deletion in our sequence of 66 bp. This virus was detected in two black rats trapped on a pig farm and in one brown rat trapped on an industrial location, which, to our knowledge, is the first time that rat HEV has been detected in wild rats in the Netherlands. Rat HEV has been detected in both brown and black rats in other European countries [72] and has recently also been found in humans [73]. The rat HEV-positive rats were trapped in 2014 and 2016. Therefore, it would be interesting to include rat HEV in current surveillance studies to investigate the infection prevalence in rats and the risk for public health.

Conclusions

Using 16S rRNA gene amplicon sequencing, we identified possible zoonotic bacteria in rat kidney samples, and we obtained a better overview of the rat kidney bacterial composition and the apparent domination of certain bacteria. Prevalence estimates and subsequent correlation analyses using both 16S rRNA gene amplicon sequencing and qPCR data were not significantly different, indicating that 16S rRNA gene amplicon sequencing could also be a suitable tool to give indications of population pathogen prevalence and correlations. Moreover, using VirCapSeq-enriched metagenomic sequencing, we detected rat HEV in wild rats.

Although 16S rRNA gene amplicon sequencing has the advantage to detect multiple pathogens at once and certainly has the potential to be a suitable tool for detection of new zoonotic pathogens and potentially pathogen surveillance, there are several limitations that should be considered when using this method. First, 16S rRNA gene amplicon sequencing can in most cases reliably identify bacteria to genus level only, so subsequent identification

or confirmation to species level should be performed when identifying potentially zoonotic bacteria. Second, the low numbers of reads from low microbial biomass samples make it more difficult to distinguish between bacteria present in the sample and contaminating bacteria. Therefore, it is important to use sufficient control and mock samples in future studies to be able to better distinguish them. Future studies could also investigate the effects of using pooled samples (either from multiple animals or from multiple tissues per animal) to decrease laboratory effort and costs. Currently, we recommend to still use more established methods such as (q)PCR for pathogen detection and to use 16S rRNA gene amplicon sequencing as a complementary or pre-screening method. Especially when only a limited number of pathogens are of interest, qPCR is currently still less time-consuming and less expensive. When more reliable identification to species level would be possible in the future, 16S rRNA gene amplicon sequencing could be a very promising technique for the surveillance of zoonotic bacteria.

Acknowledgements

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Supplementary data

Table S1. *Characteristics of the wild rats used in this study*

		Rattus norvegicus	*Rattus rattus*
Total		147	42
Location type	Urban	52	0
	Rural	83	9
	Agriculture	1	13
	Industry	11	20
Municipality	Amsterdam	35	20
	Appeltern	20	0
	Baarle-Nassau	0	8
	Gemert-Bakel	0	2
	Leudal	22	11
	Maastricht	20	0
	Nederweert	0	1
	Nijmegen-Doetinchem	25	0
	Roermond	25	0
Sex	Male	84	21
	Female	61	21
Weight	Average (95 % CI)	265.3 g (172.0 – 358.7)	156.7 (102.1 – 211.3)[a]
Age class	Juvenile	6	5
	Sub-adult	19	7
	Adult	122	30

[a]Black rats average weight was calculated based on 29/42 rats because the weight from 13 adult rats was not measured. Therefore, the average weight for black rats in our sample is likely underestimated.

Table S2. *Mitochondrial 16S rRNA amplicon sequences from other mammals used during blocking primer development.*

Animal	**Accession number**	**Animal**	**Accession number**
Bison bonasus	KX553931	*Microtus arvalis*	NC038176
Bos taurus	DQ124418	*Mus musculus*	JQ003190
Capreolus capreolus	KJ681491 and NC020684	*Mustela nivalis*	NC020639
Cervus elaphus	KP172593 and NC007704	*Myodes glareolus*	KM892809 and NC024538
Crocidura russula	NC006893	*Ovis aries*	NC001941 and KR868678
Dama dama	NC020700	*Rattus norvegicus*	NC001665
Erinaceus europaeus	NC002080	*Rattus rattus*	NC012374
Homo sapiens	DQ112955	*Sciurus vulgaris*	NC002369
Lepus europaeus	NC004028 and KY221030	*Sus scrofa*	FJ236995
Martes martes	NC021749	*Talpa europaea*	NC002391 and MF958963
Meles meles	NC011125	*Vulpes vulpes*	NC008434

Table S3. *Prevalence estimates of* Leptospira *and* Bartonella *carriage based on 16S rRNA gene amplicon sequencing and qPCR results, shown per internal or external factor. More than 2 reads was considered positive for 16S rRNA gene amplicon sequencing. An asterisk (*) depicts a significant difference within or correlation with a factor using the 16S rRNA gene amplicon sequencing or qPCR dataset analyzed using GLMMs; * = p < 0.05, ** = p < 0.01 and *** = p < 0.001.*

Prevalence (%)			***Leptospira***		***Bartonella***	
		n	***16S rRNA***	***qPCR***	***16S rRNA***	***qPCR***
Total		*189*	54.5	45.5	17.5	23.3
Species	*Rattus norvegicus*	*147*	58.5	56.5	21.7*	23.1*
	Rattus rattus	*42*	40.5	9.5	2.4*	2.4*
Location type	Urban	*52*	71.2	63.5	28.8	25.0
	Rural	*92*	51.1	52.2	17.4	22.8
	Agriculture	*14*	85.7	7.1	7.1	0.0
	Industry	*31*	22.6	16.1	3.2	3.2
Sex	Male	*105*	53.3	43.8	17.1	20.0
	Female	*82*	54.9	47.6	18.3	17.1
Weight	Coefficient estimate	*189*	0.0080***	0.0068**	0.0022	-0.0004

Table S4. *Results from SIMPER beta-diversity comparisons that were significantly different between groups according to Kruskal-Wallis tests using fdr-adjusted* p*-values > 0.05.*

Factor	**SIMPER comparison**	**Mean abundance group A**	**Mean abundance group B**	**Contribution to ordination (%)**	**p-value**	**Taxa**
Species	Black rat (A) vs Brown rat (B)	0.08	0.31	18.3	0.02	*Streptococcus*
		0.04	0.18	11.1	0.03	*Leptospira*
		0.18	0.01	10.3	< 0.01	*Streptococcus*
		0.12	0.01	7.2	0.04	*Streptococcus*
		0.00	0.03	2.0	0.04	*Mycoplasma*
Location type	Industry (A) vs Urban (B)	0.07	0.34	20.5	< 0.01	*Streptococcus*
		0.01	0.23	13.6	< 0.01	*Leptospira*
		0.15	0.00	9.1	0.04	*Streptococcus*
	Industry (A) vs Rural (B)	0.43	0.12	23.0	< 0.01	*Mycoplasma*
		0.01	0.17	9.6	0.01	*Leptospira*
		0.15	0.00	8.6	< 0.01	*Streptococcus*
	Industry (A) vs Agriculture (B)	0.43	0.15	24.1	0.01	*Mycoplasma*
		0.01	0.06	3.7	0.03	*Leptospira*
		0.00	0.05	3.2	0.03	*Proteobacteria*
	Urban (A) vs Rural (B)	0.22	0.12	16.1	< 0.01	*Mycoplasma*
	Urban (A) vs Agriculture (B)	0.34	0.12	20.0	0.03	*Streptococcus*
		0.00	0.05	3.1	0.01	*Proteobacteria*
	Rural (A) vs Agriculture (B)	0.02	0.15	9.3	0.01	*Streptococcus*
		0.00	0.05	3.0	< 0.01	*Proteobacteria*
Age class	Adult (A) vs Sub-adult (B)	0.17	0.08	13.0	0.02	*Leptospira*
	Adult (A) vs Juvenile (B)	0.17	0.00	9.2	0.02	*Leptospira*

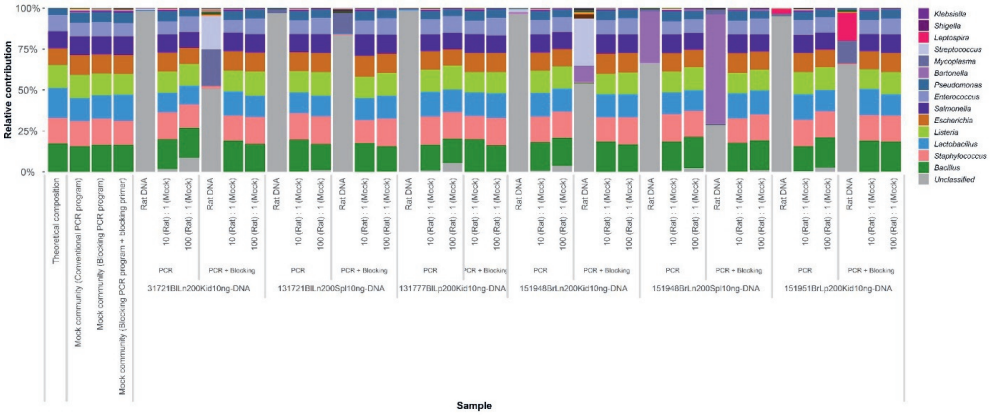

Figure S1. *Relative contribution of the blocking primer to the blocking of host DNA and increased yield of bacterial 16S rRNA amplicon sequences. Individual rat samples are described here as 'Rat DNA'.*

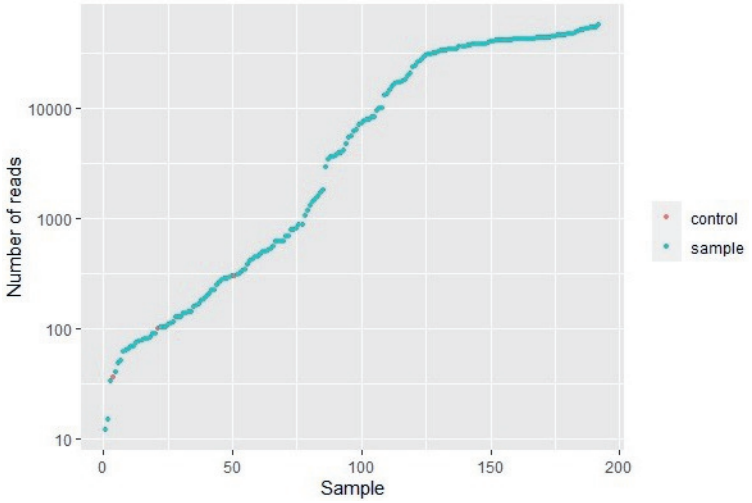

Figure S2. *Raw number of reads per sample. Each dot (blue) represents one sample. Negative control samples are depicted in red.*

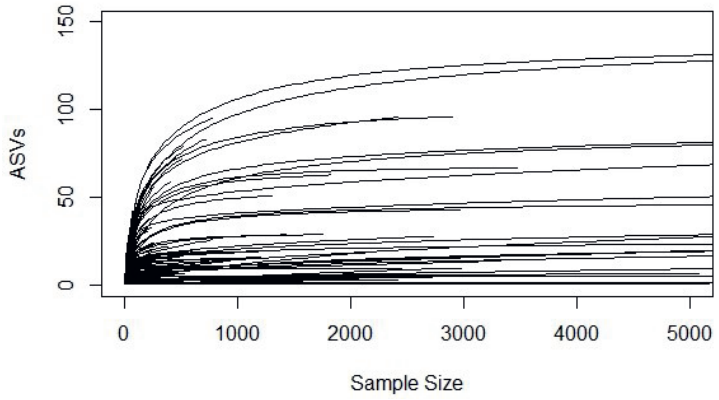

Figure S3. *Rarefaction curves showing the number of detected ASVs per number of reads sampled per sample.*

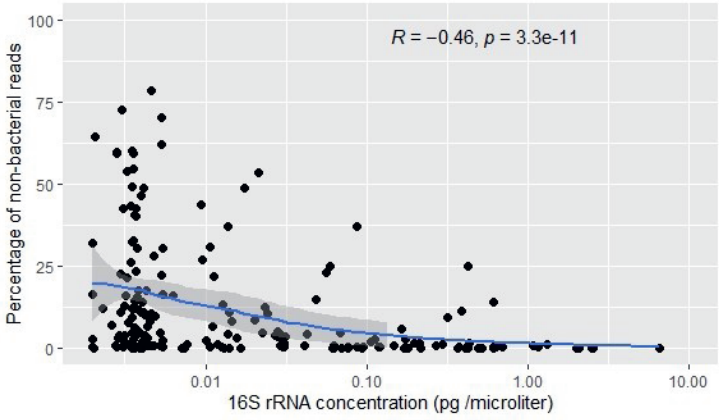

Figure S4*. Percentage of non-bacterial reads per sample versus the 16S rRNA gene concentration (pg/µL) after the use of blocking primers. Correlation was tested using Spearman correlation coefficients (R = -0.46;* p = $3.3e^{-11}$*).*

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Café

Chapter 4

Higher rat abundance in greener urban areas

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Abstract

Urban greening has become an increasingly popular strategy to improve urban life and human health. However, there are indications that the presence and extent of urban greenness may increase the abundance of wild rats. Therefore, we investigated which environmental and socio-economic factors are associated with rat abundance, with a focus on factors related to urban greenness. We systematically trapped rats (222 *Rattus norvegicus* and 5 *Rattus rattus*) in parks and residential areas in three cities in the Netherlands. We modelled the relative abundance of rats against various environmental and socio-economic variables. In addition, we compared municipality rat complaint data with our trapping data and analysed trap success over time. We observed positive relationships between the relative abundance of rats and both greenness (NDVI) and different proxies for food resources (restaurants and petting zoos). In addition, there were more municipality rat complaints in residential areas compared to parks, while there was a higher relative abundance of rats in parks. Our findings corroborate that greenness is associated with a higher abundance of wild rats, and that municipality rat complaints may underestimate the abundance of rats in greener urban areas. This study provides new insights on factors affecting relative rat abundance in cities and can guide policy makers and city planners how to minimize rat nuisance in the greener parts of cities. By taking these potential effects of urban greenness on rat abundance into account, measures can be taken that on the one hand maintain the beneficial effects of urban greening, but at the same time reduce the carrying capacity for rats.

Introduction

The anthropophilic nature of wild brown rats (*Rattus norvegicus*) and black rats (*Rattus rattus*) has made them some of the most successful and abundant mammal species in urban areas [1]. They are considered pest species that need to be controlled to prevent gnawing damage, mental stress, and zoonotic pathogen transmission [2-4]. However, in practice rats are rather difficult to control, in part due to their neophobic behaviour towards new things, high adaptability to new environments, and level of cognition [1, 5, 6]. In order to prevent uncontrolled growth of rat populations and to optimize control measures, insight is needed in the environmental and socio-economic factors that promote rat abundance in urban areas.

Previous studies showed that rat abundance can be explained by the presence of (food) waste, the maintenance/age/ownership of buildings and houses, the presence of impervious surfaces, the number of restaurants in the area, socio-economic status of inhabitants, the type of sewage system present and human population density [7-10]. These environmental and socio-economic factors either directly or indirectly affect the availability of food and/or shelter. For example, income and the percentage of owner-occupied houses have been shown to be negatively related to rat abundance, probably because areas with higher socio-economic status have less (food) littering in public areas and improved residential upkeep, limiting shelter opportunities [11, 12]. Shelter can vary from natural soil for making burrows to badly maintained buildings, or sewers [7, 8]. The condition of sewers can deteriorate over time, e.g., by cracks and blind ends, increasing the likelihood of rats using them as shelter. Combined sewage systems are particularly at risk, as they are generally older than separated sewage systems [8]. However, the relative importance of these environmental and socio-economic factors seems to vary even within cities over very short geographical distances [7, 9].

Another potentially important but understudied factor for explaining rat abundance is the extent of vegetation or "greenness" of urban areas. Urban green spaces could provide food and shelter, two important requirements for rats, besides water [11, 13]. Previous studies found fewer rats when vegetation was absent and pavement was present [7, 10, 11], which suggests that rat abundance is positively correlated with greenness, but detailed studies are lacking. A better understanding of the relationship between rat abundance and greenness is important, as urban greening is an increasingly popular strategy to increase mental health, biodiversity, and water retention, and to reduce urban heat island effects [14-16]. However, its potential negative effects, such as potential increases in the abundance of pest species, have not been thoroughly investigated. We hypothesize that greener areas can provide a high availability of human food waste (which can get easily stuck or hidden in dense vegetation), and natural food resources (e.g., fruit and nut trees). Moreover, greener areas may provide a more natural environment for digging burrows, potentially increasing shelter opportunities for rats. This could in turn result in decreased predation risk, all of which may lead to a higher abundance of rats. Alternatively, predation risk might also be higher in greener urban areas due to higher predator abundance, which would negatively affect rat abundance. The net effect of these opposing forces remains unclear. Increasing our knowledge about the relationship between rat abundance and

greenness, in relation to other environmental and socio-economic factors may enable smarter city design, and more effective preventive and control measures.

Here, we investigated the associations between rat abundance and various environmental and socio-economic factors in urban areas, with a focus on greenness. We systematically trapped rats in three cities, and modelled the relationship between these factors and relative rat abundance. Data on relative rat abundance was also compared with municipality rat complaint data to examine how well municipality rat complaint data reflects actual rat abundance. Furthermore, we investigated which factors affected trapping success over time to optimize rat trapping.

Materials & Methods

Ethics statement

This study was approved by the Dutch Animal Experiments Committee (DEC) (project number AVD3260020172104).

Study area & rat trapping

Rats were trapped in the cities of Amsterdam, Eindhoven, and Rotterdam, the Netherlands (Fig. 1). Amsterdam (219 km^2) is the capital and the largest city of the Netherlands (41,850 km^2), and is inhabited by approximately 870,000 people. Rotterdam (324 km^2; approximately 590,000 inhabitants) and Eindhoven (89 km^2; approximately 240,000 inhabitants) are respectively the second and fifth largest cities in the Netherlands [17]. Both Amsterdam and Rotterdam have a river running through the city, in contrast to Eindhoven. Brown rats are present in all three cities, but only in Eindhoven black rats are also present in the city. All cities have a similar temperate maritime climate and a similar composition of urban wildlife species. Trapping locations were divided into parks and residential areas. In each city, trapping locations were selected based on the percentages of greenness, according to the 'Green map' of the Netherlands [18]. In residential areas, we selected locations with varying percentages of greenness (about half of the locations < 40 % greenness and half > 40 % greenness) to ensure sufficient variation in the percentage of greenness between trapping locations (Fig. 2). The minimum distance between locations was 200m.

Wild brown rats (*Rattus norvegicus*) and black rats (*Rattus rattus*) were trapped using snap traps (AF Rat Box together with either the Snap-E Rat trap (Killgerm, the Netherlands) or the Gorilla rat trap (Futura, Germany)). Relative abundance of rats can also be assessed using live traps [7, 12], but we chose to use snap traps because this study was part of a larger study in which rats were tested for zoonotic pathogens [19]. Rats were trapped alternatingly in Amsterdam and Eindhoven between May and October 2020, and one year later in Rotterdam in the same months as Amsterdam the year before. In total, rat trapping took place in 48 different locations (18 in Amsterdam, 18 in Rotterdam, and 12 in Eindhoven) divided over 16 parks and 32 residential areas (Fig. 1 and Table S1).

Per trapping location, 20 traps were placed within an area of approximately 100x100m. In most cases, trapping took place in public areas. When public areas were not available,

we used front gardens from private properties after consent from the owners. Traps were randomly placed and evenly distributed (minimum distance of 10 m between traps) along walls and fences or under vegetation. Furthermore, traps could only be positioned in places where they did not obstruct sidewalks and where they could be secured using ground anchors to prevent theft. One trapping period lasted four consecutive weeks, consisting of two weeks of pre-baiting to reduce neophobic behaviour [6] followed by two weeks of trapping. The traps were pre-baited with a 1:1:1 mixture of oats, peanuts, and cocktail nuts. During the two weeks of trapping, traps were set and checked each weekday around the same time. When bait had been eaten, the traps were resupplied. On weekends, the traps were baited but non-active. On Mondays, the traps were activated again, resulting in 7 or 8 active trapping days per trap. For each rat, the date and location (GPS coordinates) of trapping was recorded. Rats were classified into age categories based on their bodyweight. For males we used: juvenile (< 100 g), subadult (101-200 g), and adult (> 200 g), and for females we used: juvenile (< 100 g), subadult (101-175g), and adult (> 175 g) [20].

Relative rat abundance

For each trapping day and location, we recorded the number of traps set, the number of rats trapped, and the number of traps triggered for other reasons (e.g., trapping non-target species or traps damaged by people). To compare wild rat abundance between locations, we calculated the relative rat abundance per location using a trap success index [9]. From now on, we will use the term relative rat abundance to refer to this trap success index. This index was calculated using the formulas below [21, 22]. Stolen or damaged traps were excluded from these calculations. We subtracted half of the traps triggered by other reasons because it is not possible to know whether they were inactive from the beginning, during, or at the end of the trapping night. Therefore, it is assumed that on average these traps were inactive half of the time [21].

$$\text{trap index} = \#\text{ rats trapped} * \frac{100}{(\#\text{ effective traps} * \#\text{ trapping nights})}$$

$$\#\text{ effective traps} = \#\text{ fully functional traps} - 0.5 * \#\text{ traps sprung for other reasons}$$

Municipality rat complaint data

Besides quantifying relative rat abundance using snap or live traps, rat complaint data is used as a proxy for rat abundance [8, 23]. While it has been shown that rat complaint data has the potential to adequately reflect rat abundance, it is prone to bias caused by various factors, such as the knowledge of citizens on how to file a rat complaint, and the tolerance level of citizens for rat nuisance in order to actively engage in filing a rat complaint [23]. We wanted to test how well municipality rat complaint data reflected relative rat abundance measured using snap traps. We received rat complaint data from Amsterdam, Rotterdam and Eindhoven, which is comprised of the total number of rat complaints filed by citizens both online and by phone. For our analyses we used the total number of rat complaints in a radius of 150m around each trapping location three and six months prior to rat trapping.

Environmental and socio-economic variables

The environmental factors considered in this study included: greenness, presence of fruit- and nut-bearing vegetation, distance to the nearest water body, amount of (food) waste present, type of sewage system, number of restaurants, and presence or absence of petting zoos (Table 1). We quantified greenness using the Normalized Difference Vegetation Index (NDVI). NDVI quantifies vegetation greenness in a satellite image by measuring the difference between near-infrared (reflected by vegetation) and red light (absorbed by vegetation) in a range from 0 (no vegetation present) to 1 (only vegetation present). Water surfaces were excluded from the NDVI map and from subsequent NDVI calculations, using ArcGIS (ESRI ArcGISTM version 10.8, CA, USA). NDVI was calculated using satellite maps from June 2020 and 2021, depending on the trapping year per location [24]. Fruit- and nut-bearing vegetation included apple (*Malus domestica*), pear (*Pyrus communis*), cherry (*Prunus avium*), plum (*Prunus domestica*), blackberry (*Rubus plicatus*), wild strawberry (*Fragaria vesca*), dog rose (*Rosa canina*), chestnut (*Castanea* spp.), hazel (*Corylus avellana*), and walnut (*Juglans regia*). We measured the number of (food) waste items, the number of waste bins, the number of restaurants, and the presence of petting zoos, as proxies for the availability of food resources (Table 1). Food waste items included actual food items, while waste items included all waste excluding food items (e.g., plastic and paper wrappings). The variables NDVI, number of restaurants, and the presence of petting zoos were calculated within a circular buffer with a 150m radius around each trapping location, representing the average home range of rats [25-29] using QGIS version 3.16 [30]. To calculate the shortest distance between trapping sites and the nearest water body (m) in QGIS, we used a shapefile of national water bodies, which includes natural public water bodies such as rivers, canals, lakes, streams, ponds and ditches [31]. All other environmental variables were measured within the trapping locations (100x100m). We also included the type of sewage system (combined versus separated) [8].

The socio-economic variables considered in this study included: mean yearly income, human population density, and the percentage of owner-occupied houses (Table 1). To correct for trapping city and trapping season, we included city and season as fixed factors in the model. Seasons were defined as follows: spring (May), summer (June, July, and August) and autumn (September and October).

Data analysis

All statistical analyses were conducted in R studio version 4.0.3 [32]. All numerical variables were standardized using a z-transformation with two standard deviations [33]. Collinearity between predictor variables was assessed using the *corrplot* package. In case of highly correlated predictor variables ($r_s > 0.7$), only one of the two predictor variables was retained. Multicollinearity was tested using the variance inflation factor (VIF). Variables with a VIF score > 5 were excluded from the model. We checked the model assumptions with functions from both the *DHARMa* and *performance* package. Results were considered significant when $p < 0.05$.

Table 1. *Overview of variables included in the models*

Variable			Definition	Spatial resolution	Data source
Environmental	Distance to nearest water body		Distance (m) to the nearest Dutch natural public water body (incl. rivers, canals, lakes, streams, ponds, and ditches)	20 cm	[31]
	Greenness (NDVI)		NDVI measured in a 150m radius buffer around each trapping location	10 m	NDVI maps from June 2020 and June 2021 [24].
	Fruit- and nut-bearing vegetation		Presence/absence of fruit- and nut-bearing vegetation within the trapping location (100x100m)	20 cm	On-site observations combined with municipality tree species maps [34-36]
	Waste	Waste general	Number of general waste items counted within the trapping location (100x100m) (waste > 5x5 cm)		On-site observations, measured four times
		Food waste	Number of food waste items within the trapping location (100x100m)		On-site observations, measured four times
		Waste bins	Number of above-ground waste bins within the trapping location (100x100m)		On-site observations
	Combined sewage system		Presence/absence of a combined (versus separated) sewage system within the trapping location (100x100m)	Per individual sewage pipe	Data for Amsterdam (via Waternet) and Rotterdam was supplied by the municipalities. For Eindhoven it was available online [37].
	Restaurants		Number of restaurants and cafeterias in a 150m radius buffer around each trapping location		Google maps
	Petting zoos		Presence or absence of petting zoos in a 150m radius buffer around each trapping location		Google maps
Socio-economic	Mean yearly income		Mean yearly income (€/year/family)	Neighbourhood level	[38]
	Percentage of owner-occupied houses		Within the trapping location (100x100m)	100 m	[39]
	Human population density		Number of inhabitants per km^2 per trapping location	100 m	[40]

Relative rat abundance model

We modelled the relationship between relative rat abundance and environmental and socio-economic predictor variables. As our data contained a lot of zero's (42 %), we analysed our data using zero-inflated negative binomial (ZINB) models with the *glmmTMB* package. ZINB models consists of a count part to model the values including true zero values (e.g., no rats present at the site), and a zero-inflated part to model the false zero values (e.g., when rats are present but not captured). The ZINB model used "the number of rats trapped per location" as the count outcome variable, offset by "the number of effective trapping nights". All environmental and socio-economic predictor variables (Table 1) were included in the count part of the initial full multivariate model. We corrected for city and season by including them as fixed factors in the model. The zero-inflated part of the model accounts for the presence of both true zeros (no rats present) and false zeros (rats present but not captured). As the incentive of rats to enter traps can be influenced by the availability of food resources (e.g., rats being less prone to enter the traps when there are ample other food resources available) [5, 6], we decided to include the food-related variables (e.g., number of restaurants, presence of waste, presence of petting zoos, and presence of fruit- and nut bearing vegetation) in the zero-inflated part of the model. Variables that were highly correlated ($r_s > 0.7$ or VIF > 5) were excluded from the final model. Post-hoc tests were performed based on the Tukey method using the *Emmeans* package. For the trapping locations defined as "parks" (n = 16/48), we created an additional univariate negative binomial model to analyse the relationship between relative rat abundance and park size (km^2; data from Google).

Comparing relative rat abundance and municipality rat complaint data

We used Kendall correlation tests to assess how well municipality rat complaint data reflected relative rat abundance. Similarities and discrepancies between both datasets were also tested and visualized per location type (park versus residential area).

Comparing rat trap success over time between parks and residential areas

In each trapping location, rats were trapped during eight trapping nights. We modelled the relationships between relative rat abundance per trapping night and the following variables: trapping night (night 1 – night 8), temperature, precipitation, season, and location type (park versus residential area). For temperature we included the minimum temperature (°C) of the day preceding the trapping night, and for precipitation we included the mean precipitation (mm) of the day preceding the trapping night [41]. This was modelled by using a ZINB model with "the number of rats trapped per location per trapping night" as count outcome variable, offset by "the number of effective traps per location per trapping night". All variables were included in the count part of the initial full multivariate model. We included location type in the zero inflated part of the model, because we know from municipality pest controllers that control is less frequent in parks compared to residential areas. This might lead to an increased aversion of rats from residential areas to traps (e.g., false zeros) compared to rats from parks which have been less exposed to traps and might therefore more easily enter these traps [5, 42]. We included city and trapping location as nested random factors in the model. Variables that

were highly correlated (VIF > 5) were excluded from the final model. Post-hoc tests were performed based on the Tukey method using the *Emmeans* package.

Results

In total, 227 rats were trapped during this study, consisting of 222 (98 %) brown rats and 5 (2 %) black rats. Due to the low number of black rats trapped, we excluded black rats from subsequent analyses. In Amsterdam we trapped 137 brown rats, and in Rotterdam 85 brown rats. In Eindhoven we trapped only five black rats, no brown rats. Of the 222 trapped brown rats, 129 were female (59 %), 91 were male (41 %), while the sex could not be determined for two rats. There were 26 % adults (58/222), 29 % sub-adults (65/222), and 45 % juveniles (99/222). The highest number of rats were trapped in two parks in Amsterdam (Fig. 1 and Table S1).

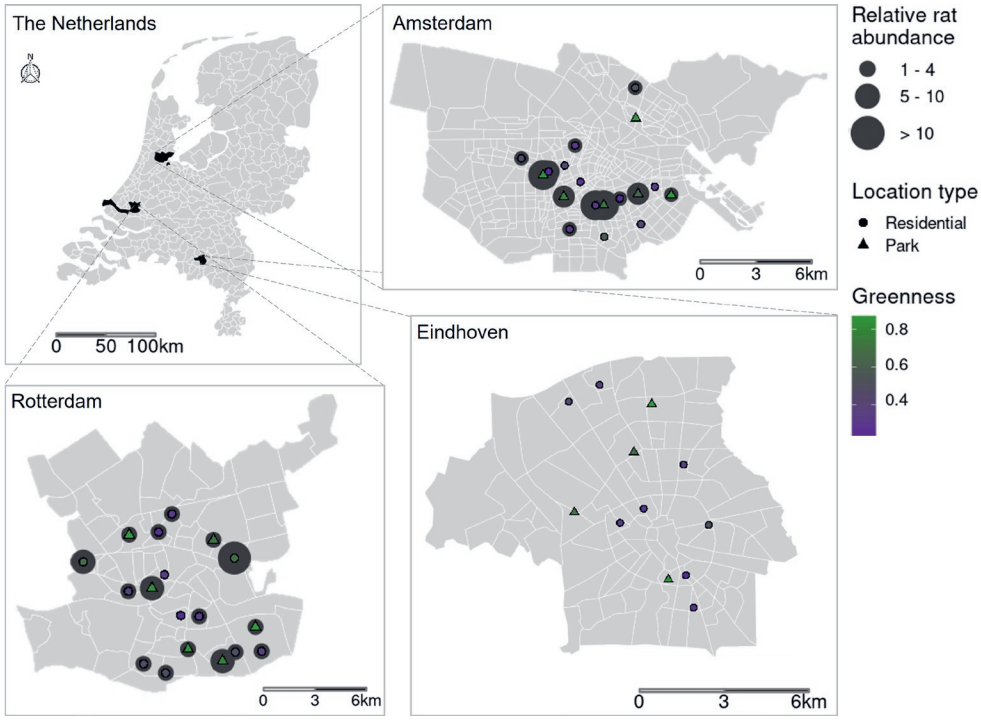

Figure 1. *Relative brown rat abundance per trapping location. Triangles and dots represent trapping locations in parks and residential areas, respectively. The size of the grey circles around each dot represents the relative abundance of brown rats. Dots or triangles without circles represent a relative rat abundance of zero. The degree of greenness per location is visualized by the NDVI gradient ranging from 0.23 (purple) to 0.87 (green). An overview of the exact relative rat abundance per trapping location can be found in Table S1.*

Relative rat abundance is related to greenness

We tested the relationship between relative rat abundance and various socio-economic and environmental variables (Fig. 2). The variable "human population density" was excluded from the model due to high correlation with "greenness" (r_s = -0.77, p < 0.001), and the variable "number of food waste items" was excluded due to high correlation with "number of waste items" (r_s = 0.73, p < 0.001). The variable "number of waste bins" was also excluded. from the count part of the model due to high multicollinearity (VIF > 5). The variable "fruit-

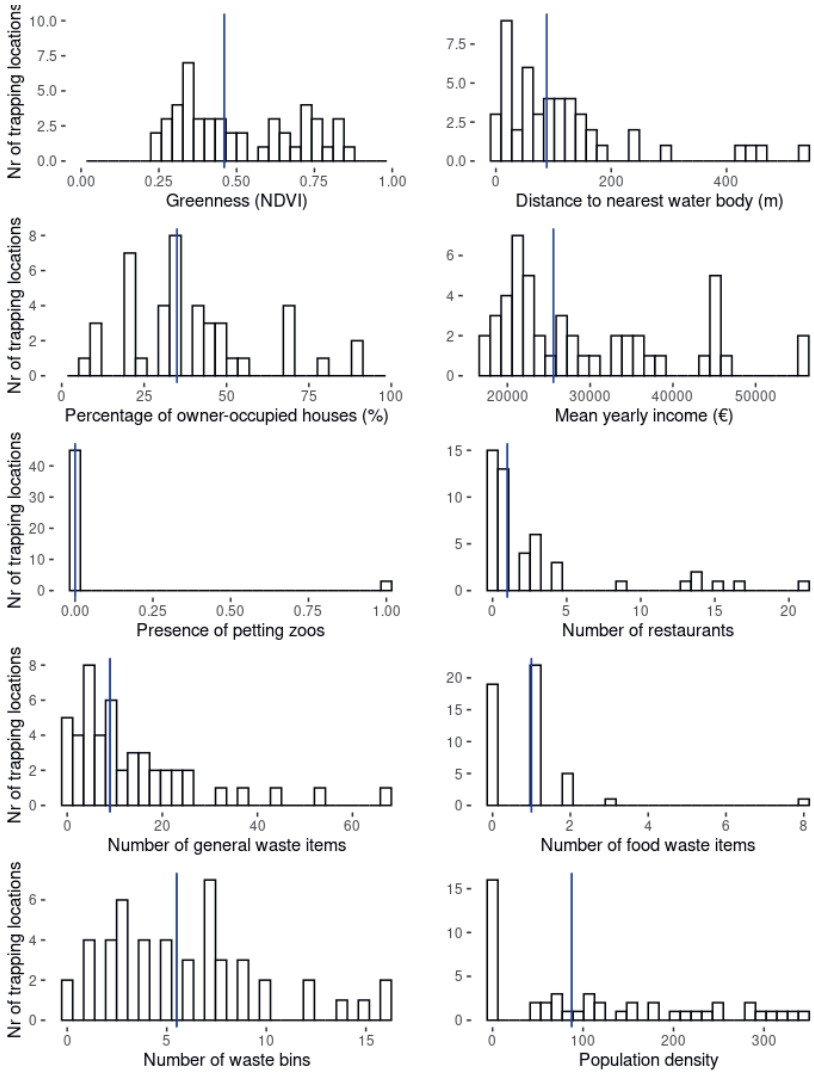

Figure 2. *Distribution patterns of numeric predictor variables (e.g., greenness, distance to nearest water body, percentage of owner-occupied houses, mean yearly income, presence of petting zoos, number of restaurants, number of general waste items, number of food waste items, number of waste bins, and population density. The blue line represents the median value per predictor.*

Table 2. *Results from the final multivariate zero-inflated negative binomial (ZINB) relative rat abundance model (n = 48 trapping locations).*

Model part	Variable	β	SE	p-value
Count part	Presence of petting zoos	1.90	0.81	**0.019**
	Greenness	1.70	0.57	**0.003**
	Number of restaurants	1.29	0.31	**< 0.001**
	Fruit- and nut-bearing vegetation	1.26	0.76	0.097
	Mean income per neighbourhood	0.81	0.41	0.052
	Distance to water	0.78	0.93	0.400
	Number of waste items	0.77	0.40	0.052
	Percentage of owner-occupied houses	0.58	0.43	0.178
	Mixed sewage system	0.04	0.43	0.919
	Season *spring / summer*	0.20 / 0.27	0.39 / 0.41	0.603 / 0.505
	City *Eindhoven / Rotterdam*	-23.83 / -0.12	12,760/ 0.45	0.999 / 0.794
Zero-inflated part	Presence of petting zoos	-8.75	9,600	0.999
	Number of waste items	-1.14	1.44	0.430
	Number of restaurants	0.77	0.87	0.374

and nut-bearing vegetation" was excluded from the zero-inflated part of the model due to model convergence problems. The final multivariate ZINB model included 11 variables in the count part of the model and three variables in the zero part of the model (Table 2). The final model showed positive relationships (in order of decreasing effect size) between relative rat abundance and the presence of petting zoos (β = 1.90, $p = 0.019$), greenness (β = 1.70, $p = 0.003$), and number of restaurants (β = 1.29, $p < 0.001$; Table 2 and Fig. 3). There were also positive trends, albeit not significant, between relative rat abundance and mean income (β = 0.81, $p = 0.052$) and the number of waste items (β = 0.77, $p = 0.052$; Table 2 and Fig. 3). There was a significant negative, albeit weak, correlation between the number of waste items and greenness ($r_s = -0.41$, $p = 0.003$; Fig. S1), and no correlation between income and greenness ($r_s = 0.10$, $p = 0.480$). In a separate model including only the rats

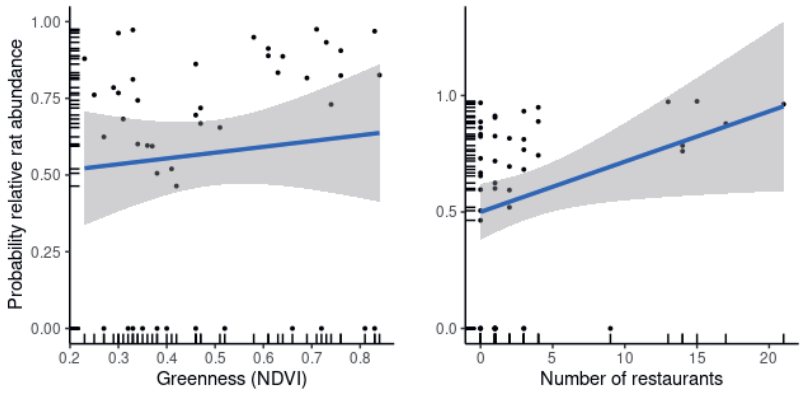

Figure 3. *Expected changes in relative rat abundance based on the probabilities of relative rat abundance and significant numerical predictor variables resulting from the model (e.g., greenness and number of restaurants; Table 2). Trendlines are added in the plots.*

trapped in parks as the outcome variable, we observed no significant relationship between relative rat abundance and park size (β = -0.60, SE = 0.90, p = 0.506; Table S1)

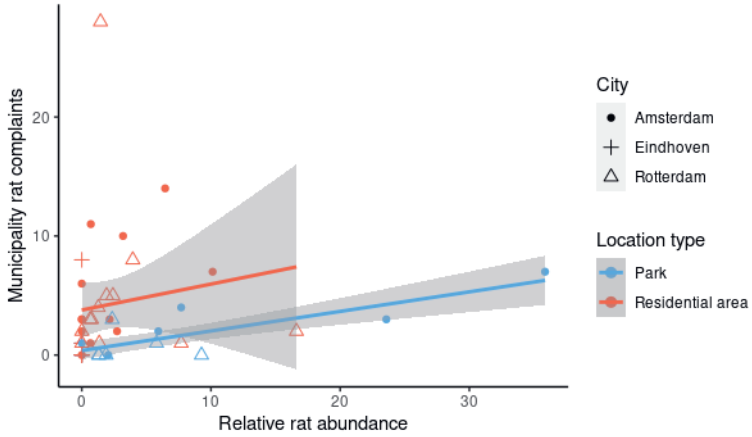

Figure 4. *Relationship between relative rat abundance per location and the number of municipality rat complaints in the six months prior to rat trapping per location type (e.g., parks in blue, and residential areas in red). Locations within different cities are visualized with different shapes (e.g. a dot for Amsterdam, a plus sign for Eindhoven, and a triangle for Rotterdam).*

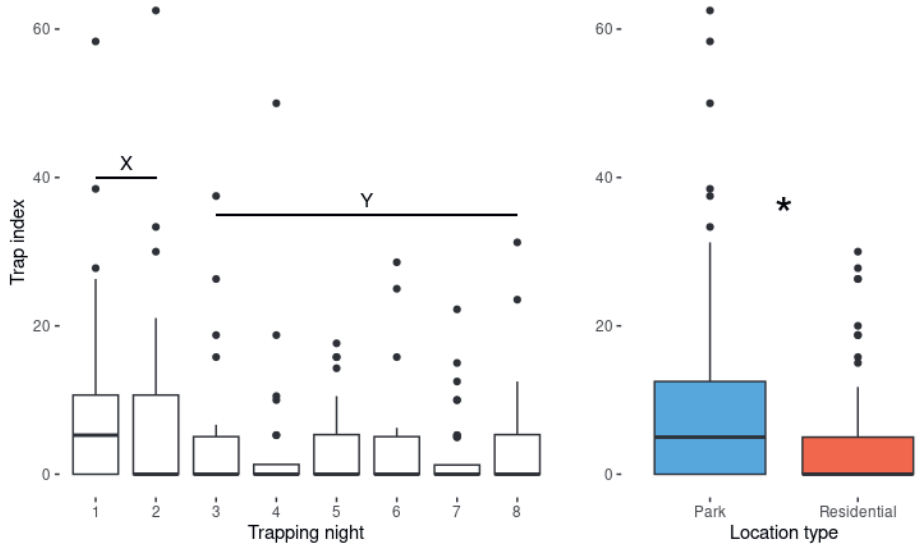

Figure 5. *A. Relative rat abundance per trapping night (1-8). Letters X and Y indicate significant differences between trapping nights ($p < 0.05$). B. Total relative rat abundance per location type (e.g., park and residential area). The asterisk (*) indicates a significant difference between location types ($p < 0.05$). The horizontal lines within bars show the median.*

Fewer rat complaints in parks

We compared the relative rat abundance per location with the total number of municipality rat complaints three and six months prior to trapping. We found significantly positive, albeit weak, correlations between relative rat abundance and the number of municipality rat complaints three and six months prior to trapping ($\tau = 0.23$, $p < 0.05$, and $\tau = 0.30$, $p < 0.007$, respectively). When we excluded the parks and only looked at the correlation between municipality rat complaints (6 months prior to rat trapping) and relative rat abundance in residential areas, we observed a significantly positive and slightly higher, albeit still weak, correlation ($\tau = 0.40$, $p < 0.004$). For parks only, this relationship was slightly stronger compared to residential areas ($\tau = 0.49$, $p < 0.02$). Overall, there seemed to be less municipality rat complaints in parks compared to residential areas (Fig. 4 and Table S1). Thus, the number of rat complaints in parks may be an underestimation of actual rat abundance.

Trap success decreases over time and is higher in parks compared to residential areas

In total, we had 6846 effective trapping nights during this study. In 222 (3 %) of these trapping nights a rat was trapped. With a ZINB model, we examined the relationship between relative rat abundance and the following predictor variables: trapping night (1-8), temperature (°C), precipitation (mm), and location type (park versus residential area). We observed a significantly lower relative rat abundance in residential areas compared to parks ($\beta = -1.19$, SE = 0.49, $p = 0.015$; Fig. 5B and Table S2). We also found that relative rat abundance significantly decreased after the second trapping night (Fig. 5A, Table S2 and S3).

Discussion

In this study, we investigated which environmental and socio-economic factors are related to urban rat abundance, with a focus on greenness. We observed significant positive relationships between relative rat abundance and both greenness and factors related to food availability. In addition, municipality rat complaints were lower in parks compared to residential areas, while the relative abundance of rats was higher in parks.

Positive relationship between relative rat abundance and greenness

We observed a strong positive relationship between relative rat abundance and greenness. This is in line with previous research that reported a higher abundance of rats in urban green spaces [7, 10, 11]. Our result suggests that greenness enables rat populations to grow into larger numbers, potentially by providing suitable habitat (e.g., food and shelter), and thereby allowing a higher rat carrying capacity. This, in combination with no or little pest control in public green spaces such as parks (personal communication with municipality pest control technicians), could facilitate the growth and maintenance of rat populations. However, these results should be interpreted with caution, as food availability and differences in avoidance behaviour of rats towards traps could differ between greener and less green areas, which we discuss in more detail below. Moreover, the term greenness encompasses a wide range of plant species and structural variations, which could have

varying impacts on rat abundance but were not considered in this study. For example, certain vegetation types may provide rats with more coverage to hide, as rats avoid open space [43]. Traweger et al. (2006) studied the relationship between rat abundance and vegetation types, and observed significant positive relationships between rat abundance and the presence of bushes, trees, ruderal vegetation, vegetation with fruits, riverbank vegetation, conifers, and deciduous trees, and a significant negative relationship between rat abundance and the presence of evergreens. In contrast, we did not observe a positive relationship between rat abundance and vegetation with fruits. We hypothesize that when ample other (human-provided) food resources are available, the abundance of rats will be influenced more by those food resources than by the presence of vegetation with fruits or nuts. In addition, fruit or nut vegetation only provides food during specific times of the year, which might be insufficient and too unreliable to support large rat populations whole year round.

Relationships between relative rat abundance and variables other than greenness

We observed significant positive relationships between relative rat abundance and the number of restaurants and the presence of petting zoos, and an almost significant relationship between relative rat abundance and the number of waste items. Previous studies showed a positive relationship between rat abundance and animal feed, which could explain the observed positive relationship with petting zoos [7, 11, 44]. The number of restaurants and waste items could also be proxies for food availability. The positive relationship with these variables is in line with previous studies, which found rat abundance to be associated with food waste [7, 11]. Distance to water was not a significant variable in our model, but was significant in previous studies [7, 13]. This could be explained by the fact that all of our parks contained water bodies within the 100x100m trapping locations, and that Amsterdam and Rotterdam are both cities characterized by a significant presence of water, such as canals and rivers. As a result, distance to water was not a limiting factor, and therefore relatively less important when compared to the other variables.

It should be noted that rats were trapped in 2020 and 2021 during the Covid-19 pandemic, which could have slightly altered their abundance and distribution compared to other years. For example, the temporary closing of restaurants may have decreased food availability, forcing rats to seek food resources elsewhere. At the same time, people more frequently visited urban parks [45], which may have resulted in increased littering and thus food availability for rats in parks. Both of these effects may have contributed to higher rat abundances in parks.

Snap trap bias?

By systematically trapping rats, we were able to compare the relative abundance of rats between locations. However, as with most abundance measurements, the outcome needs to be interpreted with caution [46]. From what we observed in the field together with previous research, we hypothesize that trap success is influenced by food availability and rat behaviour. When more food (waste) is available, there is less incentive for rats to enter the traps for food. Rats are neophobic animals, i.e. they avoid unfamiliar objects and will

probably prefer food resources that are familiar or perceived as less dangerous [5, 6]. This could lead to relatively lower trap success and thus lower abundance estimates in areas with high food (waste) availability, and could therefore underestimate the relationship between waste and relative rat abundance. To what extent neophobia influences rat' behaviour towards traps (e.g., avoidance) is difficult to predict, as it depends among other things on their previous experiences with traps [5, 42]. Therefore, rats living in different environments (e.g., parks versus residential areas) may show different behaviour towards traps based on previous encounters, which could influence trap success and thus abundance estimates. For example, rats in parks might be more prone to enter traps because they have had less previous negative experiences with traps compared to rats in residential areas, as pest control is, in general, less intensive in parks compared to residential areas in the Netherlands. This could lead to an overestimation of rats in parks compared to residential areas. However, we did not observe significant differences between the response of rats to the traps (when looking at the trap success over time) in parks versus residential areas, which might indicate that the behaviour of rats from both areas is not that different. To account for the effect of neophobia in different locations, a suggestion for future research is to combine the use of snap traps with additional techniques to estimate rat abundance, such as chew cards or (short focal) camera traps [46, 47]. In this study we tried to deploy camera traps in parallel to rat trapping, but this proved to be challenging. Especially in residential areas it was hard to install a camera trap without serious risk of theft(data not shown). Therefore, chew cards or deep learning-based systems to detect and analyse ultrasonic vocalizations, such as DeepSqueak, might be better alternatives [48].

Fewer rat complaints yet more rats trapped in parks compared to residential areas

The number of rat complaints was lower than the number of rats trapped in parks compared to residential areas. Rats could be perceived less of a problem in public space compared to the home environment, or rats can hide better when there is more vegetation, which makes them less visible, and which could lead to fewer rat complaints. The relatively lower number of complaints in parks may lead to less intensive pest control, which may contribute to rats being more abundant in these greener urban areas. Nonetheless, it is important to be aware of a higher rat abundance in parks despite a low number of complaints, because we hypothesize that these rat populations might serve as source populations for neighbouring residential areas.

The correlation between the number of rat complaints and the number of rats trapped per trapping location was positive, but weak. In contrast, previous research in Chicago found a strong positive relationship between rat complaints and rat abundance [23]. However, they used the total number of rat complaints in the last 12 months prior to trapping and in a 1 km radius of each trapping location, which is both a longer time period and larger area than we used here. It is known that rat complaints can be biased by certain factors such as knowledge on how to file a complaint and individual rat tolerance level or attitude [3, 23]. In addition, the municipality rat complaint dataset is probably not totally accurate, due to some misclassifications of citizens (e.g., filing a brown rat

complaint while it is actually a mouse). Societal changes can also lead to changes in reporting complaints. For example, during the Covid-19 pandemic, various municipalities - including the municipality of Amsterdam – experienced surges in complaints about garbage piling up followed by surges of rat complaints in those same locations, mainly residential areas (personal communication with Amsterdam's municipality pest control and [49]). Although the additional garbage piling up could have attracted more rats, the increase in rat complaints might also be caused by the fact that people were at home much more, thereby increasing the chance of spotting the rats that were already there [3].

In addition, we observed that most rats were trapped in the first days of trapping, after which the number of trapped rats declined. This is in line with previous research [9]. These results suggest that for pest control it might be more effective to have a high trap effort for a few days instead of lower trap effort for a longer period.

In this study we trapped five black rats, but no brown rats in Eindhoven, while we know from local pest control technicians and from previous unpublished research that brown rats are present. Whether this difference reflects actual differences in rat abundance (a potential relationship between the size of both the city and the rat population), or is caused by behavioural differences, or other unknown differences between Eindhoven and the other cities remains unclear. The fact that no black rats were trapped in Amsterdam and Rotterdam is not surprising, since the distribution of black rats is mostly limited to the south of the Netherlands and to harbour areas.

Conclusions

We observed a significant positive relationship between the relative abundance of rats and both greenness and different proxies for food resources (restaurants, waste items, and petting zoos). This suggests that, in addition to greenness, food availability is one of the driving factors for rat abundance in Dutch cities, which is largely influenced by human behaviour. While this study shows that greenness may be important for rat abundance, there might be other factors contributing to this observed relationship, such as the altered behaviour of rats towards traps in greener versus less green areas, which might be influenced by variation in food resource availability and differences in avoidance behaviour towards traps based on previous encounters. In addition, greenness is a broad concept encompassing a wide range of vegetation types, which calls for more precise studies to disentangle the effects of different vegetation types on rat abundance. To minimize rat abundance, the general public, policy makers and city planners need to collaborate to limit the availability of food and shelter using a multifactorial approach with measures that take into account both human awareness and city design, especially in greener urban areas. Measures that can be implemented could focus on limiting the availability of food resources in greener urban areas, for example by avoiding easy access to food waste through increasing the number of waste bins, increasing the frequency of garbage collection, altering the design of waste bins to make them less accessible for rats to enter, and by increasing human awareness towards food waste. Measures could also focus on adapting urban green spaces by choosing different vegetation types (e.g., vegetation types that provide less shelter for rats), but this should first be evaluated in future studies. Likely, the absence of either or both food and shelter resources in greener

urban areas will limit the abundance of rats. By taking measures to reduce the abundance of rats, the positive effects of urban greening can be retained, while its negative effects associated with rats and their risks posed to human health can be reduced.

Acknowledgements

We would like to thank Jan Buijs (municipality of Amsterdam), Peter Muilwijk (municipality of Rotterdam) and Sierk Visser together with Ellen van Rosmalen (municipality of Eindhoven) for giving us permission to execute this research, for providing us with city-specific information, and for additional tips and help. We would also like to thank all residents who gave us permission to place a rat trap in their front yard. Lastly, we would like to thank groenmonitor.nl for providing us with the NDVI data, and Sara Wijburg for her help with coding the maps in R.

Table S1. *Overview of the number of Rattus norvegicus trapped, the trap index per trapping location and the number of municipality rat complaints per location (total number of complaints during last six months prior to trapping).*

Location type	City	Trapping location	Number of rats trapped	Effective number of traps	Trap index	Number of municipality rat complaints	Park size (km²)
Park	Amsterdam	Sarphatipark	45	126	36	7	0.05
Park	Amsterdam	Rembrandtpark	31	132	24	3	0.45
Residential	Rotterdam	Kralingen oost	26	157	17	2	
Residential	Amsterdam	Hercules seghersbuurt	16	158	10	7	
Park	Rotterdam	Het Park	12	130	9	0	0.28
Park	Amsterdam	Vondelpark	10	130	8	4	0.47
Residential	Rotterdam	Oud mathenesse	9	117	8	1	
Residential	Amsterdam	Balboaplein	10	155	6	14	
Park	Amsterdam	Oosterpark	8	135	6	2	0.12
Park	Rotterdam	Spinozapark	9	156	6	1	0.70
Residential	Rotterdam	Agniesebuurt	6	152	4	8	
Residential	Amsterdam	Slotermeer zuid	5	157	3	10	
Residential	Amsterdam	Prinses Irenebuurt	4	147	3	2	
Residential	Rotterdam	Bloemhof	3	125	2	5	
Park	Rotterdam	Vroesenpark	3	128	2	3	0.16
Residential	Amsterdam	Swammerdam- buurt	3	139	2	3	
Park	Amsterdam	Flevopark	3	147	2	0	0.42
Residential	Rotterdam	Zuidwijk	3	157	2	5	
Park	Rotterdam	Zuiderpark	2	107	2	0	2.15
Residential	Rotterdam	Oude noorden	2	138	1	28	
Residential	Rotterdam	Delfshaven	2	148	1	1	
Park	Rotterdam	Park de twee heuvels	2	151	1	0	0.50
Park	Rotterdam	Park Rozenburg	2	155	1	0	0.03
Residential	Rotterdam	Lombardijen	2	158	1	4	
Residential	Rotterdam	Groot IJsselmonde	1	132	1	3	

Residential	Amsterdam	Staatsliedenbuurt Noordoost	1	143	1	11	
Residential	Amsterdam	Banne Noordwest	1	150	1	1	
Residential	Rotterdam	Pendrecht	1	156	1	3	
Park	Amsterdam	Noorderpark	0	154	0	1	0.40
Residential	Amsterdam	Geuzenhofbuurt	0	139	0	0	
Residential	Amsterdam	Helmersbuurt oost	0	133	0	3	
Residential	Amsterdam	Noordwest-kwadrant Indische buurt zuid	0	157	0	6	
Residential	Amsterdam	Tuindorp amstelstation	0	158	0	2	
Residential	Amsterdam	Veluwebuurt	0	134	0	3	
Park	Eindhoven	Stadswandelpark	0	144	0	0	0.16
Park	Eindhoven	Philips van Lennep park	0	136	0	0	0.20
Park	Eindhoven	Lieven de Key park	0	137	0	1	0.07
Park	Eindhoven	Henri Dunant Park	0	131	0	0	0.22
Residential	Eindhoven	Achtse barrier-hoeven	0	150	0	0	
Residential	Eindhoven	Hemelrijken	0	150	0	0	
Residential	Eindhoven	Joriskwartier	0	137	0	1	
Residential	Eindhoven	Kerkdorp acht	0	147	0	8	
Residential	Eindhoven	Kerstroosplein	0	127	0	0	
Residential	Eindhoven	Oude gracht-west	0	157	0	1	
Residential	Eindhoven	Schoot	0	151	0	0	
Residential	Eindhoven	Villapark	0	155	0	0	
Residential	Rotterdam	Rotterdam centrum	0	132	0	1	
Residential	Rotterdam	Tarwewijk	0	131	0	2	

Table S2. *Model output of the zero-inflated negative binomial model for trap index*

Model part	Predictor variable	β	SE	p-value
Negative binomial (count part)	Temperature	-0.32	0.23	0.169
	Precipitation	-0.16	0.17	0.344
	Location type [residential area]	-1.19	0.49	**0.015**
	Season [spring]	-0.27	-0.45	0.650
	Season [summer]	0.46	0.77	0.440
	Trapping night [2]	-0.15	0.23	0.601
	Trapping night [3]	-1.04	0.29	**< 0.001**
	Trapping night [4]	-1.10	0.32	**< 0.001**
	Trapping night [5]	-1.22	0.32	**< 0.001**
	Trapping night [6]	-1.26	0.33	**< 0.001**
	Trapping night [7]	-1.43	0.33	**< 0.001**
	Trapping night [8]	-0.96	0.29	**0.001**
Zero-inflation (zero part)	Location type [residential area]	-2.98	3816.23	0.999

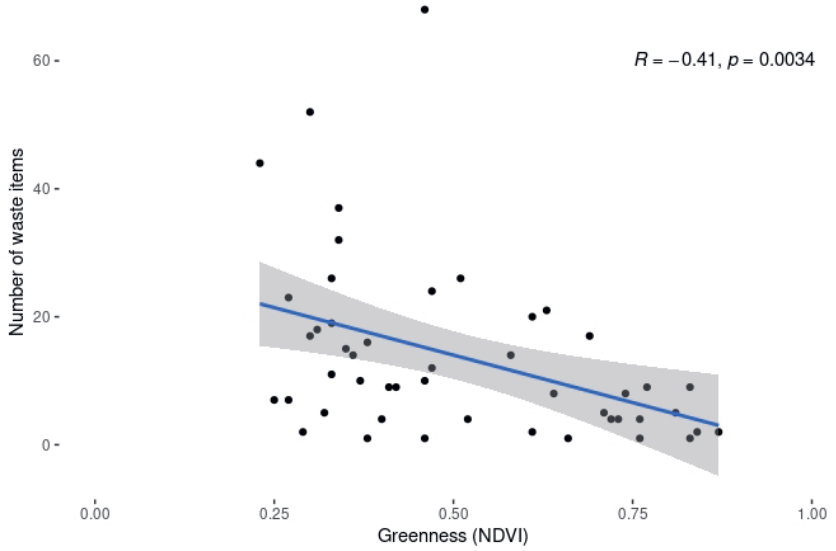

Figure S1. *Number of waste items plotted against the degree of greenness (NDVI) per location. The line represents the trendline. The R and* p*-value are displayed in the upper right corner.*

Table S3. *Pairwise comparisons of number of rats trapped (trap index) per trapping night using the Tukey post-hoc test. Significant results (p < 0.05) are indicated in bold.*

Pairwise post-hoc comparisons	Estimate	SE	p-value
Trapping_night1 - Trapping_night2	0.12	0.23	> 0.999
Trapping_night1 - Trapping_night3	10.43	0.29	**0.008**
Trapping_night1 - Trapping_night4	10.98	0.32	**0.015**
Trapping_night1 - Trapping_night5	12.23	0.32	**0.004**
Trapping_night1 - Trapping_night6	12.60	0.33	**0.004**
Trapping_night1 - Trapping_night7	14.27	0.33	**< 0.001**
Trapping_night1 - Trapping_night8	0.96	0.29	**0.025**
Trapping_night2 - Trapping_night3	0.92	0.29	**0.031**
Trapping_night2 - Trapping_night4	0.98	0.31	**0.035**
Trapping_night2 - Trapping_night5	11.01	0.31	**0.010**
Trapping_night2 - Trapping_night6	11.38	0.32	**0.012**
Trapping_night2 - Trapping_night7	13.05	0.31	**0.001**
Trapping_night2 - Trapping_night8	0.84	0.27	**0.040**
Trapping_night3 - Trapping_night4	0.05	0.32	> 0.999
Trapping_night3 - Trapping_night5	0.18	0.30	0.999
Trapping_night3 - Trapping_night6	0.22	0.31	0.997
Trapping_night3 - Trapping_night7	0.38	0.32	0.935
Trapping_night3 - Trapping_night8	-0.08	0.30	> 0.999
Trapping_night4 - Trapping_night5	0.13	0.33	> 0.999
Trapping_night4 - Trapping_night6	0.16	0.33	> 0.999
Trapping_night4 - Trapping_night7	0.33	0.34	0.980
Trapping_night4 - Trapping_night8	-0.13	0.32	> 0.999
Trapping_night5 - Trapping_night6	0.04	0.31	> 0.999
Trapping_night5 - Trapping_night7	0.20	0.33	0.999
Trapping_night5 - Trapping_night8	-0.26	0.31	0.991
Trapping_night6 - Trapping_night7	0.17	0.34	> 0.999
Trapping_night6 - Trapping_night8	-0.30	0.32	0.984
Trapping_night7 - Trapping_night8	-0.46	0.32	0.836

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Restaurant

Chapter 5

Determinants of small mammal presence in private gardens in the Netherlands

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Abstract

Urban greening is a common strategy to counter negative effects of urbanization and was shown to increase the abundance of small mammals in urban areas. However, which factors determine the presence of small mammals is poorly known, yet important for disease risk and the implementation of preventive measures. Here, we assessed which predator, environmental and socio- economic factors are associated with the presence of brown rats and micromammals (i.e., mice, voles, and shrews) in private gardens in the Netherlands. We studied their presence in 1000 private gardens from 26 municipalities with varying degrees of greenness present, using camera traps. The presence of brown rats and micromammals was modelled using generalized linear mixed models and occupancy models. We observed negative relationships between the presence of brown rats and domestic cats, and both negative and positive relationships between the presence of micromammals and domestic and wild predators. We did not find a relationship between the degree of greenness and the presence of brown rats and micromammals, while we did observe a positive relationship between micromammals and the degree of greenness in the occupancy model. Our results suggest that greenness is less important than the presence of predators for the mere presence of brown rats and micromammals in private gardens in urban environments. The observed frequent presence of small mammals in private gardens highlights the potential risk of pathogen transmission to humans and domestic animals in urban environments.

Introduction

Urban greening is a common strategy to counter some negative side-effects of urbanization, such as air pollution, decreased water quality and heat island effects [1]. Urban greening aims to increase the amount of natural, semi-natural, and artificial green space in cities, both in public and private properties, which has positive effects on biodiversity in urban environments [2, 3]. The mammal species that supposedly are facilitated by urban greening include rodents, such as rats, mice and voles, and shrews, which can thrive in urban areas due to their adaptability to new environments and their anthropophilic nature [4]. They also seem to do especially well in urban green space because vegetation can provide shelter and food, and can decrease noise from human activities [5, 6]. A recent study in the Netherlands found that the abundance of rats increased with the greenness of public urban areas [7].

A rise in the abundance of small mammals is of concern. It might not only lead to increased gnawing damage, food contamination, and negative impacts on human mental health [8, 9], but may also result in increased density-dependent transmission of pathogens between small mammals [10], and thus increased transmission of (zoonotic) pathogens to humans and other wild or domestic animals [10, 11]. Brown rats, black rats and house mice pose a high risk for pathogen transmission to humans, most frequently via contact with urine or faeces [12]. Especially in areas near human habitation with potential high exposure, such as private gardens, this could lead to an increased zoonotic disease risk for humans. Therefore, it is important to understand which factors determine the presence and occupancy of small mammals in private gardens to improve disease surveillance and preventive measures.

One important determinant of small mammal presence is food availability. Living close to humans provides small mammals with abundant food resources that are not affected by seasonal fluctuations, such as household waste, pet and bird food, chicken coops and compost heaps [13-17]. These type of food resources are especially suitable for rats, as they are opportunistic omnivores that can make use of a large variety of food resources [17-19]. The occurrence of rats seems to be influenced more by the amount of available food resources than predation, since their numbers are largely controlled bottom-up [4]. The diet of mice (including house mice and wood mice) is also subject to availability, but mostly consists of more natural food resources such as plants (mainly seeds) and invertebrates (including beetles, moths, insect larvae and spiders) [20, 21]. Voles are regarded as herbivores, mostly feeding on plant-based food resources [20, 21].

Another important factor affecting small mammal abundance is shelter availability. In urban environments, rats and house mice can find shelter in woodpiles, natural vegetation, and old or poorly maintained buildings [14-17]. The observation that rats seem to occur more frequently in lower-income neighbourhoods, in contrast to mice, which seem to occur more frequently in higher-income neighbourhoods [15], may be associated with the types of shelter that are available in these different neighbourhoods. Wood mice, voles and shrews prefer natural vegetation for shelter and nesting [22, 23].

In addition, the presence and abundance of small mammals can also be influenced by the presence of predators. In urban areas, these mainly consist of domestic cats and dogs [24-26] rather than wild predators such as martens, stoats, weasels, foxes, and birds of prey [27, 28]. Although cats probably have little impact on the rat population size [29], as they mostly seem to prey on juvenile rats [30, 31], rats do seem to avoid cats, as do house mice [32, 33]. Cats do prey on mice, voles, and shrews, and have a negative effect on their abundance [24, 31, 34]. Also, cats, like red foxes, are eclectic feeders that can adapt to local prey availability [35]. The extent of predation of wild predators on small mammals remains unclear, as predators in urban areas shift their diet to a wider range of (easy accessible) food resources, which do not require them to show their natural hunting behaviour [35, 36]. In general, small mammal populations, with the exception of rats, are affected more by top-down control (by predators) and their population growth is rarely limited by food resources (bottom-up control) [4, 37]. However, this might vary depending on predator pressure, which may differ between urbanized and more natural environments [35].

Here, we aimed to get more insight into factors determining the presence of small mammals in private gardens. We measured the presence and occupancy of brown rats and micromammal species (mice, voles, and shrews) in 1000 private gardens in neighbourhoods with varying degrees of greenness across 26 different municipalities, using camera traps. We investigated whether predators and environmental (including the degree of greenness) and socio-economic factors were associated with the presence of brown rats and micromammals. We hypothesized that greenness (as proxy for both food and shelter resources) would be the most important factor for determining the presence of brown rats and micromammals in private gardens.

Materials & Methods

Study area

Camera traps were deployed in private gardens in 26 municipalities across the Netherlands (c. 41,850 km^2, 50.7°N to 53.5°N and 3.2°E to 7.2°E; Fig. 1) from 2016 until 2022. This data collection was part of a larger ongoing citizen science project aimed at monitoring wildlife in private gardens called 'Zoogdieren in de achtertuin' (Camera trap – mammals in the backyard), set up by the Dutch Mammal Society (DMS), Silvavir ecological consultants, and Wageningen University (WUR) [38, 39].

Data collection and photo analysis

Multiple types of passive infrared (PIR) motion detection camera traps were deployed: Reconyx HC500, Reconyx HC600, Reconyx HS2X, Bushnell agressor, Bushnell core DS Low Glow, Browning Strike force HD-X, Browning 2020 Spec Ops Edge, and Spypoint Force-Dark infrared motion sensor cameras. All camera traps were positioned with an unblocked view of at least 1.5 meters, with the camera lens located 20 cm above ground, as to increase the likelihood of detecting small mammals. Camera traps were aimed to be deployed for a minimum of three weeks. A leaking can of sardines was used as lure and attached to a tree or pole at a height of 15 cm above ground and at a distance of

1.5 - 2 m from the camera trap [39, 40], as to increase the likelihood of mammals present in the garden to appear in front of the camera, and to level out differences in sensitivity between camera models. The number of photos per burst was typically set to ten with no pause between bursts. To create a record of camera operation time (effort), time-lapse photos were taken every 12 hours. We used the software Agouti (https://www.agouti.eu/) [41] to store and process the camera trap data. Agouti automatically groups photos and bursts into sequences by the software at a threshold difference of 120 seconds between sequences. The sequences were annotated manually (97.3 %) or by AI (2.7 %), and time-lapse photos were excluded from annotation.

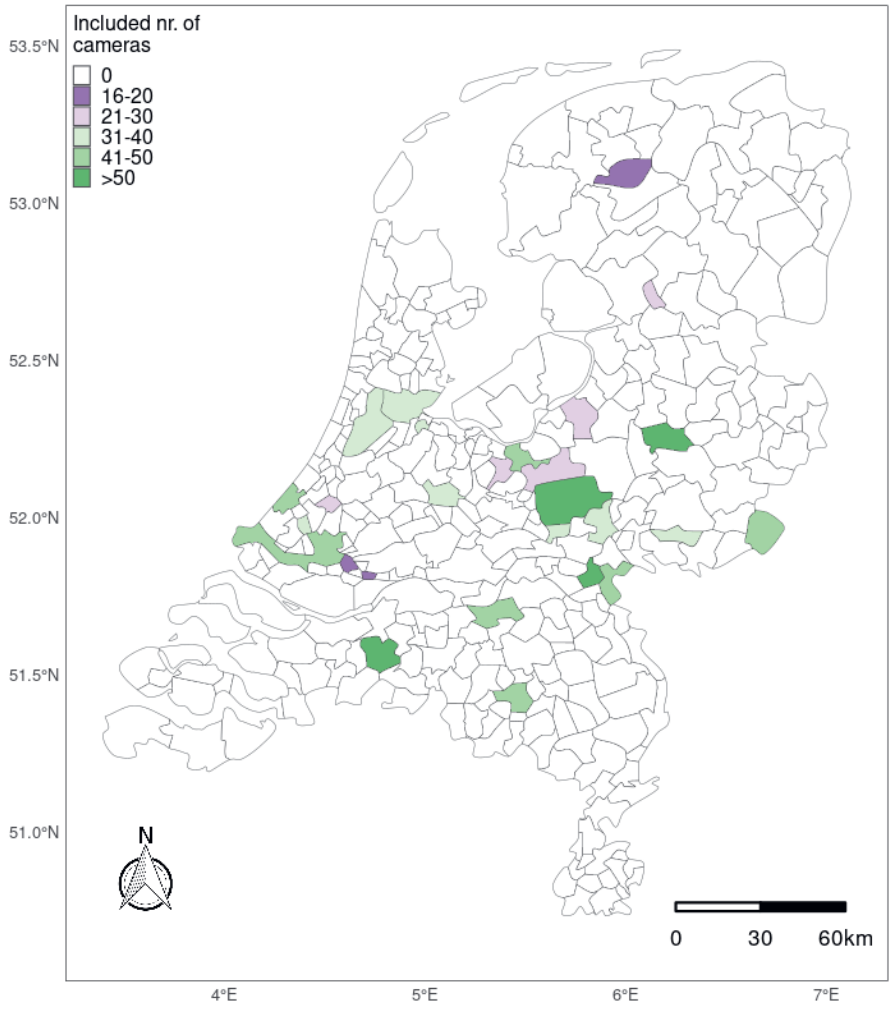

Figure 1. *Map of municipalities across the Netherlands in which private gardens were sampled with camera traps (min = 16, max = 73 gardens per municipality)*

Surveyed gardens with incompletely annotated deployments, invalid camera set-up, or incorrect coordinates were excluded from this study. As identification of certain small mammals (e.g., mice, voles, and shrews) to species level was unreliable, sightings of these species were grouped together and will hereafter be referred to as "micromammals". This group includes: *Apodemus sylvaticus, Apodemus flavicollis, Crocidura leucodon, Crocidura russula, Micromys minutus, Microtus agrestis, Microtus arvalis, Microtus oeconomus, Microtus subterraneus, Mus musculus, Myodes glareolus, Neomys fodiens, Sorex cf. araneus* and *Sorex minutus* [42]. Gardens were considered positive for brown rat or micromammal presence in case of photographing at least one occasion of these target species.

Model covariates

We derived covariates regarding the presence of predators, the environment, and the socio-economic status of the area to explain the presence of small mammals in private gardens (Table 1). Wild predators of the brown rat (*Rattus norvegicus*) are cats (*Felis catus*), dogs (*Canis lupus familiaris*), mustelids (*Mustelidae*), and red foxes (*Vulpes vulpes*) [25, 26, 43]. Micromammals, such as the house mouse (*Mus musculus*) and the wood mouse (*Apodemus sylvaticus*), are, in addition to the aforementioned predators, predated by brown rats [43]. The presence of predators was expressed as the proportion of sampling days in which a predator was photographed (effort). Next, the geographical coordinates of camera traps were used as centroids to create circular buffers with a radius of 150m for rats and a radius of 50m for micromammals, representing their average home range [44, 45]. To quantify greenness per buffer area, we used the Normalized Difference Vegetation Index map from 2021 (NDVI; 100 m^2 resolution). The NDVI is a dimensionless index between 0 and 1, which visualises the difference between near-infrared and visible reflectance of vegetation cover [46]. Prior to calculating the greenness within the buffer zones, water bodies were masked from the NDVI map using ArcGIS (ESRI ArcGIS™ version 10.8, CA, USA). Based on data from Wageningen Environmental Research's national land-use database (LGN2020), the landscape composition was determined (25 m^2 resolution; [47]). To calculate water and road density, we georeferenced the LGN categories fresh water and infrastructure, respectively, within the buffer areas (i.e., 150m for rats, and 50m for micromammals) surrounding each camera trap. Four-digit (PC4) and six-digit (PC6) postal codes were assigned to camera trap geolocations to subtract data available on PC4 or PC6 level. Human population density was collected on PC6 level. The percentage of buildings built before 1945 and the median disposable income were collected on PC4 level.

Data analyses

Small mammal presence in relation to predictive variables was analysed using a generalized linear mixed modelling (GLMM) framework and occupancy modelling framework. Prior to these analyses, the following pre-processing steps were conducted. Firstly, to increase the robustness and reliability of the results, five municipalities with fewer than 15 surveyed gardens in total were excluded. Secondly, numerical variables ranging between 0 and 1 (e.g., variables regarding predator presence, and the degree of greenness) were logit-transformed. If values were exactly equal to zero or one, a constant value (i.e., half of the minimum value) was added or subtracted, respectively, to allow transformation. Thirdly,

Table 1. *Description of included covariables.*

Model	Type of variable	Variable	Description	Spatial resolution of map	External data source
Variables	Predator	Domestic cats	Proportion (%) of days a domestic cat is detected per deployment.	Individual garden	-
	Predator	Red foxes	Proportion (%) of days a red fox is detected per deployment.	Individual garden	-
	Predator	Mustelids	Proportion (%) of days a mustelid is detected per deployment.	Individual garden	-
	Predator	Dogs	Proportion (%) of days a dog is detected per deployment.	Individual garden	-
	Predator	Brown rats	Proportion (%) of days a brown rat is detected per deployment.	Individual garden	-
	Environmental	Greenness	Degree of greenness present within buffer areas based on NDVI data (0 - 1) from 2021, corrected for the presence of water.	100 m^2	[48]
	Environmental	Road density	Total density of roads within a buffer area derived from the LGN2020 spatial layer.	25 m^2	[47]
	Environmental	Water density	Total density of fresh water within a buffer area derived from the LGN2020 spatial layer.	25 m^2	[47]
	Environmental	Building period < 1945	Proportion (%) of buildings built before 1945.	PC4	[49]
	Socio-economic	Human population density	Human population density per PC6 area.	PC6	Statistics Netherlands (CBS)
	Socio-economic	Median disposable income	Median disposable income per PC4 area.	PC4	Statistics Netherlands (CBS)
	Time	Season	Season of deployment start date (winter, spring, summer, autumn)	-	-
Random effect [a]	Location	Municipality	Municipality in which the camera trap was deployed.	-	-

[a] Year was not included as a random factor due to high overlap with municipality (Supplemental Table S1).

all other numerical variables (e.g., road density, water density, building period < 1945, human population density, and median disposable income) were standardized by subtracting the mean and dividing by the standard deviation (e.g., to a mean of 0 and a

standard deviation (SD) of 1). Lastly, deployments containing missing values for any of the predictor variables were excluded.

Time-to-event (survival) analysis

A time-to-event analyses was used to establish the minimum deployment period needed to have a reliable indication of small mammal presence/absence in a garden and to right-censor the number of observation days in case of extremely large deployment durations (i.e., duration cap). Here, an event was defined as the initial photographic sighting of one of our target species (i.e., brown rats, micromammals) and their predators. [50]. Then, based on visual inspection of the Kaplan-Meier survival curve, the time point at which in approximately 75 % of gardens the target species had been observed was estimated and was defined as the minimum deployment period. The data was right censored at the time point at which the target species had been detected in almost 100 % of the gardens in which it was present. The generated timeframe was used for subsequent GLMM analyses.

Generalized linear mixed models

A GLMM with municipality as random factor was used to investigate associations between the presence or absence of small mammals and potential explanatory variables (Table 1). Separate models were created for the presence or absence of brown rats [0, 1] and micromammals [0, 1]. To assess the robustness of identified associations, both models were developed with and without a duration cap (Supplemental analysis 1) derived from the Kaplan-Meier survival curves. Predictor variables were evaluated for multicollinearity by using the variance inflation factor (VIF < 5). Interactions were tested between predators and greenness in the rat and micromammal models. Significant interactions were retained in the models. *P*-values below 0.05 were considered significant. To assess the strength of associations between presence of the target species and predictor variables, the log odds and corresponding 95 % confidence intervals (CIs) were calculated.

Occupancy models

We used an occupancy model to estimate occupancy and the detection probability of our species of interest [51]. Occupancy was defined as the likelihood that a rat or micromammal occupied a garden throughout the deployment period [52], and the detection probability as the chance of detecting a species in a garden given that the species was present [51]. As camera trap detection data frequently exhibits significant heterogeneity, we decided to use the Royle-Nichols (RN) occupancy model variant as this model is expected to outperform standard occupancy models in such situations [53]. In this analysis we used the "unmarked" package (1.2.5) and its occuRN function [54].

Occupancy models require that (semi)-continuous data is reformatted into presence/absence data per time interval. To determine the time interval that should be used as well as the minimum number of intervals that a location needs to have to be included in the analysis, we used the method and R-script supplemented by De Jager et al. (unpublished). Using the estimated time intervals, we created the presence/absence matrices for rats and micromammals. The number of detections (1) and non-detections (0) per interval were used to generate a vector containing zeros and ones per target species per camera

trap. This data was compiled within a matrix containing all valid sites (camera traps) and used to estimate occupancy and detection probability in our single-species occupancy models [51]. The deployment duration per camera trap was used to determine the number of repeated surveys [51]. Based on the estimated required minimum number of intervals and number of repeated surveys per camera trap, only those locations with sufficient data were included in the analysis. We estimated occupancy with the biological, environmental, and socio-economic covariates (Table 1). Detectability was assumed to be equal across all gardens.

R version 4.3.1 (2023-06-16) was used for data extraction and all statistical analyses [55].

Results

In total, valid deployments of camera traps were obtained for 1000 household gardens in 26 different municipalities across the Netherlands (Table 2). These gardens were surveyed for 30.0±9.8 days, ranging from 1 to 85 days (Supplemental Fig. S1 and Table S2). In total, the cameras recorded 118,354 detections of 117 species on 28,481 camera trapping days. This included 11,007 sightings of either brown rats (n = 1,288) or micromammals (n = 9,719; Supplemental Table S2). The percentage of gardens in which at least one rat was detected ranged from 0 % to 32 % per municipality, and for micromammals from 0 % to 64 % (Table 2).

During the study, the number of deployed camera traps varied across seasons, with the highest number of cameras deployed in autumn (n = 358), followed by spring (n = 237), winter (n = 205), and summer (n = 200; Fig. 2 and Supplemental Table S3). Brown rat sightings were most frequent in winter (median = 4, interquartile range (IQR) = 21.5, total = 214) and autumn (median = 4, IQR = 14, total = 787), followed by summer (median = 3, IQR = 6, total = 64) and spring (median = 1, IQR = 6, total = 223; Fig. 2a). Sightings of micromammals occurred most often during autumn (median = 8 detections per camera trap, IQR = 29, total = 4072). Following autumn, most sightings occurred in winter (median = 5, IQR = 28, total = 3152), spring (median = 5, IQR = 13, total = 1872), and summer (median = 3.5, IQR = 8, total = 623; Fig. 2a). Amongst detected species, domestic cats were by far the most observed species in these gardens (91.6 %), followed by micromammals (38.4 %), dogs (24.7 %), mustelids (23.2 %), brown rats (9.5 %) and red foxes (8.7 %; Fig. 2b).

Correlates of brown rat and micromammal presence in gardens

Deployment selection and censoring

Across all 1000 gardens, about 75 % of the brown rat, micromammal and predator detections happened within the first 20 days of camera deployment (Kaplan-Meier survival analysis; Supplemental Fig. S2 - S4). After approximately 50 days, there were no new sightings of brown rats and micromammals (Supplemental Fig. S2 - S4). As a result, a minimum deployment duration of 20 days was established, and deployments were censored at 50 days. A total of 862 gardens were included in subsequent GLMM analyses (with duration cap). In gardens that were excluded based duration was too short (n = 136), eight gardens (6 %) had brown rat detections, whereas 44 gardens (32 %) had detections of micromammals.

Table 2. *Overview of camera trapping data and the detection of rats and micromammals per municipality*

Municipality	# Gardens	Detections of brown rats (%)	Detections of micromammals (%)
's-Gravenhage	45	11.1	26.7
's-Hertogenbosch	41	7.3	58.5
Amersfoort	24	4.2	25
Amsterdam	38	31.6	21.1
Arnhem	33	12.1	42.4
Barneveld	24	4.2	33.3
Berg en Dal	42	11.9	57.1
Breda	61	31.1	50.8
Delft	34	5.9	47.1
Deventer	59	6.8	64.4
Doetinchem	39	7.7	17.9
Ede	73	6.8	43.8
Eindhoven	41	7.3	61
Haarlemmermeer	40	12.5	35
Meppel	29	3.4	34.5
Nijkerk	43	9.3	9.3
Nijmegen	60	6.7	45
Nunspeet	29	0	41.4
Papendrecht	17	11.8	11.8
Ridderkerk	16	12.5	56.2
Rotterdam	50	12	36
Smallingerland	16	0	12.5
Utrecht	35	2.9	11.4
Wageningen	35	0	0
Winterswijk	47	2.1	53.2
Zoetermeer	29	6.9	41.4
Total	**1,000**	**9.5**	**38.4**

Generalized linear mixed models

To understand the drivers of brown rat and micromammal presence in private gardens, we included predator presence, as well as environmental and socio-economic variables as predictor variables in our analyses (Table 1). Details on the distribution of these variables can be found in Fig. 3. Greenness by itself did not predict brown rat and micromammal presence (Fig. 4). For brown rats specifically, we identified a negative association between the presence of brown rats and the percentage of domestic cats (Log odds = -0.23 (-0.37 – -0.09), $p < 0.001$). We further detected a positive correlation with the season Spring (Log odds = 0.95 (0.07 – 1.84), $p = 0.033$; Fig. 4a and Fig. 5), and a positive interaction between the degree of greenness and the percentage of domestic cats (log odds = 0.20 (0.00 – 0.41), $p = 0.049$). Regarding micromammals, we found negative relationships with human population density (Log odds= -0.22 (-0.40 – -0.04), $p = 0.017$), the percentage of detected foxes (log odds = -0.41 (-0.65 – -0.17), $p = 0.001$), and the percentage of detected dogs (log odds = -0.12 (-0.24 – 0.00), $p = 0.041$). In addition, positive correlations were observed between the presence of micromammals and the percentage of detected brown rats (Log

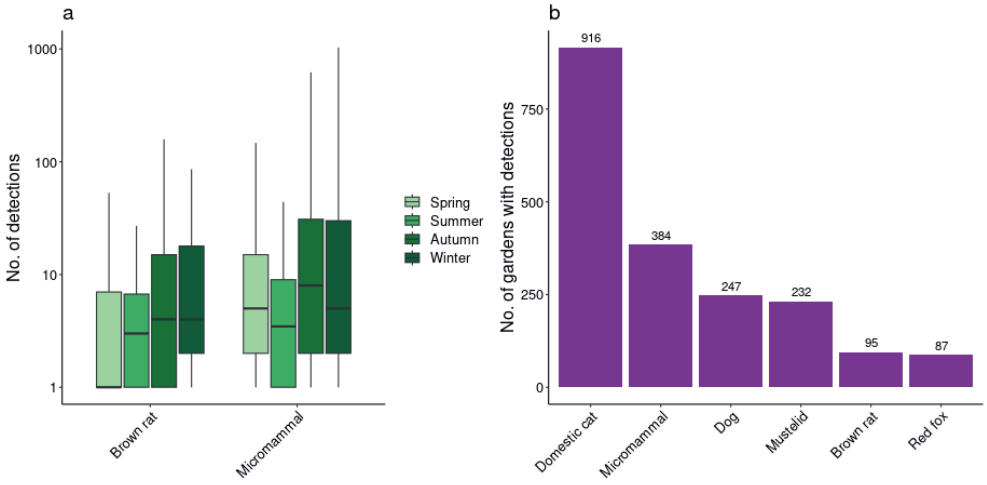

Figure 2. *(a) Number of detections of brown rats and micromammals per garden per season (e.g., spring, summer, autumn, winter); (b) Number of gardens with at least one sighting of the species of interest (e.g., domestic cats, micromammals, dogs, mustelids, brown rats, and red foxes; total number of gardens = 1,000).*

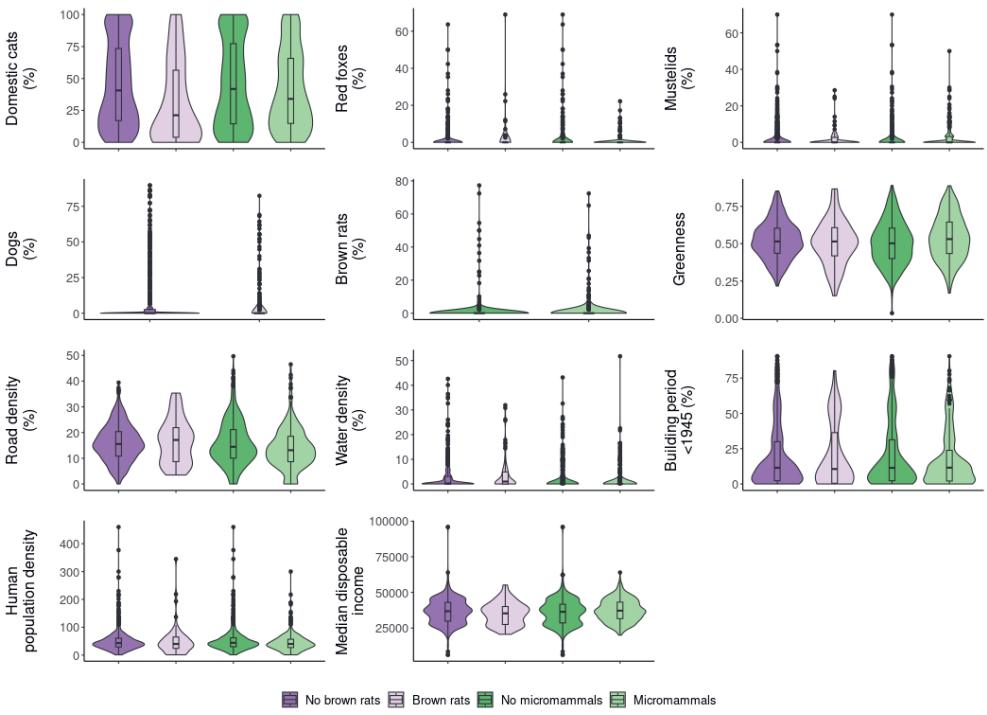

Figure 3. *Distribution of predictor variables included in GLMM with duration cap (n = 849; 13 gardens were excluded due to missing data)*

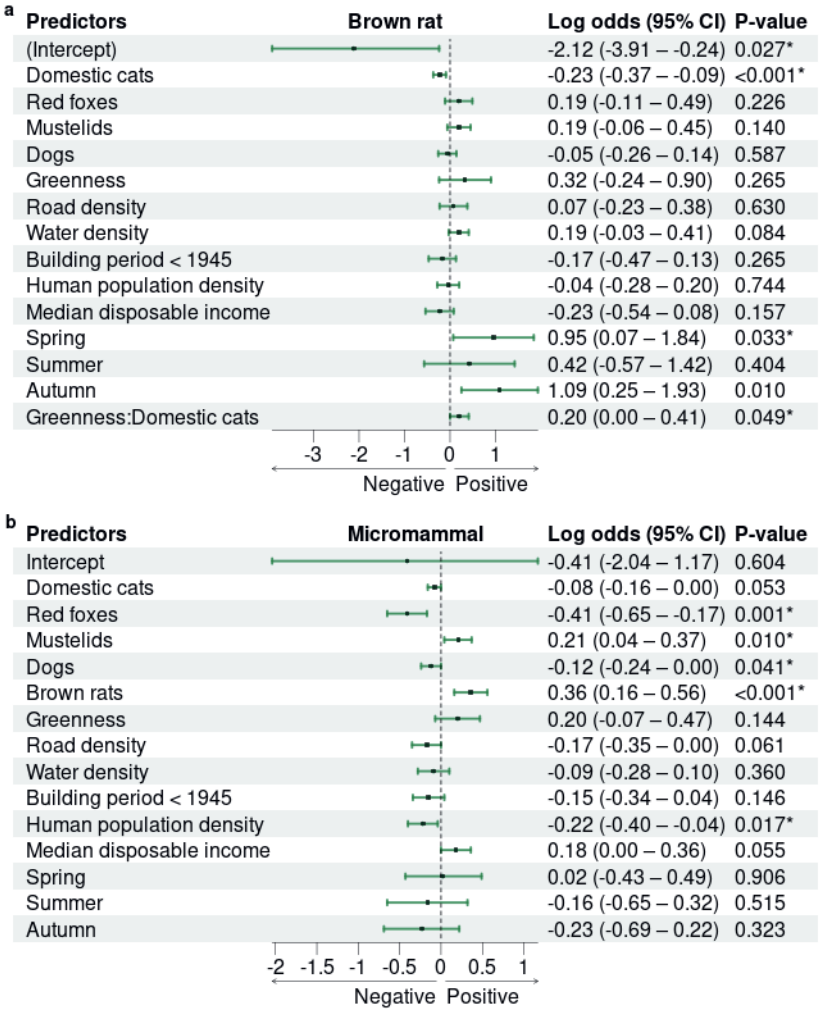

Figure 4. *Model output of the presence of brown rats (Fig. 4a) and micromammals (Fig. 4b) in relation to predictor variables (i.e., domestic cat presence, mustelid presence, red fox presence, brown rat presence, dog presence, greenness, road density, water density, building period < 1945, human population density, median disposable income and season (reference variable = winter, n = 849). *Statistically significant.*

odds = 0.36 (0.16 – 0.56), $p < 0.001$), as well as with the percentage of detected mustelids (Log odds = 0.21 (0.04 – 0.37), $p = 0.010$). We observed negative trends, albeit not significant, with the percentage of domestic cats, road density and median disposable income (Fig. 4b and Fig. 6). Due to multicollinearity among predictors (VIF > 5) the interaction between mustelids and greenness was not considered in the final micromammal model (Supplemental Table S4 and S5). The proportion of variance explained by both the fixed and random effects was 27.6 % for the brown rat model, and 28.8 % for the micromammal model. The results of the GLMMs without censoring can be found in the Supplemental analysis 1.

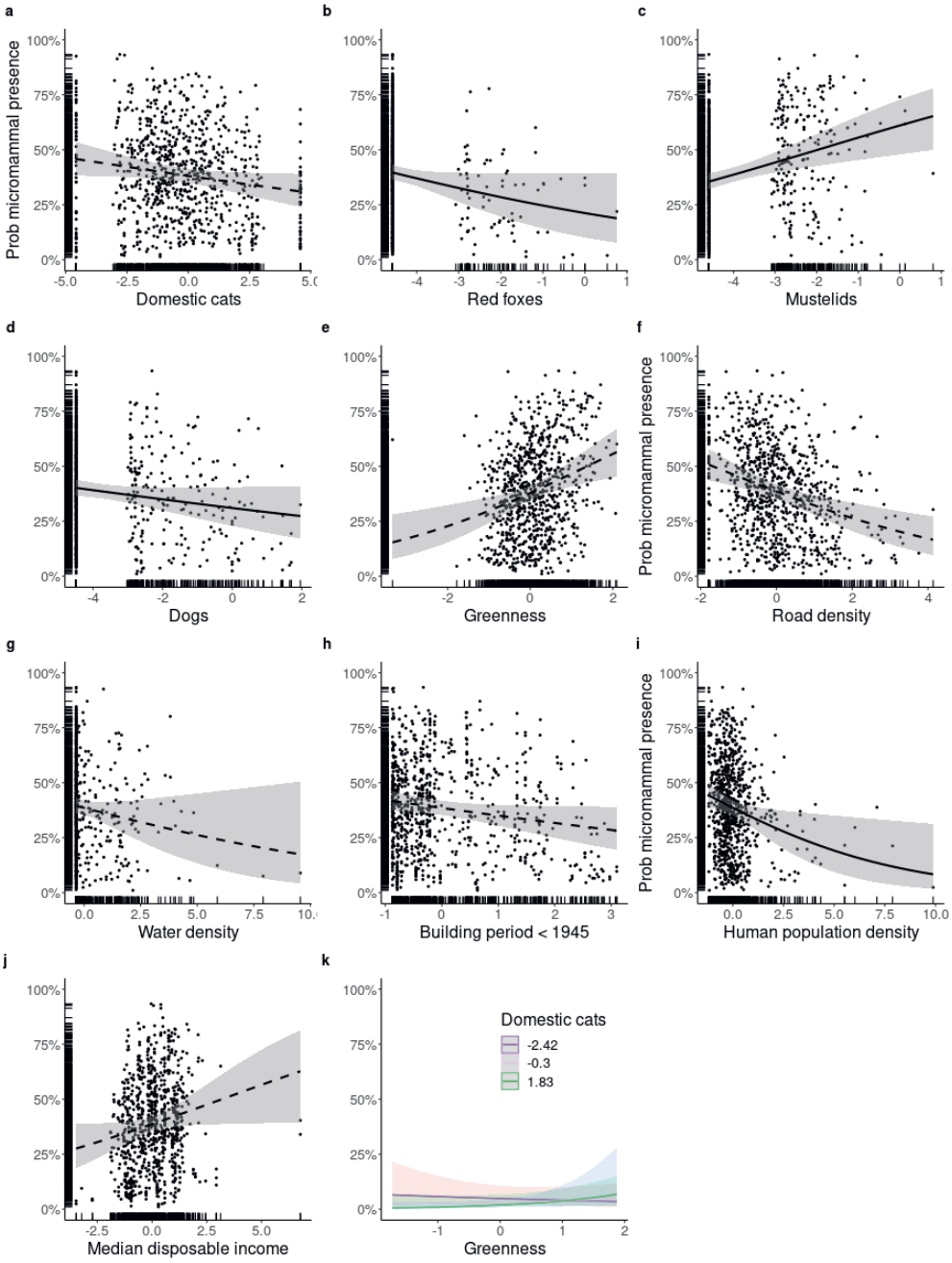

Figure 5. *Predicted probability of brown rat presence per environmental variable (a-j). Significant associations are displayed with solid lines, non-significant associations are displayed with dashed lines. Interaction between greenness and domestic cats is shown in Fig. 5k.*

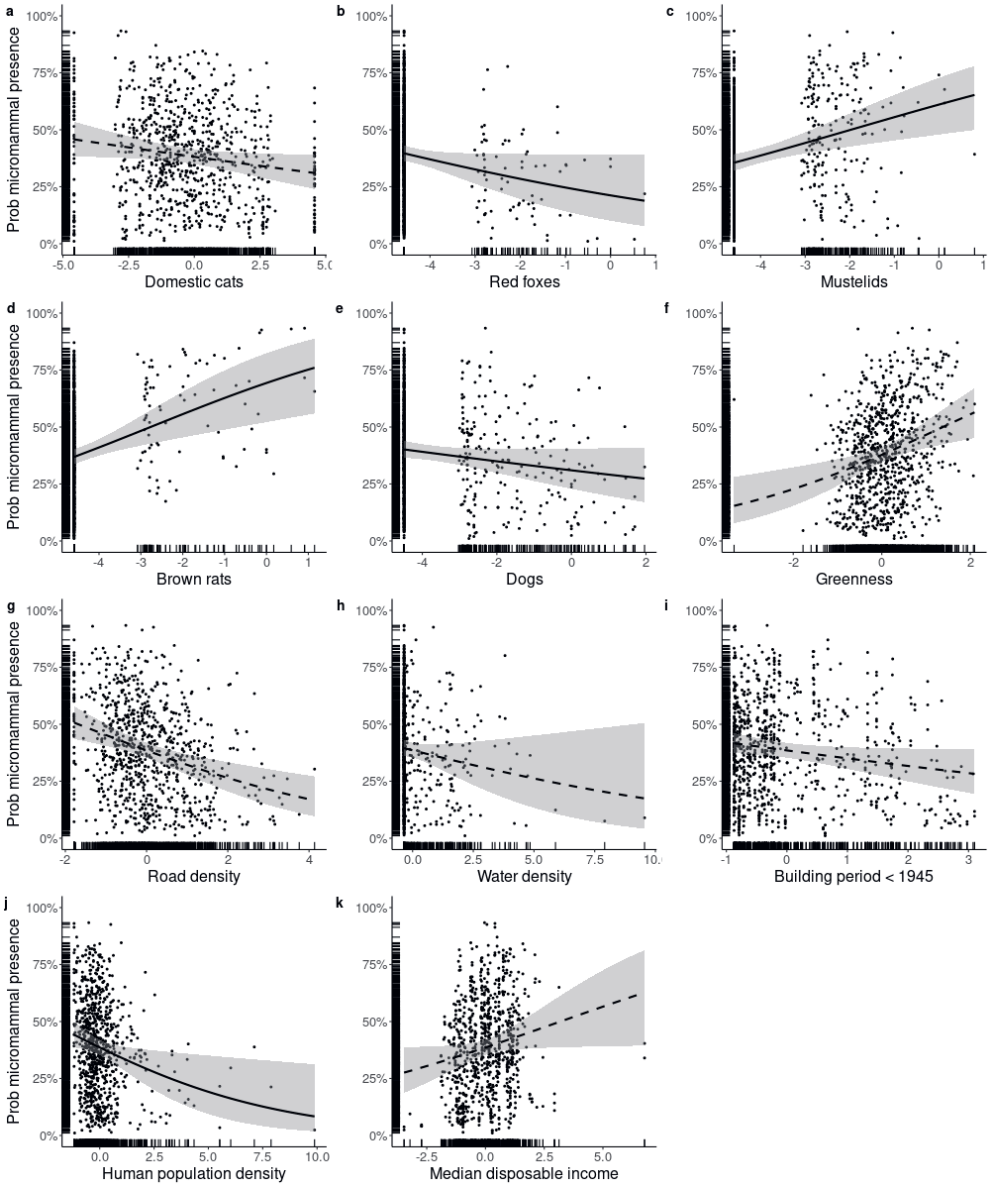

Figure 6. *Predicted probability of micromammal presence per environmental variable. Significant associations are displayed with solid lines, non-significant associations are displayed with dashed lines.*

Occupancy modelling

For micromammals, the optimal combination of dT and nT was dT = 3 and nT = 3 (Fig. S6). Due to the relatively low detection of brown rats throughout the study (< 10 % of the gardens; Fig. S5), it was not possible to establish an optimal interval size (dT) or to

determine the minimum number of intervals (nT). As a result, occupancy modelling for brown rats could not be performed as the results would not be meaningful. The occupancy model for micromammals showed an average occupancy of 0.62 (95 % CI: 0.56 – 0.69) and a detection probability of 0.28 (95 % CI: 0.26 – 0.30). Like micromammal presence in the GLMM (including duration cap), micromammal occupancy was associated with the percentage of mustelids (positive association), the percentage of brown rats (positive association), the percentage of red foxes (negative association), the percentage of dogs (negative association), and human population density (negative association; Table 3). In addition, the micromammal occupancy model identified associations with the percentage of cats (negative association), road density (negative association), and greenness (positive association; Table 3). We observed similar trends, albeit not significant, for these three variables in the micromammal GLMM (Table 3).

Table 3. *Summary statistics for the micromammal occupancy model (n = 956) and mixed logistic regression model with duration cap (n = 849).*

Predictors	Occupancy model			Mixed logistic regression model	
	Estimate	**SE**	**p-value**	**Log odds(95 % CI)**	**p-value**
(Intercept)	-0.81	0.45	0.073	-0.41 (-2.04 – 1.17)	0.604
Domestic cats	-0.09	0.02	< 0.001*	-0.08 (-0.16 – 0.00)	0.053
Red foxes	-0.30	0.07	< 0.001*	-0.41 (-0.65 – -0.17)	0.001*
Mustelids	0.15	0.04	< 0.001*	0.21 (0.04 – 0.37)	0.010*
Dogs	-0.11	0.03	0.002*	-0.12 (-0.24 – 0.00)	0.041*
Brown rats	0.19	0.40	< 0.001*	0.36 (0.16 – 0.56)	< 0.001*
Greenness	0.15	0.08	0.041*	0.20 (-0.07 – 0.47)	0.144
Road density	-0.15	0.05	0.004*	-0.17 (-0.35 – 0.00)	0.061
Water density	-0.04	0.05	0.417	-0.09 (-0.28 – 0.10)	0.360
Building period < 1945	-0.08	0.05	0.139	-0.15 (-0.34 – 0.04)	0.146
Human population density	-0.22	0.06	< 0.001*	-0.22 (-0.40 – -0.04)	0.017*
Median disposable income	0.08	0.05	0.097	0.18 (0.00 – 0.36)	0.055
Spring	0.02	0.13	0.884	0.02 (-0.43 – 0.49)	0.906
Summer	-0.12	0.14	0.404	-0.16 (-0.65 – 0.32)	0.515
Autumn	-0.10	0.13	0.447	-0.23 (-0.69 – 0.22)	0.323

*Statistically significant.
Green and purple highlighted cells represent similarities and differences, respectively, between the occupancy model and mixed logistic regression model output.

Discussion

Which factors determine the presence of small mammals in urban areas is poorly known, yet important for disease risk and the implementation of preventive measures. In this study we investigated whether predators, environmental factors and socio-economic factors could predict the presence of brown rats and micromammals in private gardens in the Netherlands. Our analyses relied on camera trapping data, which we analysed using GLMMs and occupancy models. We found that the presence of predators was a more important predictor of both the presence and occupancy of brown rats and micromammals in private gardens than the degree of greenness or socio-economic factors.

Relationships between small mammals and predators

Domestic cats are known to prey upon micromammals and on juvenile rats [24, 27, 30-32, 34]. In case of brown rats, we observed a negative relationship between their presence and the presence of domestic cats. This finding coincides with previous research in which rats seemed to avoid cats [32]. In addition, we identified a positive interaction between domestic cats and greenness, which implied that the negative effect of cats on rats is larger in greener areas and smaller in less green areas. This might indicate that rats in urbanized areas are bolder than rats in greener areas [56]. Schell et al (2021) found a similar negative relationship between predator pressure and rat boldness.

In contrast to brown rats, we did find that domestic cat presence was negatively associated with micromammal occupancy. This suggests that cats may not determine the mere presence or absence of micromammals but do affect their abundance. The stronger inverse relationship between brown rats and domestic cats could be related to the more cautious or neophobic behaviour of rats in response to cat presence compared to micromammals' behaviour [57-59]. Moreover, the grouping of mice, voles and shrews into one group (micromammals) likely weakened the relationship between micromammals and cats because voles and shrews are less preferred prey to cats, compared to mice [60]. The negative relationships between the presence and occupancy of micromammals and both red foxes and dogs could be caused by them predating on micromammals [43]. However, we suspect that the relationship with red foxes could also be caused by spatial segregation, given they were co-observed in only 29 gardens (8 %). The positive relationships we found between micromammals and both mustelids and brown rats could be caused by predator (e.g., mustelids and brown rats) movement upon prey (e.g., micromammal) availability [61]. However, mustelid predation might also be lower because certain mustelid species (e.g., stone martens) can shift their diet, by for example eating less mammals in urban areas compared to rural areas [35]. Besides that, micromammals and rats can compete for the same resources, which might also explain their positive relationship [62].

Relationships between small mammals and the degree of greenness

Although the occupancy of micromammals showed a positive relationship with greenness, the presence of both brown rats and micromammals was poorly explained by the degree of greenness, which is in disagreement with previous studies [7, 17, 63]. One reason may be that we measured presence-absence (non-detection) and occupancy of small mammals, whereas previous studies focused on rat abundance (measured using either snap traps, or live traps), or municipality rat complaint data. Possibly, the degree of greenness does not influence the mere presence or absence of small mammals but does influence their abundance, for which the positive relationship observed in the occupancy model might be an indication.

Unlike previous studies that lacked data on predators, our study suggests that predators are important predictors for the presence and/or occupancy of brown rats and micromammals in gardens, more than greenness. Small mammal predation is often however insufficient to reduce small mammal populations for effective biological control

[64]. Only when small mammal population sizes are very small, predators may be able to prevent re-infestation [64]. Besides that, predator species can also carry and introduce zoonotic pathogens [65-67]. In that way, using predators to reduce small mammal populations to limit the potential exposure to zoonotic pathogens might not prevent zoonotic pathogen transmission, but replace the type of zoonotic pathogens from small mammals by the ones carried by their predators.

Relationships between small mammals and other environmental and socio-economic variables

Micromammal presence and abundance were negatively associated with human population density, in contrast with a prior study [68]. Antonelli et al., however, only investigated house mice, which prefer to live close to humans, while other mice species, voles and shrews prefer more natural environments [22, 23]. The grouping of all mice, voles, and shrews into 'micromammals' may thus have resulted in this reversed relationship. Furthermore, we observed a positive correlation of micromammal occupancy with median disposable income and a negative association with road density, along with corresponding non-significant trends in presence. The positive relationship with income might be linked to the luxury effect, a pattern of higher biodiversity in affluent neighbourhoods [69]. This could be associated with environmental characteristics such as a higher plant diversity, bigger gardens, and maintenance of green space. The negative relationship with road density might be related to the negative relationship with human population density (although both predictors were not highly correlated). A higher road density might lead to more habitat fragmentation and decreased habitat quality, which could negatively affect micromammals [70]. Other environmental variables (e.g., water density and building period < 1945) were not found to be significantly associated with the presence and/or occupancy of small mammals.

Limitations and strengths

One limitation of this study was the grouping of mice, voles, and shrews into "micromammals" because of difficulties in reliably identifying each micromammal to species level from camera trap images. Mice, voles, and shrews differ in terms of ecology. For example, wood mice prefer more vegetation cover and greenness compared to house mice, which prefer to live in human population dense areas [68, 71]. Grouping may thus have masked important differential relationships between individual micromammal species and certain predictor variables. Second, the measure that we used for greenness (NDVI) can consist of many different combinations of plant species and structures, which could have different effects on the presence and/or occupancy of small mammals. Unfortunately, we were not able to include that specific information in this study. A third limitation of this study was the lack of data on the availability of food resources such as bird feed, garbage and compost heaps, which is likely an important determinant of rodent occurrence in gardens [4, 72]. Lastly, the lure we used (a leaking can of sardines) differentially influenced the detection probability of animal species. Predators were more attracted by the lure and therefore had a greater likelihood of detection when present than rats and micromammals [43]. False negatives of rat and micromammal presence –

species less attracted by the lure - may have weakened the relationships.p

This study comprises a very large dataset, which makes the observed relationships quite reliable. In addition, we based the minimal duration period of the camera traps in the GLMMs on Kaplan-Meier survival analyses of actual detections of rats, micromammals, and their predators in the dataset. We observed that rats were detected in only 8 out of 136 (6 %) gardens that were excluded from the GLMMs due to the duration cap, which shows that the excluded data did not contain many rat detections. A recent study determined that a duration period of three to four weeks was needed to obtain precise detection estimates, which matches with the minimal duration period of 20 days often used in other studies and resulting from the Kaplan-Meier curves seen here [13, 73, 74]. In addition, the GLMMs we ran with the minimal duration period of 20 days resulted in fewer significant relationships compared to the GLMMs without the minimal duration period of 20 days. This highlights the importance of using a sufficiently long duration period to avoid false negatives (e.g., gardens with no detections of rats and micromammals, while they might have been present there) and thereby erroneous relationships with predictor variables. Moreover, future research might even strive for a minimum duration longer than 20 days (e.g., approximately 50 days) to get more reliable estimates of animal presence and fewer false negatives. Furthermore, by using a new method to determine the optimal occupancy interval criteria (De Jager et al., unpublished), we observed that the camera trap data for brown rats was unsuitable (too few brown rat observations) to reliably model occupancy. This highlights the importance of checking the suitability of your data before analysis.

Conclusions

We conclude that the presence of predators, in particular domestic cats, is a better predictor for the presence of brown rats and micromammals in private gardens in the Netherlands than urban greenness. However, it remains unknown to what extent these wild predators reduce the population size of small mammals in urbanized areas. As urban greenness only showed a positive relationship with the occupancy of micromammals and not with their presence, this may indicate a potential positive relationship between greenness and micromammal abundance. Since small mammal populations may contribute to zoonotic disease risk in urban environments, it remains important to monitor these populations and to further increase our understanding of the factors influencing their presence and behaviour.

Acknowledgements

We would like to thank all citizens that gave us permission to monitor wildlife in their gardens with our camera traps; all participating municipalities for their contributions to the data collection in this project; the students and other volunteers that deployed camera traps and/or annotated photos, including Veerle Haak, Maarten Matla, Jeroen van Wijk, Wout van Zeeburg, Marielle Vriends, Nienke Heida, Leanne Eenink, Marijn Teunizen, Kaylee Springer, Anouk van Heumen, Maaike van Overstraten, Jordi Timmermans, Kaylee Springer, and others; and Wageningen University and Yorick Liefting for hosting this project in Agouti.

Supplementary data

Table S1. *Number of deployments (n = 1,000) per municipality (n = 26) per year (2016 – 2022).*

Municipality	2016	2017	2018	2019	2020	2021	2022
's-Gravenhage	0	0	32	13	0	0	0
's-Hertogenbosch	0	18	23	0	0	0	0
Amersfoort	16	8	0	0	0	0	0
Amsterdam	0	0	0	0	38	0	0
Arnhem	0	0	0	13	16	4	0
Barneveld	0	0	0	7	14	3	0
Berg en Dal	0	0	4	38	0	0	0
Breda	0	1	0	12	41	7	0
Delft	0	0	26	8	0	0	0
Deventer	23	36	0	0	0	0	0
Doetinchem	0	0	0	2	17	20	0
Ede	0	0	0	0	0	49	24
Eindhoven	0	0	0	22	19	0	0
Haarlemmermeer	0	24	16	0	0	0	0
Meppel	0	22	7	0	0	0	0
Nijkerk	0	0	0	0	0	43	0
Nijmegen	7	3	0	0	29	21	0
Nunspeet	0	0	29	0	0	0	0
Papendrecht	0	0	0	17	0	0	0
Ridderkerk	0	0	0	0	0	0	16
Rotterdam	0	37	13	0	0	0	0
Smallingerland	0	0	0	16	0	0	0
Utrecht	0	0	35	0	0	0	0
Wageningen	0	0	0	0	0	35	0
Winterswijk	0	0	0	22	25	0	0
Zoetermeer	0	0	0	0	27	2	0

Table S2. *Descriptive statistics per year (2016 – 2022).*

Year	No. of gardens	No. of trapping days	No. of detections¥	No. of brown rat detections	No. of micromammal detections
2016	46	1,640	6925	24	937
2017	149	5,035	20,015	229	2,411
2018	185	5,336	23,890	185	1,022
2019	170	5,344	19,108	368	2,721
2020	226	5,565	23,081	326	1,896
2021	184	4,289	19,324	117	409
2022	40	1,272	6,011	39	323
Total	1,000	28,481	118,354	1,288	9,719

¥Excluding observations of humans.

Table S3. *Number of deployments (n = 1,000) per municipality (n = 26) per season (spring, summer, autumn and winter).*

Municipality	Spring	Summer	Autumn	Winter
's-Gravenhage	17	7	9	12
's-Hertogenbosch	10	14	7	10
Amersfoort	5	8	6	5
Amsterdam	0	0	38	0
Arnhem	5	6	12	10
Barneveld	5	0	8	11
Berg en Dal	13	5	11	13
Breda	19	9	17	16
Delft	12	7	7	8
Deventer	14	9	25	11
Doetinchem	11	6	12	10
Ede	15	19	20	19
Eindhoven	11	7	11	12
Haarlemmermeer	18	8	6	8
Meppel	8	10	6	5
Nijkerk	0	0	43	0
Nijmegen	26	11	20	3
Nunspeet	0	29	0	0
Papendrecht	0	0	17	0
Ridderkerk	1	7	5	3
Rotterdam	15	17	13	5
Smallingerland	16	0	0	0
Utrecht	0	0	20	15
Wageningen	0	0	23	12
Winterswijk	6	13	16	12
Zoetermeer	10	8	6	5

Table S4. *Predictor variables of brown rat presence evaluated for multicollinearity (VIF).*

Predictor	VIF	95 % CI lower limit	95 % CI upper limit
Domestic cats	1.04	1.01	1.22
Red foxes	1.22	1.14	1.34
Mustelids	1.06	1.01	1.21
Dogs	1.06	1.01	1.21
Greenness	1.57	1.44	1.73
Road density	1.28	1.19	1.40
Water density	1.06	1.02	1.21
Building period < 1945	1.06	1.02	1.21
Human population density	1.09	1.04	1.21
Median disposable income	1.18	1.11	1.3
Season	1.04	1.01	1.24
Greenness: Domestic cats	1.38	1.28	1.52

Table S5. *Predictor variables of micromammal presence evaluated for multicollinearity (VIF).*

Predictor	VIF	95 % CI lower limit	95 % CI upper limit
Domestic cats	1.05	1.01	1.22
Red foxes	1.14	1.08	1.26
Mustelids	1.33	1.24	1.46
Dogs	1.04	1.01	1.22
Brown rats	1.04	1.01	1.22
Greenness	16.63	14.65	18.91
Road density	1.11	1.05	1.23
Water density	1.02	1.00	1.43
Building period < 1945	1.04	1.01	1.22
Human population density	1.06	1.02	1.20
Median disposable income	1.09	1.04	1.21
Season	1.03	1.00	1.27
Greenness: Mustelids	15.95	14.05	18.13

[a] Interaction Greenness:Mustelids was removed excluded from the final micromammal GLMM model with duration cap due to VIF > 5.

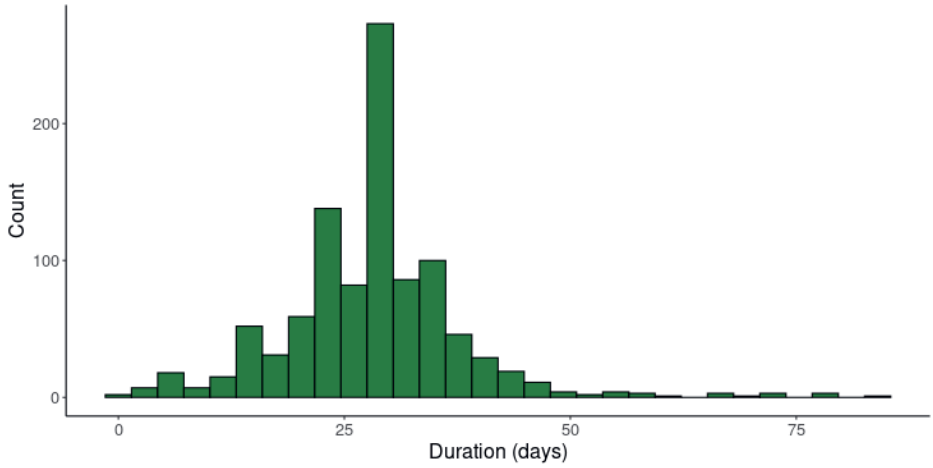

Figure S1. *Frequency distribution of uncensored camera deployment duration*

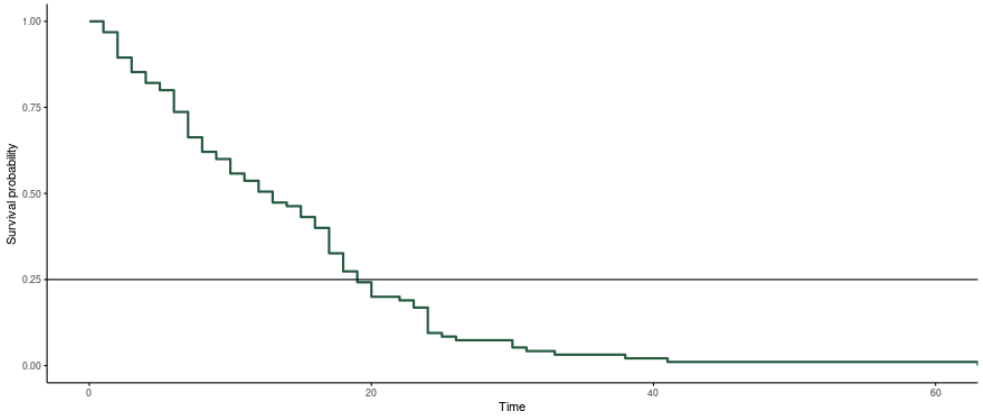

Figure S2. *Kaplan-Meier curve for brown rat detections (horizontal line = 75 % of detections (i.e., intersection = 19 days) have occurred in gardens in which at least once a brown rat was detected)*

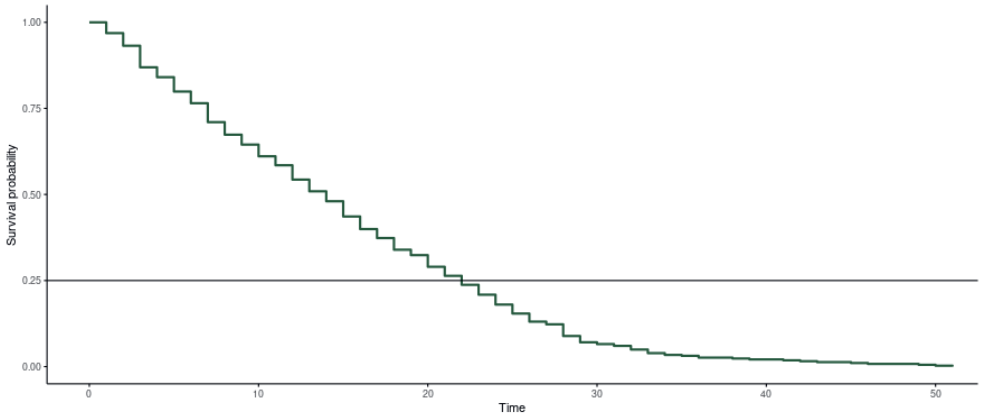

Figure S3. *Kaplan-Meier curve for micromammal detections (horizontal line = 75 % of detections (i.e., intersection = 22 days.) have occurred in gardens in which at least once a micromammal was detected)*

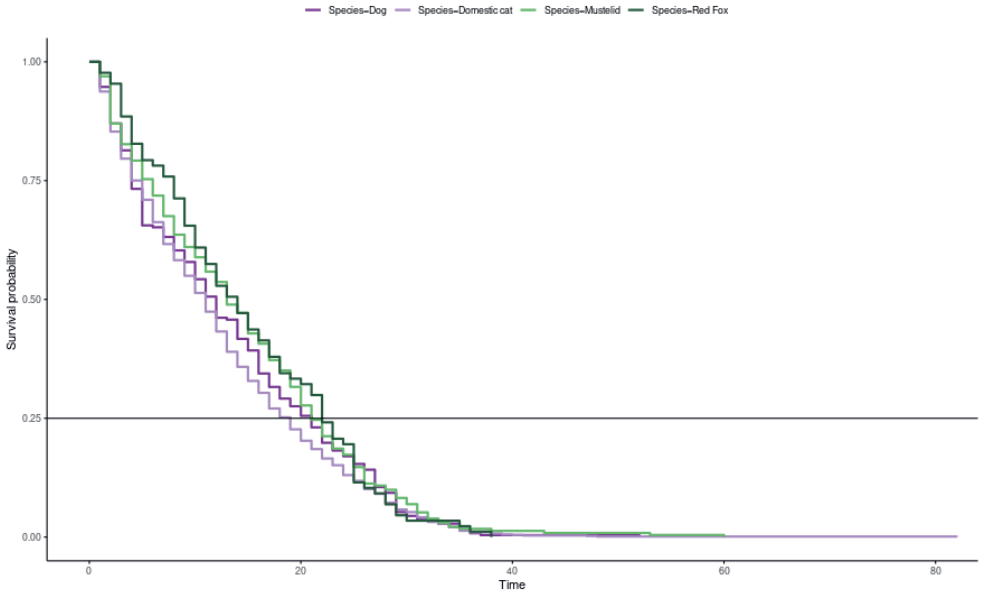

Figure S4. *Kaplan-Meier curves for the detections of dogs (dark purple), domestic cats (purple), mustelids (green), and red foxes (dark green)*

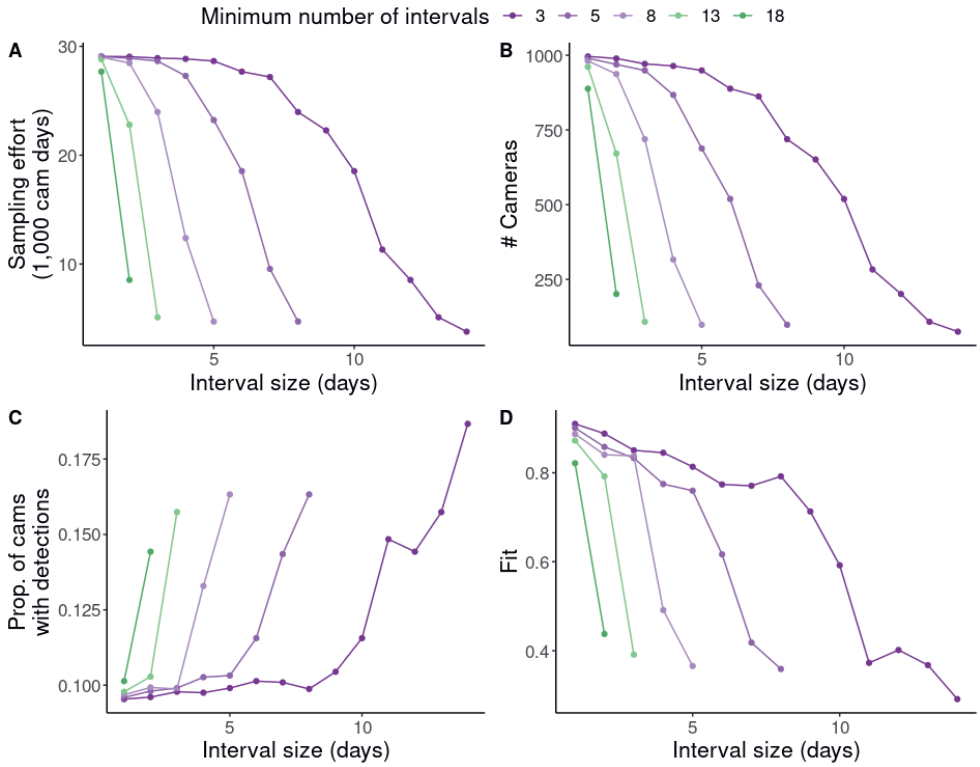

Figure S5. *Estimation of optimal interval size and minimum number of intervals needed for occupancy analysis of the rat data. Colours indicate the minimum number of intervals; for visualisation, we only plotted five different values. Total sampling effort (a) and the number of cameras (b) that are used in the analysis decrease with increasing interval size and minimum number of intervals. In case of rat recordings, the proportion of cameras with detections (c) remains low for all combinations of interval size and minimum number of intervals. Hence, the fit of the RN occupancy model (d) on this data is insufficient (Fit > 0.14) to achieve reliable results, regardless of interval size and minimum number of intervals.*

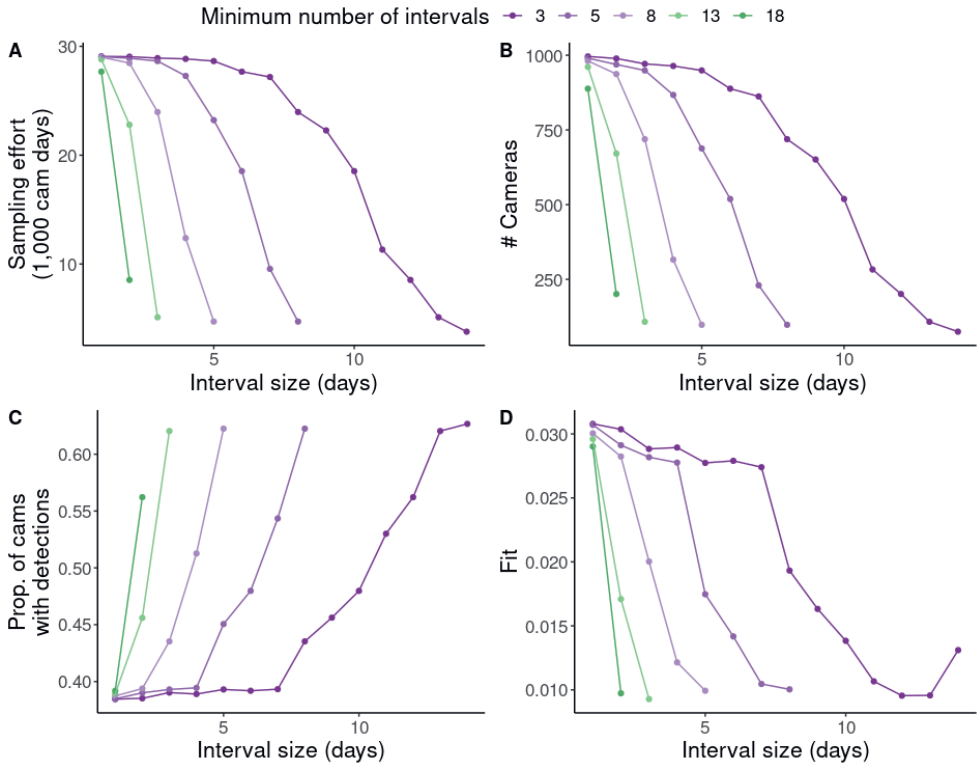

Figure S6. *Estimation of optimal interval size and minimum number of intervals needed for occupancy analysis of the micromammal data. Colours indicate the minimum number of intervals; for visualisation, we only plotted five different values. Total sampling effort (a) and the number of cameras (b) that are used in the analysis decrease with increasing interval size and minimum number of intervals. In case of micromammal recordings, the proportion of cameras with detections (c) increases with rising interval size and increasing minimum number of intervals and is sufficiently large for all combinations of interval size and minimum number of intervals. Hence, the fit of the RN occupancy model (d) on this data is sufficient (Fit << 0.14) to achieve reliable results in all cases, regardless of interval size and minimum number of intervals. The combination of interval size (dT) and minimum number of intervals (nT) that optimizes the fit (fit à 0) is dT = 3 and nT = 3.*

Supplemental analysis 1 Generalized linear mixed models excluding duration cap

We fitted the same model on the capture data without censoring deployments at a duration of 50 days. Compared to the model for brown rats with the duration cap, the model for brown rats without duration cap identified an additional significant positive association with water density, while domestic cats and the spring season were significant in both models (Supplemental Table S6). We identified additional associations with domestic cats, greenness, and median disposable income. However, the identified association with the percentage of dogs disappeared (Supplemental Table S6). The proportion of variance explained by both the fixed and random effects was 28.7 % for the brown rat, and 26.0 % for the micromammal model.

Table S6. *GLMM output for brown rat presence and micromammal presence (n = 985; timeframe (0 – 50 days observation days)).*

	Predictors	Log odds	95 % CI	p-value *
Brown rats¥	*(Intercept)*	-1.61	(-3.51 – 0.26)	0.092
	Domestic cats	-0.24	(-0.36 – -0.12)	< 0.001
	Red foxes	0.27	(-0.01 – 0.56)	0.063
	Mustelids	0.21	(-0.06 – 0.47)	0.126
	Dogs	-0.03	(-0.21 – 0.15)	0.748
	Greenness	0.42	(-0.14 – 0.99)	0.143
	Road density	0.1	(-0.17 – 0.39)	0.456
	Water density	0.22	(0.02 – 0.42)	0.035
	Building period < 1945	-0.17	(-0.45 – 0.11)	0.239
	Human population density	-0.09	(-0.34 – 0.16)	0.464
	Median disposable income	-0.2	(-0.51 – 0.1)	0.182
	Spring	0.92	(0.09 – 1.75)	0.030
	Summer	0.29	(-0.65 – 1.23)	0.548
	Autumn	0.94	(0.14 – 1.74)	0.021
	Greenness:Domestic cats	0.26	(0.08 – 0.45)	0.006
Micromammals¤	*(Intercept)*	-1.05	(-2.66 – 0.54)	0.195
	Domestic cats	-0.08	(-0.15 – -0.02)	0.010
	Red foxes	-0.42	(-0.67 – -0.17)	0.001
	Mustelids	0.18	(0.02 – 0.34)	0.032
	Dogs	-0.11	(-0.21 – 0.01)	0.072
	Brown rats	0.27	(0.09 – 0.46)	0.005
	Greenness	0.25	(0 – 0.51)	0.047
	Road density	-0.12	(-0.29 – 0.05)	0.163
	Water density	-0.07	(-0.24 – 0.1)	0.438
	Building period < 1945	-0.19	(-0.36 – 0)	0.050
	Human population density	-0.24	(-0.42 – -0.07)	0.006
	Median disposable income	0.17	(0 – 0.35)	0.047
	Spring	0.32	(-0.12 – 0.76)	0.148
	Summer	-0.09	(-0.54 – 0.36)	0.697
	Autumn	-0.2	(-0.62 – 0.22)	0.350

*Statistically significant. Green and purple highlighted cells represent similarities and differences, respectively, between the mixed logistic regression model output with and without duration cap.

¥Model random effects, σ^2 (random effect variance): 3.29; Marginal R^2 / Conditional R^2: 0.177 / 0.287

¤Model random effects, σ^2 (random effect variance): 3.29; Marginal R^2 / Conditional R^2: 0.099 / 0.260

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Café

Chapter 6

Increased rat-borne zoonotic disease hazard in greener urban areas

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Abstract

Urban greening has benefits for both human and environmental health. However, urban greening might also have negative effects as the abundance of wild rats, which can host and spread a great diversity of zoonotic pathogens, increases with urban greenness. Studies on the effect of urban greening on rat-borne zoonotic pathogens are currently unavailable. Therefore, we investigated how urban greenness is associated with rat-borne zoonotic pathogen prevalence and diversity, and translated this to human disease hazard. We screened 412 wild rats (*Rattus norvegicus* and *Rattus rattus*) from three cities in the Netherlands for 18 different zoonotic pathogens: *Bartonella* spp., *Leptospira* spp., *Borrelia* spp., *Rickettsia* spp., *Anaplasma phagocytophilum*, *Neoehrlichia mikurensis*, *Spiroplasma* spp., *Streptobacillus moniliformis*, *Coxiella burnetii*, *Salmonella* spp., methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum beta- lactamase (ESBL)/AmpC-producing *Escherichia coli*, rat hepatitis E virus (ratHEV), *Seoul orthohantavirus*, *Cowpox virus*, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), *Toxoplasma gondii* and *Babesia* spp. We modelled the relationships between pathogen prevalence and diversity and urban greenness. We detected 13 different zoonotic pathogens. Rats from greener urban areas had a significantly higher prevalence of *Bartonella* spp. and *Borrelia* spp., and a significantly lower prevalence of ESBL/AmpC-producing *E. coli* and ratHEV. Rat age was positively correlated with pathogen diversity while greenness was not related to pathogen diversity. Additionally, *Bartonella* spp. occurrence was positively correlated with that of *Leptospira* spp., *Borrelia* spp. and *Rickettsia* spp., and *Borrelia* spp. occurrence was also positively correlated with that of *Rickettsia* spp. Our results show an increased rat-borne zoonotic disease hazard in greener urban areas, which for most pathogens was driven by the increase in rat abundance rather than pathogen prevalence. This highlights the importance of keeping rat densities low and investigating the effects of urban greening on the exposure to zoonotic pathogens in order to make informed decisions and to take appropriate countermeasures preventing zoonotic diseases.

Introduction

Urban greening is the process that changes the urban living environment by replacing built-up or paved areas with green space. These changes have been associated with positive effects on mental health, water retention and biodiversity [1-3]. Urban greening is also increasingly applied as a measure to sustainably counteract the negative effects of urbanization, such as air pollution, reduced water quality and heat island effects on environmental and human health [4].

However, little is known about the deleterious effects urban greening might have on human health, particularly the effects on wildlife-borne zoonotic pathogens. Increased urban greening could alter wildlife host populations, microclimate and pathogen transmission cycles [5], and could hence influence pathogen transmission to humans. Concurrently, the considerable overlap in space use of urban green spaces by humans, domesticated animals and wild animals increases the chance of pathogen spill-over [6]. Therefore, it is important to investigate the effects of urban greening on the occurrence and diversity of zoonotic pathogens.

Wild rats are ubiquitous in urban areas and are able to host a multitude of zoonotic pathogens [7]. Changes in their abundance and living environment (e.g. through urban greening) may have a significant impact on zoonotic infectious diseases. Previous studies have found a positive association between urban greenness and the abundance of wild rats [8-10], which suggests that an increase in greenness might lead to an increase in rat-borne disease hazard, provided that there is no decrease in pathogen prevalence through a dilution effect. Disease hazard posed by wild rats is the product of rat population density and pathogen prevalence [11]. For example, high rat abundance can increase density-dependent pathogen transmission, resulting in higher numbers of infected rats [12]. However, the strength of the relationship between rat density and pathogen prevalence may vary depending on the location or pathogen considered [13, 14].

To provide greater insight into the potential zoonotic disease hazard associated with urban greening, we investigated the relationship between urban greenness and zoonotic pathogen prevalence and diversity in wild rats. We screened wild brown rats (*Rattus norvegicus*) and black rats (*Rattus rattus*) from three urban areas in the Netherlands for a total of 18 zoonotic pathogens, including bacteria, viruses and parasites, and we modelled the relationships between urban greenness and pathogen prevalence and diversity.

Materials & Methods

Ethics statement

This study was approved by the Dutch Central Animal Experiments Committee (CCD) (project number AVD3260020172104).

Sample collection

Part of the rats were systematically trapped during fieldwork using snap traps (20 traps

per location) in 48 locations (16 parks and 32 residential areas) in Amsterdam, Rotterdam and Eindhoven between May and October (2020 and 2021; Fig. 1). The residential areas were selected based on the percentage of greenness present (about half of the locations < 40 % greenness and half of the locations > 40 % greenness) to ensure enough variation in the percentage of greenness between locations (see de Cock et al. (under review) for further details). In addition, we received freshly trapped (< 24 h) dead rats from 45 locations in Amsterdam and Rotterdam collected between March and December 2021 by municipality pest controllers (Fig. 1). After an initial short storage at −20 °C, rats were transferred to −80 °C until further investigation. Before necropsy, rats were thawed at 4 °C. Sex, species (based on external morphology), body weight (g), body length (cm), tail length (cm), number of skin wounds and the number of specimens of ectoparasites (fleas, ticks and mites) were recorded. Ectoparasites (excluding mites) were identified to species level based on external morphology. During necropsy, multiple tissue samples were collected (Table S1) and stored at −80 °C until further analysis. Heart fluid was obtained by centrifuging the hearts in 1 mL phosphate-buffered saline (PBS) to get an equivalent serum dilution of 1:25 [15]. Throat swabs and feces were collected and stored at 4 °C for 3–5 days before further testing. Lung and liver tissue samples were stored in RNAlater (Thermo Fisher, NL) for 3–5 days at 4 °C before being stored at −80 °C.

Figure 1. *Rat trapping locations in Amsterdam and Rotterdam, collected during fieldwork or by municipality pest controllers.*

Nucleic acid extractions

DNA extractions were performed on the following tissue samples: spleen, kidney, nasal septum and ear pinna. From each tissue DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol.

Total nucleic acid (tNA) extractions were performed on lung, liver, brain and salivary gland samples. Lung and liver tissues were homogenized using MagNA Lyser Green Bead tubes (Roche Diagnostics, GmbH, Mannheim, Germany) with 600 µL lysis buffer (MagNa Pure 96 Total Nucleic Acid Isolation kit, Roche) on a FastPrep-24™ 5G Homogenizer (MP Biomedicals, Germany) once (40 s. at 6 m/s). Then, tNA was isolated using the MagNa Pure

96 Total Nucleic Acid Isolation kit (Roche) on the MagNA pure 96 platform (Roche). Quality control of the lung tNA isolation and inhibition control was performed with a β-actin real-time PCR (qPCR). Brain tNA was extracted as previously described in 300 µL tissue lysis buffer, resulting in 40 µL of processed sample mixed with 500 µL external lysis buffer and 450 µL medium [16]. Salivary gland tNA was extracted using NucleoMag® VET (Macherey-Nagel, Düren, Germany), per kit instructions, on a KingFisher™ Flex Purification System (Thermo Fisher Scientific, MA, USA).

Zoonotic pathogen analyses

Rats were screened for 18 pathogens (Table S1) using molecular detection methods, either direct conventional polymerase chain reaction (PCR), qPCR, combined with reverse transcription (RT) for RNA viruses (RT-PCR/ RT-qPCR), cultivation of bacteria, or serological methods, as described in Table S1. The qPCR results were considered positive by inspecting multiple elements: sigmoid curve presence, fluorescence, amplification difference and quantification of cycle (Cq) values (< 40, except for *Leptospira* spp. and *Bartonella* spp. < 45, *Toxoplasma gondii* < 41 and SARS-CoV-2 < 36). Tick-borne pathogens were tested in multiplex qPCRs. qPCR-positive samples were subjected to conventional PCR followed by Sanger sequencing (Baseclear, Leiden, the Netherlands). Obtained sequences were assembled, trimmed and used for species level typing in BioNumerics version 7.6.3 (bioMérieux, Marcy-lÉtoile, France) using UPGMA multiple alignment. ESBL *E. coli* beta-lactamase genes obtained from the rats were compared with data from the Dutch human population [17].

Predictor variables

Separate binomial generalized linear mixed models (GLMMs) per pathogen were created, which are further explained in the next section. We included both rat-specific and location-specific variables in these pathogen models. In each pathogen model, we included pathogen presence/absence as the dependent variable, a set of predictor variables and random factors. The following predictor variables were included: greenness, distance to water, rat age, sex, infestation (0/1) of ticks and of fleas, and season. The greenness of the trapping location was measured using the Normalized Difference Vegetation Index (NDVI). NDVI quantifies vegetation greenness in a satellite image by measuring the difference between near-infrared (reflected by vegetation) and red light (absorbed by vegetation) in a range from 0 (no vegetation present) to 1 (only vegetation present). NDVI was calculated using satellite maps from June 2020 and 2021, depending on the trapping year per location, with a resolution of 10 × 10 m [18]. Water surfaces were excluded from the NDVI map and from subsequent NDVI calculations, using ArcGIS (ESRI ArcGIS™ version 10.8, CA, USA). We calculated the mean NDVI in a 150 m circular buffer around each trapping location, representing the average home range of rats [19], in QGIS version 3.16 [20]. To calculate the shortest distance between trapping sites and the nearest water body (m) in QGIS, we used a shapefile of national water bodies, which includes natural public water bodies such as rivers, canals, lakes, streams, ponds and ditches [21]. A proxy for rat age was used based on the body length to mass ratio, body length (cm)/ weight (g) * 10 [22]. Infestation with fleas or ticks was scored as 1 in case fleas or ticks were present and as 0 if they were absent. Seasons were defined as follows: spring (March, April and

May), summer (June, July and August), autumn (September, October and November) and winter (December, January and February). City and trapping location within a city were included as nested random factors in all models.

Data analysis

All statistical analyses were conducted in R studio version 4.0.3 [23]. All numerical variables were standardized using a z-transformation with two standard deviations [24]. We created separate binomial GLMMs per pathogen using the *glmmTMB* package [25]. In each model we included pathogen absence/presence as the dependent variable, and a fixed set of predictor variables (sex, age, greenness, distance to water, the absence/presence of tick infestation, the absence/presence of flea infestation, and season). No models were created when pathogen prevalence was too low for models to properly converge. We also tested the relationship between pathogen diversity (measured as pathogen species richness) and the same set of predictor variables and random factors, using a Poisson GLMM with the number of pathogens detected offset by the number of pathogens tested. In addition, we tested the relationships between flea and tick infestation and predictor variables (sex, age, greenness, distance to water, and season) using binomial GLMMs. City and trapping location were included as nested random factors in all models. Multicollinearity was tested using the variance inflation factor (VIF). Variables with a VIF score > 5 were excluded from the model. Model assumptions were checked using the *DHARMa* package [26]. Tukey HSD post-hoc tests were performed using the *Emmeans* package [27]. For all models, individual rats with missing values for any of the variables under consideration were excluded. To measure the strength of associations between predictor variables and pathogen prevalence, the odds-ratio (OR) and the 95 % confidence intervals (CIs) were determined. Results were considered significant when $p < 0.05$.

Correlations between predictor variables were calculated using the Kendall rank correlation test (high correlation when $\tau > 0.7$). Co-infections between pathogens were calculated two-by-two using the $\chi 2$ test or Fisher's exact test to assess whether the number of observed co-infections could be explained by chance. Disease hazard was calculated by multiplying probabilities of pathogen prevalence models with the probabilities of the relative rat abundance model (data from de Cock et al., under review), which was calculated by dividing the number of trapped rats by the total number of trapping nights [10, 11].

Results

Rat population and location characteristics

In total, 412 wild rats (407 brown rats and five black rats) were collected, of which 227 were trapped during fieldwork and 185 were provided by municipality pest controllers. Of the captured rats, 40 % were male and 60 % were female. Body weight ranged from 20 to 466 g with a mean of 148 g (mean for males was 154 g and for females 143 g). 36 % of the rats were collected in parks and 64 % in residential areas. 23 % of the rats were captured in spring, 29 % in summer, 38 % in autumn and 10 % in winter. The levels of greenness

ranged from 0.09 to 0.83, with a mean of 0.45 (Fig. S1). Distance to water ranged from 0 to 397 m, with a mean of 96 m (Fig. S1). We collected ectoparasites from 117 out of 412 rats (28 %). These ectoparasites included fleas (n = 82/412; 20 %), mites (n = 42/ 412; 10 %) and ticks (n = 9/412; 2 %). Fleas included rat fleas (*Nosopsyllus fasciatus*; 85 %) and mouse fleas (*Leptopsylla segnis*; 15 %). Ticks included *Ixodes ricinus* larvae and nymphs (89 %) and one *Ixodes hexagonus* nymph (11 %). Mites were not identified to species level. Ectoparasite counts per rat ranged from 0 to 11 (Table S2).

Zoonotic pathogens detected in rats from urban areas

Five black rats were trapped in Eindhoven (3 in a park and 2 in a residential area), which did not carry any of the pathogens included in the screening. Due to the low number of black rats and their different ecology compared to brown rats, these five rats were excluded from further statistical analyses. Among the 407 brown rats, 13 zoonotic pathogens were detected (Table 1). Individual pathogen distribution maps are presented in Figs. S2-S4. The most prevalent pathogen was *Bartonella* spp. (26 %, CI: 21–30 %), followed by *Leptospira* spp. (20 %, CI: 16–24 %) and ESBL/ AmpC-producing *E. coli* (13 %, CI: 10–16 %). The prevalence of the other pathogens ranged from 0 % to 4 %. Sequences of 23 selected *Bartonella* spp. isolates from rats from different locations all had the highest similarity with *Ba. tribocorum* (98.70 %–100 % with MG027921).

Five tick-borne bacteria were detected, of which *Rickettsia* spp. was the most prevalent, detected in 16 out of 402 rats (4 %, CI: 2–6 %; Table 1). Of these, 14 were captured in Amsterdam in 2020. Ten *Rickettsia* spp. positive rats were also positive in the specific *Rickettsia* (*R.*) *helvetica* qPCR. The six rats positive in the *R. stenos* qPCR but not in the *R. helvetica* qPCR could not be further sequenced to species level. For *Borrelia* spp., identification to species level was successful in nine out of 13 samples. Eight samples had the highest similarity with *Borrelia* (*Bo.*) *afzelii* (100 % with OL848440), and one sample had the highest similarity with *Bo. bavariensis* (100 % with KX906941). We also detected the presence of *Anaplasma phagocytophilum* (n = 1), *Babesia microti* (n = 1) and *Neoehrlichia mikurensis* (n = 1; Table 1). We did not detect *Cowpox virus* (CPXV), *Seoul orthohantavirus* (SEOV), SARS-CoV-2, *Coxiella* (*C.*) *burnetii* or *Spiroplasma* spp. in any of the rats. We did detect RNA from rat hepatitis E virus (ratHEV; species *Rocahepevirus ratti*) [28] in 15 animals, but no anti-hepatitis E virus IgG antibodies (Table 1). Serological assays were all negative for CPXV, SEOV, SARS-CoV-2 and *C. burnetii*. DNA from and antibodies to *Toxoplasma gondii* were found in two and three different rats, respectively. DNA from and antibodies to *Streptobacillus moniliformis* were found in seven and 135 rats, respectively (Table 1).

Drivers of zoonotic pathogen prevalence and diversity

We could construct pathogen models for *Bartonella* spp., *Leptospira* spp., ESBL/AmpC-producing *E. coli*, *Rickettsia* spp., ratHEV and *Borrelia* spp. The prevalence of the other detected pathogens was too low to construct models. No multicollinearity (VIF < 5) was observed in the models. Greenness had a significant positive relationship with the prevalence of both *Bartonella* spp. (OR: 2.74, CI: 1.24–6.08, $p = 0.013$) and *Borrelia* spp. (OR: 27.99, CI: 1.00–782.07, $p = 0.050$; Table 2), and a significant negative relationship with the prevalence of both ESBL/AmpC-producing *E. coli* (OR: 0.23, CI:0.07–0.78, $p = 0.018$) and

Table 1. *Pathogen prevalence of the 18 zoonotic pathogens tested in brown rats. Pathogen prevalence is calculated based on the number of positives in (q)PCR or culturing. NA: no value, analyses not performed. The total number of animals tested per pathogen may differ because different organs were tested and from some rats specific organ samples could not be obtained.*

	Pathogen prevalence (%, n)			Seroprevalence		
	Parks n = 145 n (%)	**Residential areas** n = 262 n (%)	**Total prevalence** n = 407 % (95 % CI)	**Parks** n = 145 n (%)	**Residential areas** n = 262 n (%)	**Identified species**
***Bartonella* spp.**	54/128 (42 %)	44/254 (17 %)	26 % (21, 30 %)	NA	NA	*Ba. tribocorum*
***Leptospira* spp.**	35/143 (24 %)	45/262 (17 %)	20 % (16, 24 %)	NA	NA	NA
ESBL/AmpC- producing *E. coli*	12/138 (9 %)	34/229 (15 %)	13 % (10, 16 %)	NA	NA	*E. coli*
***Rickettsia* spp.**	9/143 (6 %)	7/259 (3 %)	4 % (2, 6 %)	NA	NA	*R. helvetica*
Rat hepatitis E virus	2/141 (1 %)	13/262 (5 %)	4 % (2, 6 %)	0/141 (0 %)	0/261 (0 %)	Rat hepatitis E virus
***Borrelia* spp.**	9/143 (6 %)	4/258 (2 %)	3 % (2, 5 %)	NA	NA	*Bo. afzelii* and *Bo. bavariensis*
Streptobacillus moniliformis	2/143 (1 %)	5/258 (2 %)	2 % (1, 4 %)	47/141 (34 %)	85/261 (33 %)	*S. moniliformis*
MRSA	3/136 (2 %)	1/224 (< 1 %)	1 % (0, 3 %)	NA	NA	*St. aureus*
***Salmonella* spp.**	0/138 (0 %)	2/229 (1 %)	1 % (0, 2 %)	NA	NA	*Sa.* Typhimurium (serovar)
Toxoplasma gondii	1/141 (1 %)	2/260 (1 %)	< 1 % (0, 2 %)	1/141 (1 %)	2/261 (1 %)	*T. gondii*
Anaplasma phagocytophilum	1/128 (1 %)	0/254 (0 %)	< 1 % (0, 1 %)	NA	NA	*A. phagocytophilum*
***Babesia* spp.**	1/144 (1 %)	0/262 (0 %)	< 1 % (0, 1 %)	NA	NA	*Bab. microti*
Neoehrlichia mikurensis	0/128 (0 %)	1/254 (< 1 %)	< 1 % (0, 1 %)	NA	NA	*Neoehrlichia mikurensis*
Cowpox virus	0/143 (0 %)	0/257 (0 %)	0 % (0, 1 %)	0/141 (0 %)	0/261 (0 %)	NA
Coxiella burnetii	0/143 (0 %)	0/262 (0 %)	0 % (0, 1 %)	0/141 (0 %)	0/261 (0 %)	NA
SARS-CoV-2	0/140 (0 %)	0/262 (0 %)	0 % (0, 1 %)	0/141 (0 %)	0/261 (0 %)	NA
Seoul orthohantavirus	0/140 (0 %)	0/262 (0 %)	0 % (0, 1 %)	0/97[a] (0 %)	0/40[a] (0 %)	NA
***Spiroplasma* spp.**	0/143 (0 %)	0/260 (0 %)	0 % (0, 1 %)	NA	NA	NA

[a] Only rats captured in 2020 were tested

ratHEV (OR: 0.06, CI: 0.01–0.43, $p = 0.005$; Table 2). No significant relationships were observed between greenness and *Leptospira* spp. or *Rickettsia* spp. (Table 2). In addition, no significant relationship was observed between greenness and pathogen diversity (OR: 1.26, CI: 0.91–1.72, $p = 0.159$; Table 2). Overall, rat pathogen diversity was rather low, with about half of all rats carrying none of the pathogens screened (Fig. 2A), and was distributed evenly across cities (Fig. S5).

Age had a significant positive relationship with the prevalence of *Bartonella* spp. (OR: 13.41, CI: 5.48–32.81, $p < 0.001$), *Borrelia* spp. (OR: 172.22, CI: 5.94–4991.16, $p = 0.003$), *Leptospira* spp. (OR: 4.03, CI: 1.90–8.53, $p < 0.001$) and ratHEV (OR: 14.05, CI: 2.27–86.95, $p = 0.004$; Table 2). Moreover, for these pathogens, the effect size of age was up to six times larger than the effect size of urban greenness (Table 2). Furthermore, we observed a significant positive relationship between rat age and pathogen diversity (OR: 2.98, CI: 2.18–4.09, $p < 0.001$;Table 2). There was no correlation between age and NDVI ($\tau = 0.01$, $p = 0.75$; Fig. S6).

Lastly, we observed a significant negative relationship between flea infestation and *Leptospira* spp. (OR: 0.43, CI: 0.19–0.99, $p = 0.047$; Table 2). No significant relationship was observed between flea infestation and *Bartonella* spp. (OR: 1.84, CI: 0.95–3.56, $p = 0.071$; Table 2). We also modelled the relationships between flea and tick infestation and the predictor variables, which showed a significant increase in the probability of tick infestation in greener urban areas (OR: 46.97, 3.53–624.58, $p = 0.004$), but not for flea infestation (OR:1.05, CI: 0.55–1.98, $p = 0.889$; Table S3). Moreover, we observed lower probability of flea infestations in summer (OR: 0.38, CI: 0.19–0.78, $p = 0.008$), and lower probability of tick infestations in spring (OR: 0.07, CI: 0.00–0.91, $p = 0.042$; Table S3).

Table 2. *Overview of pathogen (diversity) statistical models including predictor variables, Odds Ratios (ORs), 95 % Confidence intervals (CIs) and* p*-values. Significant values are given in bold.*

Outcome	Predictor variable	Odds Ratio (OR)	95 % CI	p-value
***Bartonella* spp.**	Greenness	2.74	1.24 – 6.08	**0.013**
	Age	13.41	5.48 – 32.81	**< 0.001**
	Distance to water	1.55	0.69 – 3.45	0.285
$\sigma^2 = 3.29$	Sex	0.83	0.47 – 1.47	0.515
Marginal $R^2 = 0.337$	Flea infestation	1.84	0.95 – 3.56	0.071
Cond $R^2 = 0.424$	Tick infestation	0.89	0.17 – 4.61	0.889
$ICC = 0.13$	Season *spring / summer /winter*	0.56/1.35/0.38	0.22 – 1.37 /0.54 – 3.35 /0.11 – 1.30	0.203 /0.517 /0.124
***Leptospira* spp.**	Greenness	1.96	0.74 – 5.18	0.173
	Age	4.03	1.90 – 8.53	**< 0.001**
	Distance to water	0.78	0.32 – 1.90	0.583
$\sigma^2 = 3.29$	Sex	1.51	0.83 – 2.76	0.178
Marginal $R^2 = 0.153$	Flea infestation	0.43	0.19 – 0.99	**0.047**
Cond $R^2 = 0.384$	Tick infestation	1.31	0.21 – 8.25	0.770
$ICC = 0.27$	Season *spring / summer /winter*	0.69 /0.64 /0.74	0.24 – 2.04 /0.21 – 1.96 /0.22 – 2.53	0.505 /0.432 /0.629

Table 2. *continued*

Outcome	Predictor variable	Odds Ratio (OR)	95 % CI	p-value
ESBL/AmpC-producing *E. coli*	Greenness	0.23	0.07 – 0.78	**0.018**
	Age	1.21	0.55 – 2.67	0.633
	Distance to water	0.51	0.16 – 1.65	0.260
$\sigma^2 = 3.29$	Sex	1.33	0.66 – 2.70	0.427
Marginal $R^2 = 0.179$	Flea infestation	0.47	0.16 – 1.37	0.164
Cond $R^2 = 0.352$	Tick infestation	1.73	0.16 – 19.11	0.655
ICC = 0.21	Season *spring / summer /winter*	1.92 /1.93 /0.14	0.58 – 6.41 /0.58 – 6.39 /0.01 – 1.32	0.287 /0.284 /0.086
***Borrelia* spp.**	Greenness	27.99	1.00 – 782.07	**0.050**
	Age	172.22	5.94 – 4991.16	**0.003**
	Distance to water	3.61	0.07 – 177.36	0.518
$\sigma^2 = 3.29$	Sex	1.59	0.33 – 7.61	0.564
Marginal $R^2 = 0.874$	Flea infestation	1.85	0.29 – 11.71	0.515
Cond $R^2 = 0.933$	Tick infestation	1.78	0.10 – 32.36	0.697
ICC = 0.47	Season *spring / summer /winter*	0.07 /1.32 /0.00	0.00 – 3.83 /0.08 – 21.69 /0.00 – Inf	0.194 /0.845 /0.999
Rat hepatitis E virus	Greenness	0.06	0.01 – 0.43	**0.005**
	Age	14.05	2.27 – 86.95	**0.004**
	Distance to water	1.97	0.68 – 5.64	0.209
$\sigma^2 = 3.29$	Sex	1.29	0.42 – 4.01	0.659
Marginal $R^2 = 0.849$	Flea infestation	0.78	0.21 – 2.99	0.723
Cond $R^2 = NA$	Tick infestation	0.00	0.00 – Inf	1.000
ICC = NA	Season *spring / summer /winter*	2.83 /0.65 /0.17	0.74 – 10.86 /0.11 – 3.80 /0.02 – 1.72	0.129 /0.633 /0.134
***Rickettsia* spp.**	Greenness	1.30	0.32 – 5.33	0.716
	Age	3.37	0.83 – 13.68	0.089
	Distance to water	2.20	0.48 – 10.09	0.310
$\sigma^2 = 3.29$	Sex	2.61	0.84 – 8.07	0.096
Marginal $R^2 = 0.887$	Flea infestation	2.39	0.69 – 8.31	0.172
Cond $R^2 = NA$	Tick infestation	0.00	0.00 – Inf	0.998
ICC = NA	Season *spring / summer /winter*	1.25 /0.18 /0.00	0.35 – 4.45 /0.03 – 1.10 /0.00 – Inf	0.731 /0.064 /0.996
Pathogen diversity	Greenness	1.26	0.91 – 1.72	0.159
	Age	2.98	2.18 – 4.09	**< 0.001**
	Distance to water	1.03	0.74 – 1.42	0.882
$\sigma^2 = 3.30$	Sex	1.08	0.84 – 1.37	0.557
Marginal $R^2 = 0.092$	Flea infestation	1.01	0.75 – 1.37	0.935
Cond $R^2 = 0.100$	Tick infestation	1.12	0.56 – 2.24	0.741
ICC = 0.01	Season *spring / summer /winter*	0.93 /1.07 /0.58	0.66 – 1.32 /0.76 – 1.52 /0.33 – 1.01	0.692 /0.684 /0.054

Co-infection of zoonotic pathogens

Co-infections were investigated between the most prevalent pathogens: *Bartonella* spp., *Leptospira* spp., ESBL/AmpC-producing *E. coli*, ratHEV, *Borrelia* spp. and *Rickettsia* spp. We observed significant co-infection relationships between *Bartonella* spp. and *Leptospira* spp. ($\chi 2 = 6.93$, $p = 0.008$), *Borrelia* spp. (OR: 4.40, CI: 1.17–18.03, $p = 0.013$) and *Rickettsia* spp. (OR: 4.22, CI: 1.25–15.18, $p = 0.009$), and a significant relationship between the occurrence of *Borrelia* spp. and *Rickettsia* spp. (OR: 8.72, CI: 1.38–39.83, $p = 0.011$; Fig. 2B and Table S4).

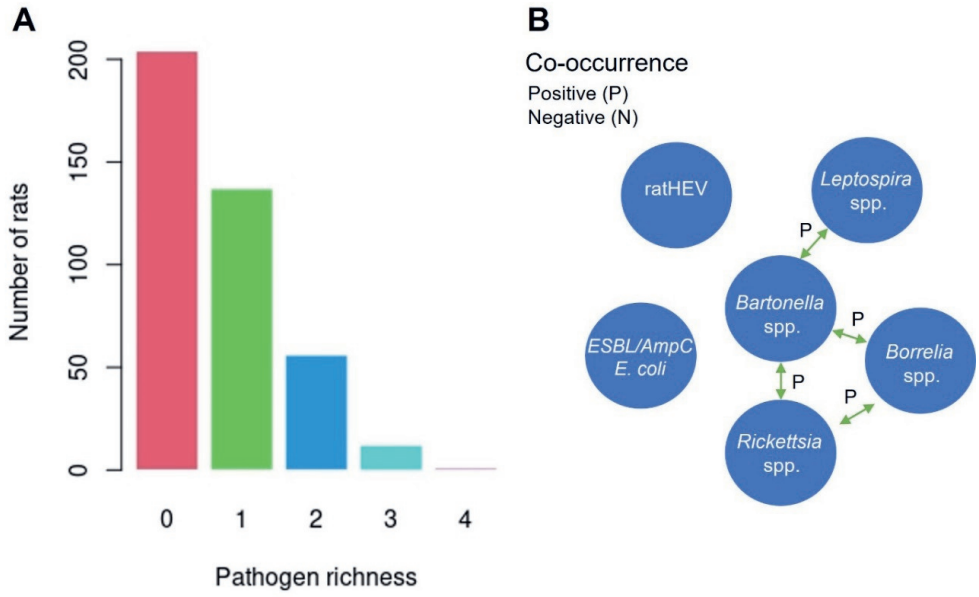

Figure 2. *A: Number of pathogens detected per rat. 2B: Coinfection patterns of the pathogens. Positive and negative associations in the coinfection patterns, which were significant in the 95 % CI (confidence interval) level are shown with green, respective red arrows.*

Human rat-borne disease hazard

For pathogens that were significantly associated with urban greenness (e.g. *Bartonella* spp., *Borrelia* spp., ESBL/AmpC-producing *E. coli* and ratHEV), we calculated the change in rat-borne disease hazard by multiplying probabilities of the relative rat abundance model with probabilities of the pathogen prevalence models (Fig. 3). We observed an increased disease hazard for both *Bartonella* spp. and *Borrelia* spp., and a decreased disease hazard for both ESBL/AmpC-producing *E. coli* and ratHEV, in greener urban areas (Fig. 3).

Typing of antimicrobial resistant bacteria detected in rats

We detected Salmonella enterica serovar Typhimurium in two rats (Table 1). MRSA and ESBL/AmpC-producing E. coli were further analysed to determine their antimicrobial resistance genes. MRSA was detected in three rats from the same park in Amsterdam. All rats carried the mecC gene. One rat from Rotterdam was also MRSA positive and carried

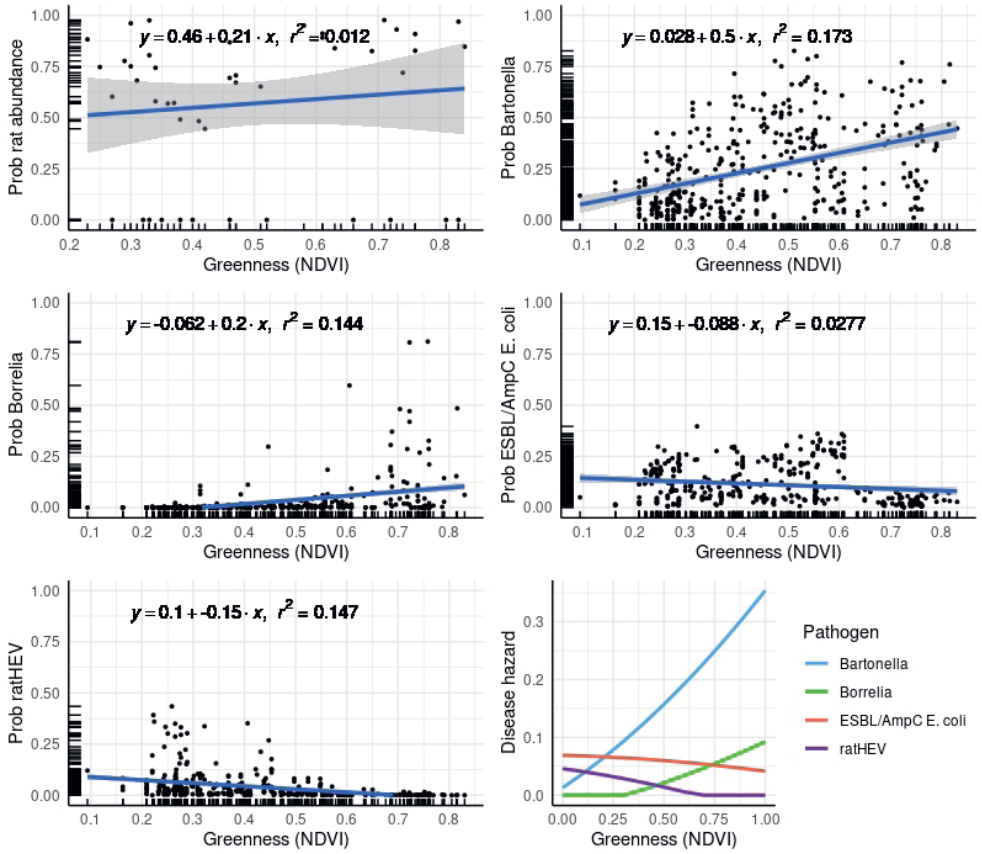

Figure 3. *Expected changes in rat-borne disease hazard with urban greenness, based on the probabilities (prob) of relative rat abundance and pathogen prevalence models (*Bartonella *spp.,* Borrelia *spp., ESBL/AmpC-producing* E.coli *and ratHEV). Trendlines (blue) and equations are added in the plots.*

the mecA gene. ESBL/AmpC-producing E. coli was detected in 46 out of 367 rats (13 %, CI: 10–16 %). Of these 46 positive rats, we obtained 66 isolates, and were able to sequence 64 of them. This resulted in 55 unique isolates from 45 rats. In total, nine different ESBL/AmpC genes were found (Fig. 4). The predominant genes were CTX-M-15, CTX-M-1 and DHA-1. One E. coli isolate contained both DHA-1 and CMY-2 genes, while all other isolates contained only one ESBL/AmpC gene. We detected multiple β-lactamase genes per isolate and per rat, including TEM-1A, TEM-1B, OXA-1 and LAP-2 (Table S5). The ESBL/AmpC genes found in these rats are similar to those previously found in the Dutch human population (Fig. 4) [17]. For both rats and humans, the dominant gene is CTX-M-15 (Fig. 4). A lower diversity of ESBL/AmpC genes were found in rats (n = 9) compared to humans (n = 14; Fig. 4). Furthermore, we also observed various high-risk E. coli sequence types (ST) in these rats, such as ST131 (n = 6 rats), ST69 (n = 3), ST10 (n = 3), ST38 (n = 2), ST648 (n = 2),cST58 (n = 1), ST117 (n = 1) and ST1193 (n = 1). A list of all isolates including their β-lactamase genes, sequence types and serotypes can be found in Table S6.

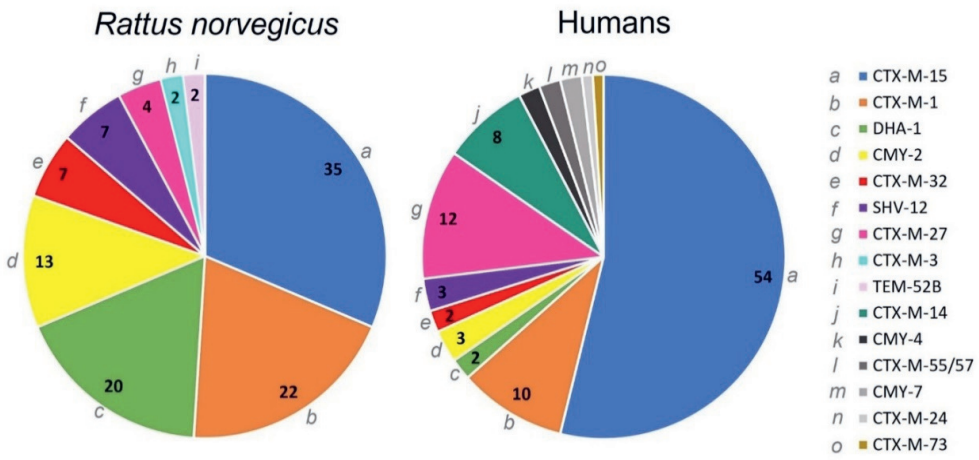

Figure 4. *Pie charts showing the percentage of ESBL and AmpC genes found in* Escherichia coli *from* Rattus norvegicus *(n = 45; left), and in* E. coli *and* Klebsiella pneumoniae *from the Dutch human population (n = 104; right) [17]. Data for humans were collected in 2015-2017. For the rats, ESBL/AmpC genes found in* K. pneumoniae *(n = 2) were not sequenced and therefore excluded here. Numbers in the pie charts represent percentage occurrence.*

6

Discussion

This study investigates the relationship between urban greenness and the prevalence and diversity of zoonotic pathogens in wild rats. The observed significant positive relationships between greenness and the prevalence of *Bartonella* spp. and *Borrelia* spp., in combination with the previously observed higher abundance of rats in greener urban areas, leads to an increased hazard for these zoonotic diseases in greener urban areas.

Urban greenness and rat-borne pathogens

We observed positive relationships between greenness and both *Bartonella* spp. and *Borrelia* spp. prevalence, and significant negative relationships with both ESBL/AmpC-producing *E. coli* and ratHEV prevalence. For ESBL/AmpC-producing *E. coli* this relationship could reflect its previously detected relationship with the presence of food vendors [14], which may be more abundant in city centers, which are often less green areas. The transmission mode of ratHEV is still unknown [29], which makes it hard to explain that relationship. Our findings do not support previous studies that reported a higher pathogen prevalence in residential areas compared to urban green spaces [30]. However, the residential areas in those studies comprised mostly urban slums, which are not comparable with urban areas in the Netherlands. Pathogen prevalence varies with pathogen type, transmission mode and host abundance. Based on the trends we observed in pathogen prevalence, vector-borne pathogens, such as *Bartonella* spp. and *Borrelia* spp., seem to be particularly sensitive to urban greening. This could be caused by a positive effect of greenness on survival of (pathogens carried by) tick and flea vector populations. *Ixodes ricinus* ticks rely on vertebrate hosts for food and on leaflitter for shelter [31], which are more likely to be found in greener urban areas. This could increase

the abundance of ticks and hence increase pathogen prevalence and tick-borne disease hazard. Fleas are permanent ectoparasites and depend on the availability of hosts. Since rat density increases with greenness [10], and consequently the number of fleas, density-dependent transmission of flea-borne *Bartonella* spp. leads to a higher prevalence. Additionally, greenness might also enhance the survival of fleas and their eggs in the environment due to more suitable microclimatic conditions [32].

We expected to find an overall higher prevalence of zoonotic pathogens in wild rats from greener urban areas, in part due to higher rat abundance and hence density-dependent pathogen transmission. However, similar to previous studies, we did not find a significant relationship between rat abundance and prevalence for *E. coli, Leptospira* spp., ratHEV, SEOV and *Toxoplasma gondii*, suggesting that for these pathogens environmental exposure may be more important than rat abundance [13, 14]. It could also be due to differences in transmission dynamics between more and less green areas. For example, rats in less green areas may have fewer shelter options available, which might force them to use or compete for the same shelter options. This could increase rat-to-rat contact and thereby enhance pathogen transmission in these areas. Possibly rats move across the urban landscape, which could weaken effects of greenness on pathogen prevalence and diversity.

Detected zoonotic pathogens in wild brown rats

The observed prevalence for *Leptospira* spp. (20 %) was comparable to the prevalence observed in urban areas in Sweden (12 %), France (15-44 %) and Canada (11 %) [33-36], and within the range of previously observed prevalence in urban and non-urban areas in the Netherlands (3-57 %) [37, 38]. While water bodies can act as an important source of *Leptospira* spp. infection [39], we did not observe a positive relationship between *Leptospira* spp. prevalence and water proximity. This may be partially due to the high availability of water sources in Amsterdam and Rotterdam, therefore not a limiting factor for *Leptospira* spp. transmission at the spatial scale we investigated. On the other hand, *Leptospira* spp. can also be transmitted directly from rat to rat (e.g. vertically, sexually and via direct contact with infected urine), which could also explain the lack of association between *Leptospira* spp. and distance to water [40, 41].

The observed *Bartonella* spp. prevalence (26 %) is comparable to a study in Belgium (37 %) [42], in which the main *Bartonella* species was also found to be *Ba. tribocorum*. While *Bartonella* is considered predominantly a flea-transmitted pathogen [43], we did not find a significant relationship with the probability of flea infestation. Fleas were found on 20 % of the rats, which is lower than frequencies observed in rats from studies in France and Canada (42-45 %) [44, 45]. The use of snap traps in our study instead of live traps could have caused fleas to leave the dead hosts, resulting in an underestimation of the actual flea infestation in wild rats [46]. Unexpectedly, we did find a negative relationship between *Leptospira* spp. and flea infestation. Whether this is related to the rat's swimming behavior [47], or whether the underestimation of flea infestation has caused a potentially non-meaningful relationship between *Leptospira* spp. and flea infestation, remains to be determined.

Likewise, we also found very few ticks (*Ixodes ricinus* and *I. hexagonus)* on the collected rats (2 %), which might also be and underestimation due to the use of snap traps. However, absence of or low infestations of ticks (0-0.7 %) on wild brown rats have been reported previously [44, 48-51]. As there are substantial numbers of ticks present in vegetation in urban areas [31, 52], this might suggest that rats are not preferred tick hosts, which could be influenced by their swimming and grooming behavior [53]. Thus, rats might not play a major role in urban transmission cycles of tick-borne pathogens. This may also explain the relatively low prevalence (< 5 %) of tick-borne pathogens (*Borrelia* spp.*, Rickettsia helvetica, Anaplasma phagocytophilum, Babesia microti* and *Neoehrlichia mikurensis*) found in the rats. Other European studies also reported low prevalences for tick-borne bacteria in rats: *Borrelia* spp. (0-7 %) [44, 51], *Rickettsia* spp. (0-1 %) [44, 54], *Neoehrlichia mikurensis* (< 1 %) [55], *Anaplasma* spp. (0-1 %) and *Babesia* spp. (0 %) [44, 55].

The prevalence (< 1 %) of *Toxoplasma* (*T.*) *gondii* is lower than expected, considering the role of rodents as intermediate hosts in the lifecycle of *T. gondii* and based on previous literature, in which a prevalence of 8 to 10 % was observed in rats captured on farms in the Netherlands [38, 56]. Moreover, a seroprevalence of 8-28 % was observed in France and Cyprus [57, 58]. However, it must be noted that both the tissue selected for PCR analyses (in this study the brain only) and the diagnostic characteristics of the selected serological test (here a specific ELISA) may have a higher specificity compared to the mentioned studies, and can therefore explain the lower prevalence. Cats, the definitive hosts of *T. gondii*, generally have a lower *T. gondii* prevalence in urban areas compared to rural or agricultural areas [59, 60], which could be due to reduced cat hunting activity in urban areas and thereby altered predator-prey dynamics that limit transmission.

We further observed a relatively low prevalence (4 %) of ratHEV compared to the European average (10-15 %) [55, 57, 61-64]. As all samples were serologically negative for HEV, we suspect that the HEV-ELISA we used is less sensitive to detect ratHEV. Similarly, we observed a low prevalence (2 %) of *Streptobacillus moniliformis* compared to previous studies (13-92 %) from Germany, the USA, South Africa and Japan [65-68]. However, we observed a higher seroprevalence of *S. moniliformis* (33 %), indicating a higher rate of previous infection. Prevalence differences may be caused by the tissue tested, e.g. a prevalence of 22 % versus 10 % was observed in oral swabs and tongue tips, respectively [68]. In this study, we tested salivary glands, which is thought to be not the most sensitive tissue and therefore might have decreased the observed infection prevalence.

Undetected zoonotic pathogens

We did not detect *C. burnetii*, CPXV, SEOV and SARS-CoV-2 in any samples. In previous studies from the Netherlands and Germany the prevalence of *C. burnetii* was 1-5 % [69, 70]. The Dutch study was performed during the largest European Q-fever outbreak ever (causative agent *C. burnetii*), which likely increased spillover from infected ruminants to rats. CPXV has previously been detected in wild rats, but only sporadically or with a low (sero)prevalence (0-0.8 %) [44, 54, 71, 72]. While SEOV has been detected in wild rats from the Netherlands, France and the United Kingdom with a prevalence of 0-19 % [15, 37, 44, 57, 73], the absence of SEOV-positive wild rats in this study is supported by other studies [37, 74]. Despite the concurrent COVID-19 pandemic, no SARS-CoV-2 was detected in our

study nor in wild rats from two other European countries [75, 76]. This while infections with specific SARS-CoV-2 variants (Alpha and Beta) have been observed in rats in laboratory settings [77, 78], and recently in wild rats from New York (Delta and Omicron variant) [79]. The discrepancy might be due to variable susceptibility of rats (naïve laboratory rats versus wild rats), to variable susceptibility to different SARS-CoV-2 strains circulating at the time of sampling (e.g. Wuhan-Hu-1, Alpha, Beta, Delta and Omicron), and to the exposed viral dose (lower concentrations of infectious virus particles in the environment).

Rat age and rat-borne pathogens

We observed positive relationships between age and the prevalence of *Bartonella* spp., *Borrelia* spp., *Leptospira* spp. and ratHEV. This relationship with age has been observed previously in rats for both *Leptospira* spp. and *Bartonella* spp. [34, 54, 64, 80-82], and in other rodent species for *Rickettsia* spp., and *Bo*. *garinii* [83, 84], but not for ratHEV. Moreover, age had a positive relationship with pathogen diversity, which implies that older rats carry more, and a higher diversity of, pathogens. Likely because of cumulative exposure combined with persistent infections.

Co-infections observed in wild rats

Co-infections between *Bartonella* spp. and other pathogens have been observed previously in rodents, including co-infections with *Cowpox virus*, *Babesia* spp. and *Mycoplasma* spp. [85]. Negative interaction is thought to be caused by competition (e.g. for specific host resources) and positive interaction by increased host susceptibility [86]. In contrast to our study, Rothenburger et al. (2019) found a negative rather than a positive relationship between infection of *Bartonella* spp. and *Leptospira* spp. Hence, we expect that factors related to the structure of the urban environment (e.g. an inner city neighborhood of Vancouver versus Dutch cities) are affecting pathogen transmission dynamics, which are underlying the observed co-infections with *Bartonella* spp. The observed co-infection between *Borrelia* spp. and *Rickettsia* spp. could be due to their shared transmission via ticks.

Antimicrobial resistant bacteria found in wild rats

Urban wildlife is considered a sentinel, used to detect risks to humans, of environmental pollution by antimicrobial resistant bacteria and the types of resistance genes [87, 88]. In this study, the prevalence of *Salmonella* (*Sa*.) enterica serovar Typhimurium was 1 %, which is comparable with that found in Germany (4 %) [70], but not from Thailand (30 %) [89]. While *Sa*. Typhimurium hosts include humans, cattle, swine, horses, sheep, poultry and wild rodents, most outbreaks of human salmonellosis have been linked to consumption of *Salmonella*-contaminated food sources of animal origin [90]. Serovar Typhimurium is also one of the two main serovars found in the Dutch human population [91]. The low *Salmonella* spp. prevalence observed in rats in Europe suggests that rats are not a major source of human infections in this region.

Similarly, we observed a low prevalence (1 %) of MRSA, which is comparable to previous studies (1-6 %) in wild rats from urban areas in Portugal, Austria, Canada and China [92-96], and to the prevalence observed in humans from other European countries [97]. In the

studies from Portugal, Austria and China the rats' MRSA resistance genes were typed and the *mecA* gene was found. This is also the most dominant gene in human MRSA isolates. The study in Portugal also detected the *mecC* gene [96]. We also detected both genes. The *mecC* gene is considered to have a broad host range, including livestock, companion and wildlife animals [98] such as black and brown rats, rabbits, hares and hedgehogs [99-101]. We expect that rats are occasional spill over hosts for MRSA, and that wild animals such as hedgehogs, in which a prevalence of up to 64 % has been observed [101], are the reservoir hosts.

We detected ESBL/AmpC-producing *E. coli* in 13 % of the rats, which falls within the observed prevalence in rats from other European countries (1-16 %) [95, 102, 103], and which is slightly higher compared to the prevalence of 7 % observed in the Dutch human population [17]. The ESBL/AmpC genes found in these rats represented those found in the Dutch human population quite well, indicating that rats, living near humans, are good sentinel animals for ESBL/AmpC-producing *E. coli*. The most frequently observed resistance genes found in humans (CTX-M-1, CTX-M-14, CTX-M-15 and CMY-2), are also the most frequently observed genes in wildlife species including birds and mammals (e.g. wild boar, roe deer, red fox, badger, hedgehog and brown and black rat) [104], in cats and dogs [105], and in brown rats from this study. This suggests a common source or potential interspecific transmission between vertebrates, including wildlife, humans and other animals. Although it is hard to determine the direction of antimicrobial resistance gene spread, wild animals, especially those living close to humans, could pose potential risks for human and animal health by contributing to the circulation and evolution of antimicrobial resistant bacteria [104].

Human rat-borne zoonotic disease hazard

Overall, the rat-borne zoonotic disease hazard increases with urban greenness, except for ESBL/AmpC-producing *E. coli* and ratHEV. For pathogens without a significant increase in prevalence in greener urban areas, the increased disease hazard is due to the increase in rat abundance. Whether this increased disease hazard actually leads to an increase in human disease risk depends on human exposure. It should be noted that these rats were trapped in 2020 and 2021 during the COVID-19 pandemic, which could have slightly altered the abundance of rats compared to other years. However, as we used the relative abundance for the abundance calculations, we expect general patterns to hold. In total, we detected 13 out of 18 assessed zoonotic pathogens in these rats, highlighting the potential of wild rats to host a great diversity of zoonotic pathogens, and the possible human exposure to these pathogens in urban areas. However, the number of rat-borne disease cases reported in humans in the Netherlands in the past years is relatively low, which could indicate low exposure to rat-borne pathogens or underdiagnosis [91].

Conclusions

This study shows that for most pathogens rat-borne disease hazard increases in greener urban areas. The overall increased disease hazard in greener urban areas is mainly caused by the increase in rat abundance rather than pathogen prevalence, as for most pathogens the prevalence did not significantly change with greenness. Therefore, it is worthwhile

to implement sustainable rat population control measures. Such measures could focus on decreasing food availability or designing urban greening in a way to make it less attractive for rats, but to still be able to profit from urban greening's beneficial effects on human health ('smart urban greening'). Still, the general term "greenness" consists of many different combinations of plant species and structures, which calls for more precise studies to distinguish the effects of different vegetation types. This study highlights the importance of investigating and considering both the positive and negative effects of urban greening on wildlife and wildlife-borne zoonotic pathogens to be able to make an informed decision on how to perform urban greening or which countermeasures to take.

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Table S1. *Detection methods used per pathogen*

	Pathogen	Tissue	(RT-) qPCR target(s)	(RT-) PCR target for typing	Serology (using heart fluid)	Culturing and typing
Bacteria	***Anaplasma phagocytophilum***	Spleen	*msp2* [106]	*groEL* [107]	-	-
	***Bartonella* spp.**	Spleen	*ssrA* [108]	*gltA* [109]	-	-
	***Borrelia burgdorferi* sensu lato complex**	Ear pinna and spleen	*ospA* and *flaB* [110]	*IGS* [111]	-	-
	Borrelia miyamotoi	Ear pinna and spleen	*flaB* [112]	-	-	-
	Coxiella burnetii	Spleen	*COM1* and *IS1111* [113]	-	Enzyme-linked immunosorbent assay (ELISA) ID Screen Q Fever Indirect Multi-species (ID. VET Innovative Diagnostics, France)[a]	-
	ESBL/AmpC-producing *E. coli*	Feces	-	-	-	Culture [17] [b,c] and typing: In-house sequencing for β-lactamase genes, serotype and sequence type[d]
	***Leptospira* spp.**	Kidney	*lipL32* [114]	-	-	-
	MRSA	Throat swab	-	-	-	Culture [115] [b] and typing: Multiplex PCR targeting *mecA, mecC*, *lukF* and *spa* genes [116]
	Neoehrlichia mikurensis	Spleen	*groEL* [117]	-	-	-
	Rickettsia helvetica	Ear pinna	*gltA* [118]	*gltA* [119]	-	-
	***Rickettsia* spp.**	Ear pinna	*gltA* [120]	*gltA* [119]	-	-
	***Salmonella* spp.**	Feces	-	-	-	Culture: ISO 6579-1 using MSRV agar [121] [b] and typing: serotyped using whole-genome sequencing [122]

Table S1. *continued*

	Pathogen	Tissue	(RT-) qPCR target(s)	(RT-) PCR target for typing	Serology (using heart fluid)	Culturing and typing
Bacteria	***Spiroplasma* spp.**	Ear pinna	*rpoB* [123]	-	-	-
	Streptobacillus moniliformis	Salivary glands	*16S* rRNA, *gyrB* and *EGFP* [68]	-	*Streptobacillus* multiplex serology[e]	-
Viruses	***Cowpox virus***	Nasal septum	*14-kD* [124]	-	Indirect fluorescent antibody test (IFA) [125]	-
	Rat hepatitis E virus	Liver	*ORF1* (RT-qPCR) [61] [f]	*ORF1* (RT-PCR) [64] [f]	ELISA ID Screen Hepatitis E Indirect Multi-species (ID. VET Innovative Diagnostics, France)[a]	-
	SARS-CoV-2	Lung	*E* gene [126]	-	Indirect ELISA and virus neutralization test (VNT) [127] [g]	-
	Seoul orthohantavirus	Lung	S-segment [128] [h]	-	Human Dobrava/Hantaan virus IgG ELISA (Progen Biotechnik GmbH, Heidelberg, Germany)[i]	-
Parasites	***Babesia microti***	Ear pinna and spleen	*ITS* [129]	-	-	-
	***Babesia* spp. (excl. *Babesia microti*)**	Ear pinna and spleen	*18S* [130]	-	-	-
	Toxoplasma gondii	Brain	529 base pair (bp) repeat [131] [j]	-	ELISA ID Screen Toxoplasmosis Indirect Multi-species (ID.VET Innovative Diagnostics, France)[a]	-

[a] All steps were carried out according to the manufacturer's instructions. Protein G labeled with horseradish peroxidase (P21041) was used as conjugate (ThermoFisher, NL).
[b] The time from taking the sample during dissection to incubation was 3-5 days.
[c] When colonies had different colors, we sequenced multiple isolates per animal. Suspicious colonies were typed using MALDI-TOF MS.

[d] Sequenced on an Illumina NextSeq 550 system. Libraries (2×150 bp reads) were prepared using the Illumina Nextera XT DNA Library Prep kit (Illumina, Inc.). Reads were processed using an in-house developed pipeline "Juno assembly" consisting of trimming and *de novo* assembly as well as quality control of the raw reads, trimmed reads and assemblies [132]. The output of the "Juno assembly" pipeline was used in two in-house developed pipelines; "Juno typing" for *in silico E. coli* serotyping and multi-locus sequence typing (MLST) analysis [133], and "Juno AMR" for detection of antimicrobial resistance determinants based on the ResFinder and PointFinder tools created by the Center For Genomic Epidemiology [134].

[e] The Streptobacillus serological assay is part of the rat multiplex serology, which comprises rat viruses, *Mycoplasma* spp., *Rodentibacter* spp. and *Streptobacillus moniliformis*. The multiplex serology is based on an immunosorbent assay in combination with the fluorescent bead technology from Luminex Corp. (Austin, TX, USA). For the analysis of antibody responses to *S. moniliformis*, bacterial membrane proteins were extracted and directly coupled to polystyrene beads with an embedded fluorescent dye (SeroMap; Luminex Corp.) before incubation with heart fluid (final dilution 1:100). The general set-up and protocol of the Streptobacillus multiplex serology is described by Schmidt et al. (manuscript in preparation). The Luminex analyzer BioPlex200 (BioRad Laboratories GmbH, Munich, Germany) was used to distinguish between the bead set and the bound antigen, and to quantify the amount of bound serum antibody by a secondary antibody (1:1000 dilution biotinylated goat anti-rat IgM/IgG, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and a fluorescent reporter conjugate (1:500 Streptavidin-R-phycoerythrin). Final antigen-specific median fluorescence intensity (MFI) values were measured of at least 75 beads per bead set and fluid sample. Samples were defined as positive if the net MFI values were above the cut-off of 400 MFI.

[f] Initially pools of max. five samples were tested and thereafter positive pools were split and tested individually. We used both the nested broad-spectrum conventional RT-PCR with primers HEV-cs, HEV-cas, HEV-csn and HEV-casn, and the RT-qPCR with primers rHEV-F and rHEV-R2 and probe rHEV-P2. Typing was based on sequence analysis of the RT-PCR product.

[g] We tested heart fluid in an indirect ELISA for the presence of antibodies directed against the nucleocapsid protein (NP) and the complete spike protein (S1-S2) of SARS-CoV-2. All heart fluid samples were incubated for 1 hour at 56 ˚C to inactivate complement prior to testing in the ELISA or VNT. ELISAs with recombinant NP and S1-S2 proteins produced in insect cells (ECD-His tagged; both from Sino biological, Eschborn, Germany) were coated overnight in 50 mM NaHCO3 (pH 9.6) at 4 ˚C in a concentration of 50 ng/100 µL. Thereafter, 96-well plates were washed in a washing machine with phosphate-buffered saline (PBS)-0.05 % v/v Tween 20 followed by incubation with 100 µL StabilBlockTM solution per well (SURMODICS: cat.nr ST-1-1000, USA) at 37° C for 1 hour. After washing as described before, plates were incubated for 1 hour at 37 ˚C with 100 µL/well of a 10- and 40-fold dilution of the heart fluid in PBS-0.05 % v/v Tween 20 containing 5 % v/v horse serum (PBS-Tw-HS). Plates were washed again and incubated for 1 hour at 37 ˚C with 100 µL/well of a 1:2000 dilution of Goat anti-Rat IgG (H+L) Secondary Antibody-horse-radish peroxidase (HRP; Invitrogen, USA) in PBS-Tw-PS. After washing, the bound rat antibody-HRP anti-rat complexes were detected using 100 µL/well of 5'-Tetramethylbenzidine (TMB) substrate solution (1-StepTM Ultra TMB-ELISA Substrate Solution). Plates were colored for 5 minutes and 100 µL/well 0.5M H_2SO_4 was added to stop the reaction. Absorption per well was measured at 450 nM using a Molecular Devices SpectraMax® ABS Plus plate reader (San José, CA, USA). Samples were considered positive in case the absorbance at 450 nM was at least 2-fold higher than the absorbance of the 1:40 dilution of the negative control serum and significantly increased in the well with the 1:10 dilution of the sample. Samples scoring positive in one or both ELISAs (NP and S1-S2) were further tested in dilutions of 1:10, 1:20, 1:40 and 1:80 in the SARS-CoV-2 virus neutralization assay (VNT) as described by Gerhards et al. (2021).

[h] Using Taqman Fastvirus 1-step master mix (Life Technologies).

[i] The ELISA was adapted to detect rat IgG antibodies by using rabbit-anti-rat IgG horseradish peroxidase labeled conjugate (Sigma–Aldrich Chemie B.V. Zwijndrecht, The Netherlands) at a 1:5,000 dilution. The optimal optical density (OD) cut-off value was determined through the use of a binary mixture model [135].

[j] We used a final reaction volume of 25 µL, using a commercial master mix (PerfeCTa ToughMix, VWR International, Darmstadt, Germany), primers (800 nM) and probes (200 nM) (MWG- Biotech, Ebersberg, Germany) in a CFX96 instrument (Bio-Rad Laboratories). The cycling conditions were 50 °C for 2 min, followed by 95 °C for 10 min and then 55 amplification cycles of 95 °C for 15 s and 60 °C for 1 min. After each cycle, the light emission by the fluorophore was measured and results were analyzed using the CFX manager software Version 1.6 (Bio-Rad Laboratories) [131, 136, 137].

Table S2. *Counts of ectoparasite specimens (species) found per rat. Mites were not determined to species level.*

Number of ectoparasite specimens per rat	**Ticks**		**Fleas**		
	Ixodes ricinus	***Ixodes hexagonus***	***Nosopsyllus fasciatus***	***Leptopsylla segnis***	**Mites**
n = 1	3	1	41	7	22
n = 2	1	0	19	1	7
n = 3	0	0	1	1	1
n = 4	1	0	5	2	1
n = 5	0	0	2	0	0
n = 6-11	1	0	5	0	7

Table S3. *Model output for the flea and tick presence/absence models. Significant values are given in bold.*

Outcome	**Predictor variable**	**Odds Ratio (OR)**	**95 % CI**	**p-value**
Flea presence	Greenness	1.05	0.55 – 1.98	0.889
	Age	1.19	0.72 – 1.99	0.498
	Distance to water	0.79	0.44 – 1.40	0.412
$\sigma^2 = 3.29$	Sex	1.37	0.83 – 2.26	0.217
Marginal $R^2 = 0.056$	Season *spring*	0.95	0.51 – 1.79	0.882
Cond $R^2 = 0.064$	Season *summer*	0.38	0.19 – 0.78	0.008
ICC = 0.01	Season *winter*	0.93	0.39 – 2.24	0.877
Tick presence	Greenness	46.97	3.53 – 624.58	0.004
	Age	8.09	0.74 – 89.06	0.088
	Distance to water	0.25	0.00 – 41.50	0.592
$\sigma^2 = 3.29$	Sex	0.85	0.18 – 3.92	0.834
Marginal $R^2 = 0.863$	Season *spring*	0.07	0.00 – 0.91	0.042
Cond $R^2 = 0.907$	Season *summer*	0.43	0.07 – 2.66	0.362
ICC = 0.32	Season *winter*	0.00	0.00 – Inf	0.996

Table S4. *Probability of co-infections occurring by chance. O = observed number of co-infections, E = expected number of co-infections.* P-*values are determined by either Chi-square test (χ^2) or Fisher's exact test. Significant values are given in bold.*

	***Leptospira* spp.**	**ESBL/AmpC-producing *E. coli***	**ratHEV**	***Rickettsia* spp.**	***Borrelia* spp.**
***Bartonella* spp.**	**O: 30/390 (7.7 %)** **E: 20.35 (5.2 %)** **χ^2 = 6.93** **df = 1** **p = 0.008**	O: 8/351 (2.3 %) E: 12.16 (3.5 %) χ^2 = 1.74 df = 1 p = 0.187	O: 6/389 (1.5 %) E: 3.78 (1.0 %) OR: 2.04 CI: 0.58 - 6.62 p = 0.222	**O: 8/386 (2.1 %)** **E: 3.52 (0.9 %)** **OR: 4.22** **CI: 1.25 - 15.18** **p = 0.009**	**O: 7/386 (1.8 %)** **E: 3.02 (0.8 %)** **OR: 4.40** **CI: 1.17 - 18.03** **p = 0.013**
***Leptospira* spp.**		O: 9/374 (2.4 %) E: 9.22 (2.5 %) χ^2 < 0.001 df = 1 p > 0.999	O: 2/411 (0.5 %) E: 3.03 (0.7 %) OR: 0.60 CI: 0.06 - 2.73 p = 0.745	O: 4/408 (1.0 %) E: 3.25 (0.8 %) OR: 1.32 CI: 0.30 - 4.51 p = 0.750	O: 4/407 (1.0 %) E: 2.65 (0.7 %) OR: 1.77 CI: 0.39 - 6.54 p = 0.312
ESBL/AmpC-producing *E. coli*			O: 2/372 (0.5 %) E: 1.85 (0.5 %) OR: 1.09 CI: 0.12 - 5.09 p > 0.999	O: 1/371 (0.3 %) E: 1.98 (0.5 %) OR: 0.46 CI: 0.01 - 3.13 p = 0.704	O: 1/370 (0.3 %) E: 1.62 (0.4 %) OR: 0.58 CI: 0.01 - 4.09 p > 0.999
ratHEV				O: 2/406 (0.5 %) E: 0.55 (0.1 %) OR: 4.44 CI: 0.44 - 23.08 p = 0.101	O: 0/406 (0 %) E: 0.48 (0.1 %) OR: 0 CI: 0 - 9.22 p > 0.999
***Rickettsia* spp.**					**O: 3/409 (0.7 %)** **E: 0.51 (0.1 %)** **OR: 8.72** **CI: 1.38 - 39.83** **p = 0.011**

Table S5. *Number of all beta-lactamase genes (n = 13) found in rat isolates (n = 55) and their co-occurrence with other ones.*

B-lactamase genes	**CTX-M-1**	**CTX-M-3**	**CTX-M-15**	**CTX-M-27**	**CTX-M-32**	**TEM-1A**	**TEM-1B**	**TEM-52B**	**OXA-1**	**SHV-12**	**DHA-1**	**CMY-2**	**LAP-2**
CTX-M-1	8						2						
CTX-M-3							1						
CTX-M-15			14			1	2		1				
CTX-M-27				1			1						1
CTX-M-32					3								
TEM-1A			1										
TEM-1B	2	1	2	1								2	
TEM-52B								1					
OXA-1			1							1			
SHV-12									1	2			
DHA-1											8	1	
CMY-2							2				1	3	
LAP-2				1									
CTX-M-15 & TEM-1B & OXA-1			2				2		2				
Total (n)	10	1	20	3	3	1	10	1	4	3	9	6	1
Perc. (%)	18.2	1.8	36.4	5.5	5.5	1.8	18.2	1.8	7.2	5.5	16.4	10.9	1.8

Table S6. *All sequenced ESBL/AmpC* E. coli *isolates per rat, including the observed beta-lactamase resistance gene(s), serotype and sequence type. ONT = no O serotype could be defined. NA = no sequence type could be defined.*

Rat ID	Isolate ID	Beta-lactamase resistance gene(s)	Serotype	Sequence type
R_2020_25	E1	DHA-1	O86:H2	349
R_2020_25	E2	DHA-1	O86:H2	349
R_2020_107	E1	CTX-M-15	O16:H5	NA
R_2020_107	E2	CTX-M-1, TEM-1B	O9:H9	10
R_2020_108	E1	CTX-M-1	O109:H51	155
R_2020_108	E2	TEM-1B, CMY-2	O25:H4	131
R_2020_109	E1	CTX-M-1	O109:H51	155
R_2020_109	E4	CTX-M-1	O109:H51	155
R_2020_122	E1	CTX-M-15	O109:H21	40
R_2020_126	E1	CTX-M-15	O16:H5	131
R_2020_126	E3	CTX-M-32	O45:H11	714
R_2020_127	E1	CTX-M-1	O153:H30	1722
R_2020_128	E1	CTX-M-1, TEM-1B	O15:H18	69
R_2020_128	E2	CTX-M-15	O16:H5	131
R_2020_128	E3	CTX-M-15, OXA-1	O148:H30	2967
R_2020_141	E1	SHV-12	O78:H21	56
R_2020_151	E1	CTX-M-15	O16:H5	131
R_2020_151	E3	CTX-M-15	O16:H5	131
R_2020_155	E1	CTX-M-15, TEM-1A	O153:H9	3268
R_2020_155	E3	CTX-M-15	O153:H30	450
R_2020_175	E1	CTX-M-3, TEM-1B	O9:H30	361
R_2020_192	E1	CTX-M-1	O8:H21	1582
R_2020_270	E1	CTX-M-15, TEM-1B, OXA-1	O25:H4	131
R_2020_287	E1	CTX-M-15, TEM-1B, OXA-1	O153:H6	648
R_2020_299	E1	CTX-M-1	O59:H34	2640
R_2020_306	E1	CTX-M-15	O15:H18	69
R_2020_306	E2	CTX-M-15, TEM-1B	O1:H6	NA
R_2021_10	E1	CMY-2	O83:H42	648
R_2021_25	E1	CTX-M-27, LAP-2	O11:H15	973
R_2021_25	E5	CTX-M-27	O15:H18	7401
R_2021_27	E1	CTX-M-1	O75:H5	1193
R_2021_35	E1	TEM-1B, CMY-2	O9:H21	120
R_2021_59	E1	DHA-1	O25:H4	131
R_2021_64	E1	CMY-2	ONT:H18	963
R_2021_65	E1	CTX-M-1	O143:H4	117
R_2021_68	E1	DHA-1	O13:H15	108
R_2021_82	E1	CMY-2	ONT:H18	963
R_2021_83	E1	DHA-1	O13:H15	108
R_2021_86	E4	CTX-M-15	O21:H21	101
R_2021_86	E7	CTX-M-15	O21:H21	101
R_2021_102	E1	DHA-1	O13:H15	108
R_2021_102	E4	DHA-1	O13:H15	108

Table S6. *continued*

Rat ID	Isolate ID	Beta-lactamase resistance gene(s)	Serotype	Sequence type
R_2021_133	E1	CTX-M-15	O7:H4	484
R_2021_151	E1	CTX-M-32	O9:H4	2041
R_2021_151	E4	CTX-M-15	O21:H21	101
R_2021_151	E7	CTX-M-15	O21:H21	101
R_2021_158	E1	SHV-12	O15:H1	362
R_2021_170	E1	CTX-M-15	O9:H9	10
R_2021_170	E4	CTX-M-15	O101:H9	10
R_2021_178	E1	DHA-1	O124:H4	2569
R_2021_178	E2	DHA-1	O124:H4	2569
R_2021_206	E1	CTX-M-15, TEM-1B	O75:H9	58
R_2021_206	E3	CTX-M-15	O39:H49	2178
R_2021_232	E1	TEM-52B	O166:H15	349
R_2021_235	E1	CTX-M-15	O17:H18	394
R_2021_240	E1	DHA-1	O153:H9	38
R_2021_240	E2	DHA-1	O153:H9	38
R_2021_242	E1	SHV-12, OXA-1	ONT:H33	10
R_2021_259	E1	CTX-M-27, TEM-1B	O16:H5	NA
R_2021_261	E1	CTX-M-15	O125:H21	442
R_2021_263	E1	DHA-1	O153:H9	38
R_2021_273	E1	CTX-M-1	O174:H19	99
R_2021_292	E1	CMY-2, DHA-1	O15:H18	69
R_2021_299	E1	CTX-M-32	O17:H1	549

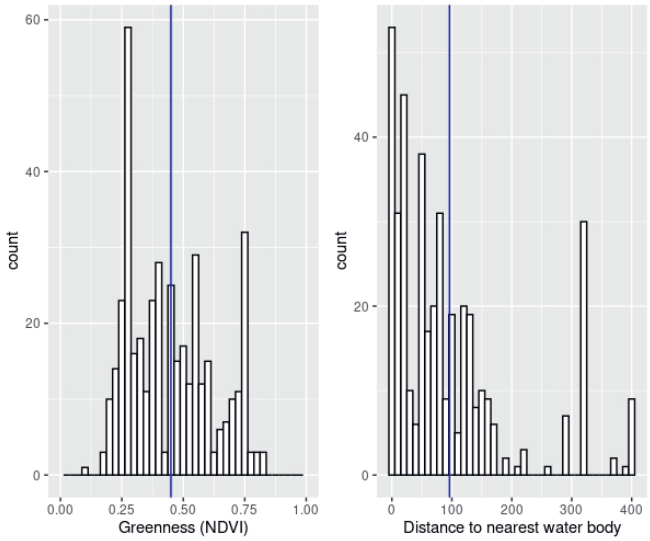

Figure S1. *Distribution of NDVI and distance to water from all trapped rats. The blue line represents the mean.*

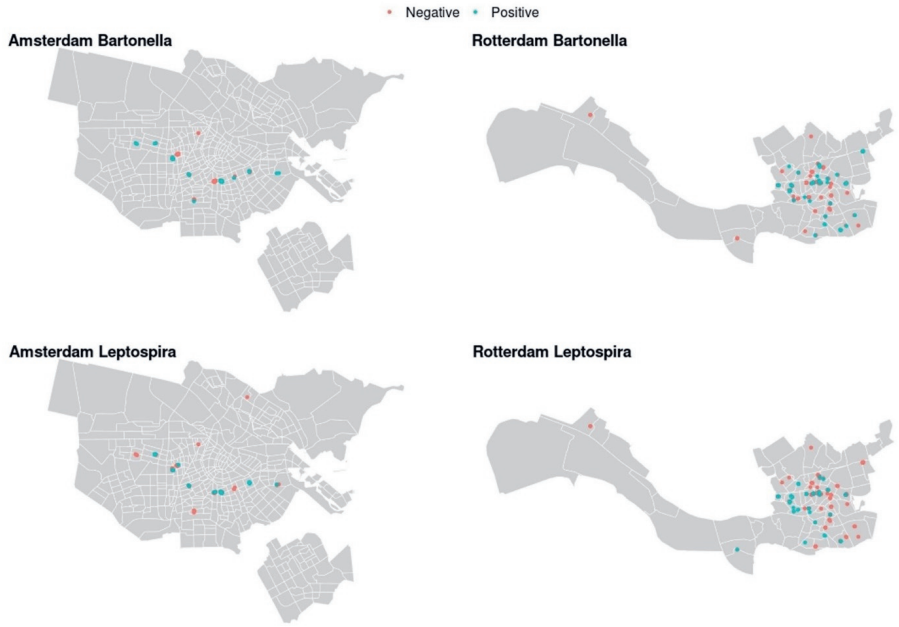

Figure S2. *Distribution maps of* Bartonella *spp. and* Leptospira *spp. positive and negative rats in Amsterdam and Rotterdam.*

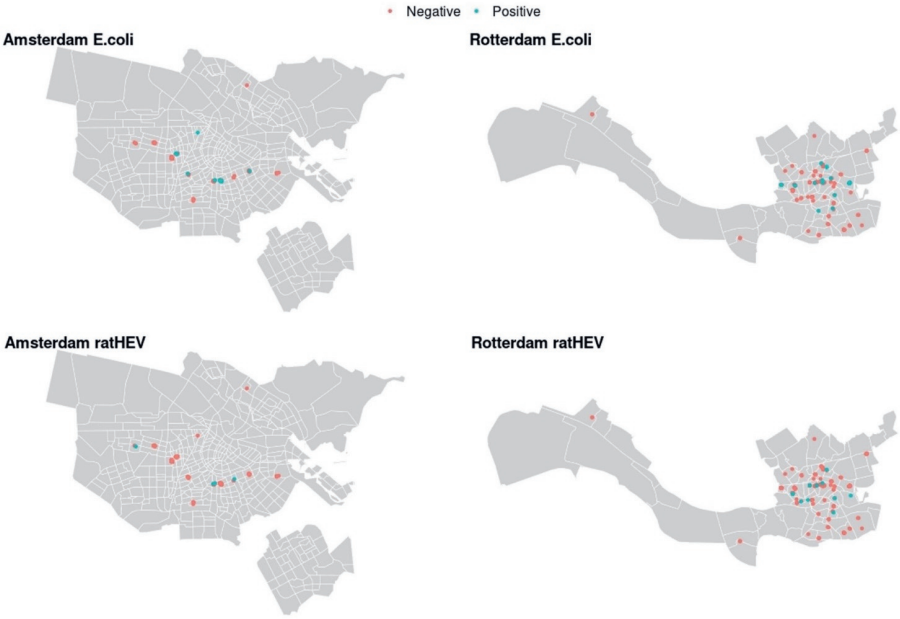

Figure S3. *Distribution maps of ESBL/AmpC-producing* E. coli *and Rat hepatitis E virus positive and negative rats in Amsterdam and Rotterdam.*

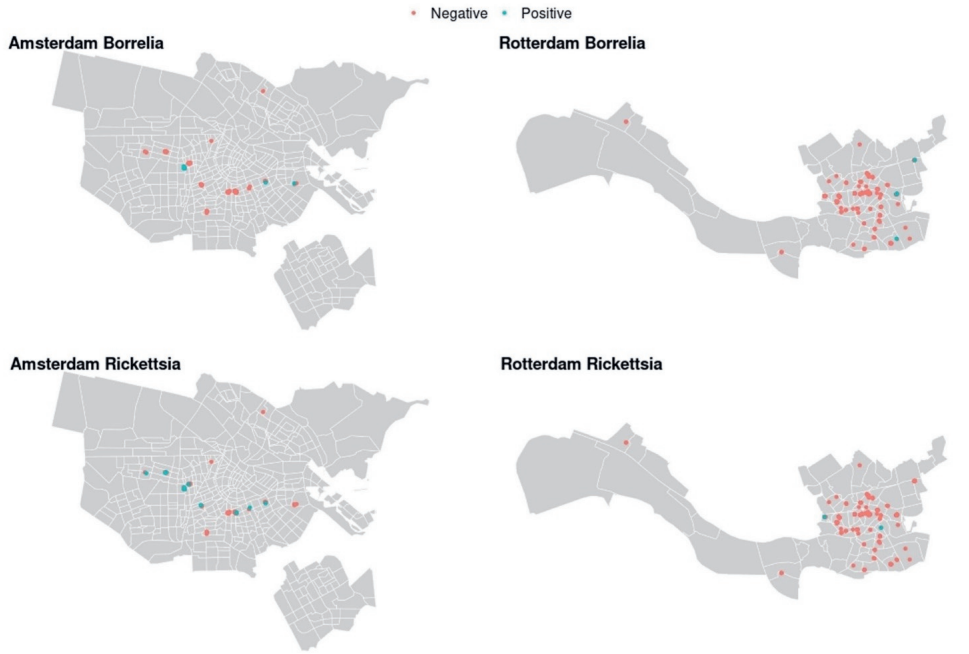

Figure S4. *Distribution maps of* Borrelia *spp. and* Rickettsia *spp. positive and negative rats in Amsterdam and Rotterdam.*

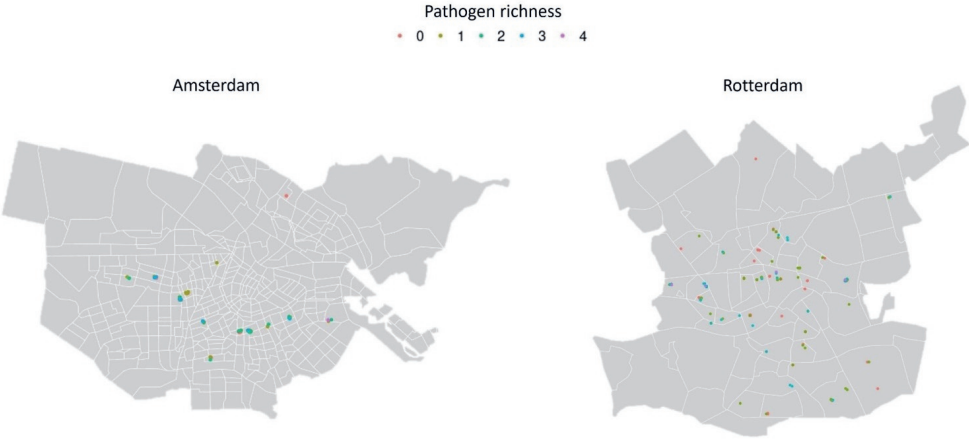

Figure S5. *Distribution map of pathogen diversity (measured using pathogen richness 0-4) in Amsterdam and Rotterdam.*

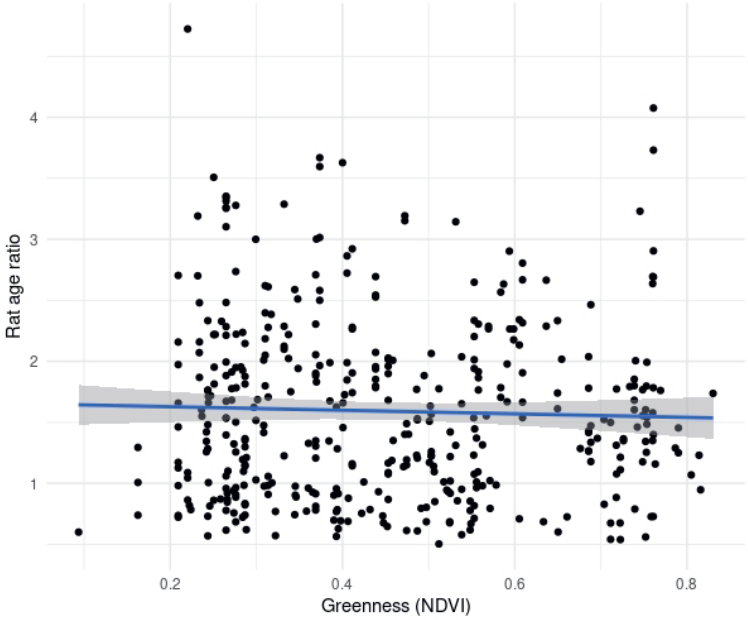

Figure S6. *Relationship between greenness (NDVI) and rat age (rat age ratio as proxy for rat age). The trendline represents the relationship.*

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Restaurant

Chapter 7

T(r)icky environments: higher prevalence of tick-borne zoonotic pathogens in rodents from natural areas compared to urban areas

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Abstract

Urban areas are unique artificial ecosystems with stark differences in species abundance and composition compared to natural ecosystems. This can affect pathogen transmission dynamics, thereby altering zoonotic pathogen prevalence and diversity. In this study, we screened small mammals from natural and urban areas in the Netherlands for up to 19 zoonotic pathogens, including viruses, bacteria and protozoan parasites. For a subset of these pathogens in two rodent species, we then tested whether pathogen prevalence and diversity were associated with habitat type (i.e., natural versus urban), degree of greenness, and various host characteristics. In total, 578 small mammals were captured, including wood mice (*Apodemus sylvaticus*), bank voles (*Myodes glareolus* syn. *Clethrionomys glareolus*), yellow-necked mice (*Apodemus flavicollis*), house mice (*Mus musculus*), common voles (*Microtus arvalis*), and greater white-toothed shrews (*Crocidura russula*). We detected a wide variety of zoonotic pathogens in small mammals from both urban and natural areas. The prevalence of tick-borne zoonotic pathogens (*Borrelia* spp. and *Neoehrlichia mikurensis*) was significantly higher in wood mice from natural areas. In contrast, the prevalence of *Bartonella* spp. was higher in wood mice from urban areas, but this difference was not statistically significant. Pathogen diversity was higher in bank voles from natural habitats, and increased with body weight for both rodent species, although this relationship depended on sex for bank voles. Additionally, we detected methicillin-resistant *Staphylococcus aureus* (MRSA), extended spectrum beta-lactamase (ESBL)/AmpC-producing *Escherichia coli*, and lymphocytic choriomeningitis virus (LCMV) for the first time in rodents in the Netherlands. The differences between natural and urban areas are likely related to differences in the abundance and diversity of arthropod vectors and vertebrate community composition. With increasing environmental encroachment and changes in urban land use (e.g., urban greening), it is important to better understand transmission dynamics of zoonotic pathogens in urban environments to reduce potential disease risks for public health.

Introduction

More than 60 % of all emerging human pathogens are zoonotic and about 75 % of these pathogens originate from wildlife [1]. Small mammals, in particular wild rodents, are reservoir hosts for a high diversity of zoonotic pathogens [2], including *Bartonella* spp., hantaviruses, and tick-borne pathogens [3, 4]. Some small mammal species are synanthropic, living in close proximity to humans, for example in urban areas [5]. Thus, there is a considerable risk of zoonotic pathogen spillover to humans in urban areas due to close contact between humans and potentially infected hosts [2]. For adequate risk management, it is important to quantify pathogen prevalence and diversity in small mammal communities in urban environments.

Urban areas represent unique artificial ecosystems that are characterized by an overall lower species diversity and an altered species composition compared to natural areas [6, 7]. More specifically, urban areas have higher relative abundances of zoonotic host species, especially for rodent communities [8]. This, in combination with increased contact rates between hosts (e.g., at supplementary feeding sites), could increase pathogen transmission in urban areas [9, 10]. For vector-borne pathogens however, transmission also depends on vector abundance. While flea infestation of small mammal communities has been found to be higher in more urbanized environments [11], tick abundance is typically lower in urban areas compared to more natural habitats [12]. What the net effect is on pathogen prevalence and diversity in urban small mammal communities remains largely unclear.

In this study, we quantified pathogen prevalence and diversity in small mammals from urban parks and residential areas of three cities in the Netherlands. Small mammals (rodents and shrews) were tested for the presence of 19 zoonotic pathogens. For two species of rodents (*Apodemus sylvaticus* and *Myodes glareolus* syn. *Clethrionomys glareolus*) and a subset of pathogens, we compared our findings with those from another study that was conducted in natural forest habitats across the Netherlands. We tested whether habitat type (urban vs natural), greenness (as measured by NDVI), and host characteristics (sex, weight, and tick presence) explained pathogen prevalence and diversity. We expected the prevalence of flea-borne and directly transmitted pathogens to be higher in urban than in natural habitats, while we expected the opposite for tick-borne pathogens. The overall diversity of zoonotic pathogens was expected to be higher in rodents from natural habitats, due to overall higher vertebrate host diversity in those areas.

Materials & Methods

Ethics statement

All handling procedures considering small mammal trapping and sample collection were approved by the Dutch Central Animal Experiments Committee (CCD; project number AVD3260020172104), the Animal Experiments Committee of Wageningen University (approval nos. 2017.W-0049.003 and 2017.W-0049.005), and the Netherlands Ministry of Economic Affairs (approval no. FF/75A/2015/014).

Sample collection

Mice, voles and shrews, hereafter referred to as 'small mammals', were collected in the Netherlands in two different studies (Figure 1). In the first study, small mammals were live-trapped in 13 natural habitats, defined as forested areas within National Parks, during August-October 2018 and March-June 2019 [13]. The second study focused on wild rats in three urban areas (Amsterdam, Rotterdam and Eindhoven) during June-October 2020 and March-October 2021, where small mammals trapped with rat snap traps were collected as by-catch [14, 15]. The urban trapping locations included urban parks and residential areas with varying degrees of urban greenness.

Necropsies and tissue sampling (Table 1) were carried out as described previously [13, 15]. Heart fluid was obtained by centrifuging the heart in 0.5 ml phosphate-buffered saline (PBS) as an equivalent to 1:25 diluted serum [16]. Information on species, sex, body weight (g), and tick presence was recorded for all individuals. Presence of fleas was also recorded for small mammals from urban areas. Ectoparasites from small mammals from urban areas were identified to species level based on external morphology using determination keys [17]. The detection of ectoparasites in small mammals from urban areas is probably underestimated due to the trapping method (i.e. snap trapping) because ticks and fleas gradually detach from dead hosts [18].

Figure 1. *Trapping locations of small mammals across the Netherlands. Small mammals trapped in natural* [13] *and urban* [14, 15] *areas are depicted as green and black circles, respectively.*

Molecular analyses

Total nucleic acid (tNA) and DNA extractions were performed as described before [13, 15]. DNA from lung (natural) or kidney (urban) tissue was used for molecular determination of small mammal species by a species-specific PCR, with slight modifications [19]. PCR mix consisted of 25 µl containing 3 µl sample, 12.5 µl Hot StarTaq Master Kit (Qiagen, Venlo, the Netherlands), 10 pmol for each forward and reverse primer, and molecular-grade water. The initial denaturation was performed at 95 °C for 15 min, followed by 50 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Species determination was performed by Sanger sequencing (Baseclear, Leiden, the Netherlands) of the obtained PCR products. Sequences were assembled, trimmed and identified to species using NCBI BLAST and BioNumerics version 7.6.3 (bioMérieux, France) using UPGMA multiple alignment. Pathogen detection was performed as described before [15], using multiple molecular methods (Table S1). For *Bartonella* spp., *Rickettsia* spp. and *Borrelia* spp., a subset of qPCR-positive samples from urban small mammals was sequenced as described in [15]. All small mammals were screened for the same set of 9 pathogens, while urban small mammals were tested for an additional 10 pathogens (Table S1).

Statistical analyses

Generalized linear mixed models (GLMMs) were used to test associations between pathogen prevalence or diversity (i.e., total number of pathogens detected per animal) and a fixed set of predictor variables, using the *glmmTMB* package in R (v4.3.1) [20, 21]. Predictor variables included sex, host weight, tick presence/absence (for models of tick-borne pathogens), and habitat type (defined as natural or urban). We also included the degree of greenness of each trapping location because of its potential influence on host and vector abundance and thus pathogen prevalence [15]. We used the Normalized Difference Vegetation Index (NDVI) as a measure of greenness, ranging from 0 (no vegetation present) to 1 (only vegetation present). NDVI was determined for a 50 m circular buffer around each location (representing the average home range of small mammals [22, 23]) in QGIS version 3.16 [24] as described before [15]. For pathogen diversity, a GLMM with a Poisson distribution was used, with the number of pathogens detected offset by the number of pathogens tested. In all models, trapping locations and plots within locations were included as nested random factors. We also included date as random factor to correct for the use of two datasets from different sampling years. Continuous variables were standardized using a z-transformation with two standard deviations [25]. Interaction terms between habitat type and greenness, and between sex and weight, were excluded from the model if not significant. Individuals with missing values for any of the included variables were excluded. Models were omitted when pathogen prevalence or the number of tested small mammals was too low for models to properly converge. For all final models, multicollinearity was tested using the variance inflation factor (VIF). Variables with a VIF score > 5 were excluded from the model. Additionally, the appropriate assumptions for each final model were checked using the *DHARMa* package. Associations were determined by calculating the Odds-Ratio (OR) and 95 % confidence intervals (CIs). Results were considered significant when $p < 0.05$.

Results

Population characteristics

In urban areas, 258 small mammals were captured from urban parks and residential areas, including 176 wood mice (*Apodemus sylvaticus*), 48 bank voles (*Myodes glareolus*, syn. *Clethrionomys glareolus*), 25 house mice *(Mus musculus)*, five common voles (*Microtus arvalis*) and four greater white-toothed shrews *(Crocidura russula*; Figure 2). In natural areas, 320 small mammals were captured, including 199 wood mice, 90 bank voles, 29 yellow-necked mice (*Apodemus flavicollis*) and two common voles (Figure 2). General characteristics of all small mammals are summarized in Table 1. The degree of greenness was significantly different between all three habitat types: natural areas, urban parks and residential areas (Figure S1).

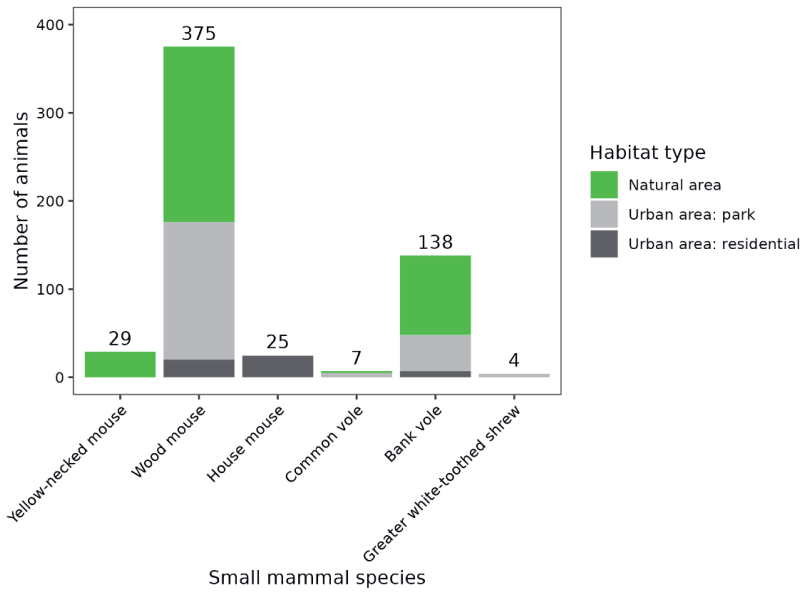

Figure 2. *Number of small mammals captured in natural and urban areas. Urban areas are divided in urban parks and residential areas. The total number of animals trapped per species is shown above the bars.*

Detected zoonotic pathogens

In total, 13 different zoonotic pathogens were detected (Table 2). Overall, the most frequently detected pathogens were *Bartonella* spp. (40 %, CI: 36-44 %), *Neoehrlichia mikurensis* (15 %, CI: 12-19 %), *Rickettsia* spp. (14 %, CI: 12-17 %) and *Borrelia* spp. (16 %, CI: 13-19 %; Table 2). We found a trend towards a higher prevalence of tick-borne zoonotic pathogens (e.g., *Borrelia* spp., *N. mikurensis*, *Anaplasma phagocytophilum* and *Babesia microti*) in small mammals from natural areas compared to urban areas (Table 2, Supplementary Table S2). In contrast, there was a trend towards higher prevalence of *Bartonella* spp. in urban areas (53 %, CI: 46-59 %) compared to natural areas (30 %, CI: 25-36 %; Table 2).

Table 1. *General characteristics, i.e. sex, weight, number trapped per year, and ectoparasite presence, of captured small mammals in natural and urban areas within the Netherlands*

		Yellow-necked mouse ***(Apodemus flavicollis)***	**Wood mouse** ***(Apodemus sylvaticus)***		**House mouse** ***(Mus musculus)***	**Common vole** ***(Microtus arvalis)***		**Bank vole** ***(Myodes glareolus)***		**Greater white-toothed shrew** ***(Crocidura russula)***
		🌲	🌲	🏠	🏠	🌲	🏠	🌲	🏠	🏠
		N = 29	N = 199	N = 176	N = 25	N = 2	N = 5	N = 90	N = 48	N = 4
Female		18	113	93[a]	13	2	2	43	31	3
Male		11	86	80	12	0	3	47	17	1
Weight (g) (range)		30.8 (10.0 – 50.0)	20.0 (10.0 – 37.0)	29.8 (13.4 - 49.5)	23.4 (12.1 - 36.3)	18.5 (18.0 – 19.0)	42.0 (26.5 - 50.7)	18.6 (9.0 – 33.0)	31.0 (13.7 - 49.9)	19.4 (10.8 - 26.5)
	Female	30.9	19.6	29.9	23.0	18.5	50.2	20.6	32.2	20.9
	Male	30.6	20.6	29.7	23.9	NA	36.5	16.7	28.7	14.8
Ectoparasite presence										
	Ticks[b]	27	154	38	0	2	0	50	5	0
	Fleas[c]			30	1		2		10	0
Year	2018	2	47			0		51		
	2019	27	152			2		39		
	2020			112	14		1		30	2
	2021			64	11		4		18	2

🌲: natural areas. 🏠: urban areas. [a] For 3 *Apodemus sylvaticus* originating from urban areas, the sex could not be determined. [b] Tick species for small mammals from natural areas were unknown, ticks of small mammals from urban areas consisted solely of *Ixodes ricinus* larvae and nymphs. [c] Presence of fleas was only recorded for small mammals from urban areas; wood mice carried mouse fleas (*Leptopsylla segnis*; n = 22), rat fleas (*Nosopsyllus fasciatus*; n = 4) or both (n = 4). Bank voles carried mouse fleas (n = 9) or rat fleas (n = 1). One house mouse and two common voles carried mouse fleas.

Table 2. *Pathogen prevalence of the 19 zoonotic pathogens (bacteria, parasites, viruses) tested in six small mammal species from natural and urban areas*

	Yellow-necked mouse *(Apodemus flavicollis)*	**Wood mouse (*Apodemus sylvaticus*)**		**House mouse *(Mus musculus)***
	(n = 29)	(n = 198)	(n = 176)	(n = 25)
Bacteria				
Anaplasma phagocytophilum	0/29 (0 %)	**2/198 (1 %)**	0/172 (0 %)	0/25 (0 %)
Bartonella spp.[a]	**13/29 (45 %)**	**55/198 (28 %)**	**110/172 (64 %)**	0/25 (0 %)
Borrelia burgdorferi s.l. complex[b]	**4/29 (14 %)**	**22/198 (11 %)**	**12/175 (7 %)**	0/25 (0 %)
Borrelia miyamotoi	**6/29 (21 %)**	**27/198 (14 %)**	**7/175 (4 %)**	0/25 (0 %)
Coxiella burnetii			0/172 (0 %)	0/25 (0 %)
ESBL/AmpC-producing *E. coli*			**2/176 (1 %)**	**1/25 (4 %)**
Francisella tularensis			0/109 (0 %)	0/14 (0 %)
Leptospira spp.	**8/29 (28 %)**	**4/198 (2 %)**	**15/176 (9 %)**	**3/25 (12 %)**
MRSA			**12/174 (7 %)**	0/25 (0 %)
Neoehrlichia mikurensis	**7/29 (25 %)**	**49/198 (25 %)**	**1/172 (1 %)**	0/25 (0 %)
Rickettsia spp.[c]	**2/29 (7 %)**	**12/198 (6 %)**	**26/176 (15 %)**	**2/25 (8 %)**
Spiroplasma spp.	0/29 (0 %)	0/198 (0 %)	**1/176 (1 %)**	0/25 (0 %)
Protozoan parasites				
Babesia microti[d]	0/29 (0 %)	**6/198 (3 %)**	0/176 (0 %)	0/25 (0 %)
Toxoplasma gondii[e]			**1/175 (1 %)**	**1/25 (4 %)**
Viruses				
Cowpox virus [f]			0/174 (0 %)	0/25 (0 %)
Hepatitis E virus			0/175 (0 %)	0/25 (0 %)
LCMV[g]			0/175 (0 %)	**1/25 (4 %)**
Puumala orthohantavirus	0/29 (0 %)	0/198 (0 %)	0/172 (0 %)	0/25 (0 %)
Tula orthohantavirus			0/172 (0 %)	0/25 (0 %)
SARS-CoV-2 [h]			0/173 (0 %)	0/25 (0 %)

: natural areas. : urban areas. MRSA: methicillin-resistant *Staphylococcus aureus*. LCMV: Lymphocytic choriomeningitis virus. Pathogen prevalence is calculated based on the number of positives in (q)PCR or culturing. Prevalence > 0 % is shown in bold. The total number of animals tested per pathogen may differ because different organs were tested and from some animals specific organ samples could not be obtained. All small mammals originating from natural areas were also tested for tick-borne encephalitis virus [13]. Pathogens that are not tested in specific small mammal species have empty cells. [a] Sequencing of 20 samples from urban areas resulted in the detection of three *B. elizabethae* (100 % identity with KT327029) in wood mice (n = 3), six *B. grahamii* (97.8-99.4 % identity with MK984789 or MZ089839) in wood mice (n = 3) and bank voles (n = 3), and 11 *B. taylorii* (96.9-100 % identity with MH932640 or Z70013) in wood mice (n = 10) and bank vole (n = 1). [b] Sequencing of two samples resulted in the detection of *B. afzelii* in two wood mice from urban areas. [c] Sequencing of 9 samples from urban areas resulted in the detection of *R. helvetica* (97.9-100 % identity with KY488349) in wood mice (n = 7) and house mice (n = 2). [d] All *Babesia* spp. positive samples were identified as *B. microti*. [e] T. gondii positives were further typed as Type II. Antibodies were detected in one wood mouse and one bank vole. [f] No antibodies were detected. [g] Only one old world Arenavirus was detected, which was further sequenced and identified as LCMV. [h] Indirect ELISA showed positive results for wood mice (n = 20) and bank vole (n = 1), but virus neutralization tests and qPCR results were negative, likely due to clearance or cross-reactivity with another coronavirus.

Common vole ***(Microtus arvalis)***		**Bank vole** ***(Myodes glareolus)***		***Greater white-toothed shrew (Crocidura russula)***
(n = 2)	(n = 5)	(n = 89)	(n = 48)	(n = 4)
0/2 (0 %)	0/5 (0 %)	**5/89 (6 %)**	0/45 (0 %)	0/4 (0 %)
0/2 (0 %)	**4/5 (80 %)**	**28/89 (31 %)**	**18/45 (40 %)**	0/4 (0 %)
0/2 (0 %)	**1/5 (20 %)**	**7/89 (8 %)**	0/46 (0 %)	0/4 (0 %)
0/2 (0 %)	0/5 (0 %)	**7/89 (8 %)**	**3/46 (7 %)**	0/4 (0 %)
	0/5 (0 %)		0/45 (0 %)	0/4 (0 %)
	0/5 (0 %)		0/48 (0 %)	0/4 (0 %)
	0/1 (0 %)		0/27 (0 %)	0/2 (0 %)
1/2 (50 %)	**1/5 (20 %)**	0/89 (0 %)	**1/48 (2 %)**	0/4 (0 %)
	1/5 (20 %)		0/47 (0 %)	0/4 (0 %)
2/2 (100 %)	0/5 (0 %)	**28/89 (31 %)**	0/45 (0 %)	0/4 (0 %)
0/2 (0 %)	0/5 (0 %)	**33/89 (37 %)**	**7/48 (15 %)**	0/4 (0 %)
0/2 (0 %)	0/5 (0 %)	0/89 (0 %)	0/48 (0 %)	0/4 (0 %)
2/2 (100 %)	0/5 (0 %)	**1/89 (1 %)**	0/48 (0 %)	0/4 (0 %)
	0/5 (0 %)		0/48 (0 %)	0/4 (0 %)
	0/5 (0 %)		0/47 (0 %)	0/4 (0 %)
	0/5 (0 %)		0/48 (0 %)	0/4 (0 %)
	0/5 (0 %)		0/48 (0 %)	0/4 (0 %)
0/2 (0 %)	0/5 (0 %)	**2/89 (2 %)**	0/44 (0 %)	0/4 (0 %)
	0/5 (0 %)		0/44 (0 %)	0/4 (0 %)
	0/5 (0 %)		0/45 (0 %)	0/4 (0 %)

We detected lymphocytic choriomeningitis virus (LCMV) in one house mouse (0.4 %, CI: 0-2 %) from a residential area in Rotterdam. In addition, methicillin-resistant *Staphylococcus aureus* (MRSA) was found in 13 out of 255 small mammals (5 %, CI: 3-9 %) that originated from two potential clusters, i.e. an urban park in Eindhoven (n = 3) and an urban park in Rotterdam (n = 10). All isolates carried the *mecC* resistance gene. We also isolated ESBL/AmpC-producing *E. coli* from three out of 258 small mammals (1 %, CI: 0-4 %), originating from residential areas in Amsterdam (n = 2) and Eindhoven (n = 1). From these three isolates, we characterized the sequence type, serotype, and beta-lactamase genes as ST155-O153:H51 carrying a CTX-M-1 gene, ST405-O102:H6 carrying a CTX-M-15 gene and ST131-O16:H5 carrying a CTX-M-27 gene, respectively.

Drivers of zoonotic pathogen prevalence

We analysed variables potentially associated with zoonotic pathogen prevalence and diversity for two rodent species that were found in large numbers in both natural and urban areas: wood mice and bank voles. For both species, models could be constructed for one flea-borne pathogen, i.e. *Bartonella* spp., and three tick-borne pathogens, i.e. *Borrelia* spp., *N. mikurensis* and *Rickettsia* spp. (Tables 3 and 4). In addition, a model for *Leptospira* spp. could be constructed for wood mice (Table 3). For wood mice, we observed a significantly higher prevalence of *Borrelia* spp. (OR: 0.18, CI: 0.04 – 0.76, $p = 0.020$) and *N. mikurensis* (OR: < 0.01, CI: 0.00 – 0.02, $p < 0.001$; Table 3) in natural areas than in urban areas. Weight was positively associated with the prevalence of *Bartonella* spp. (OR: 1.88, CI: 1.16 – 3.05, $p = 0.010$), *Borrelia* spp. (OR: 2.04, CI: 1.21 – 3.44, $p < 0.01$) and *N. mikurensis* (OR: 10.58, CI: 4.43 – 25.26, $p < 0.001$; Table 3), and we observed a higher prevalence in males compared to females for *Bartonella* spp. (OR: 2.11, CI: 1.25 – 3.56, $p = 0.005$) and *Borrelia* spp. (OR: 2.24, CI: 1.11 – 4.51, $p = 0.025$; Table 3). No associations were found between pathogen prevalence and the degree of greenness or tick presence. The prevalence of *Leptospira* spp. and *Rickettsia* spp. was not associated with any of the predictor variables. For bank voles, we observed only a positive association between *N. mikurensis* and weight (OR: 5.05, CI: 1.63 – 15.60, $p = 0.005$; Table 4), similar to what we observed for wood mice. However, no additional significant associations were found for *Bartonella* spp., *Borrelia* spp. and *Rickettsia* spp. (Table 4).

Drivers of zoonotic pathogen diversity

Coinfections of up to four different pathogens were detected in some animals (Figure S2). For both wood mice and bank voles, pathogen diversity was higher in males compared to females (OR: 1.21, CI: 1.00 – 1.46, $p = 0.050$, and OR: 1.41, CI: 1.08 – 1.83, $p = 0.011$, respectively; Table 3 and Table 4). For wood mice, pathogen diversity significantly increased with weight (OR: 1.49, CI: 1.27 – 1.74, $p < 0.001$; Table 3). For bank voles, pathogen diversity was significantly higher in natural areas compared to urban areas (OR: 0.47, CI: 0.24 – 0.92, $p = 0.028$; Table 4). Additionally, there was a significant interaction between weight and sex in the model for bank voles for males only, indicating that for males the pathogen diversity increased with weight (OR: 1.59, CI: 1.17 – 2.15, $p = 0.003$; Table 4 and Figure S3). No associations were found between pathogen diversity and the degree of greenness nor tick presence (Table 3 and 4).

Table 3. *Overview of pathogen (diversity) statistical models for A*podemus sylvaticus, *including predictor variables, Odds Ratios (ORs), 95 % confidence intervals (CIs) and* p*-values. Significant values are given in bold.*

Outcome	Predictor variable	Odds Ratio (OR)	95 % CI	p-value
***Bartonella* spp.**	Greenness	1.17	0.79 – 1.72	0.429
$\sigma^2 = 3.29$	Habitat type [a] *natural*	2.76	0.95 – 8.04	0.063
Marginal $R^2 = 0.207$	Weight	1.88	1.16 – 3.05	**0.010**
Cond $R^2 = 0.386$	Sex [a] *F*	2.11	1.25 – 3.56	**0.005**
***Borrelia* spp.** [b]	Greenness	1.49	0.85 – 2.62	0.167
	Habitat type [a] *natural*	0.18	0.04 – 0.76	**0.020**
$\sigma^2 = 3.29$	Weight	2.04	1.21 – 3.44	**0.008**
Marginal $R^2 = 0.201$	Sex [a] *F*	2.24	1.11 – 4.51	**0.025**
Cond $R^2 = NA$	Tick presence	0.64	0.28 – 1.48	0.298
***Leptospira* spp.**	Greenness	0.71	0.31 – 1.63	0.424
$\sigma^2 = 3.29$	Habitat type [a] *natural*	2.32	0.21 – 26.01	0.495
Marginal $R^2 = 0.256$	Weight	1.42	0.56 – 3.59	0.464
Cond $R^2 = NA$	Sex [a] *F*	3.50	0.97 – 12.58	0.055
Neoehrlichia mikurensis	Greenness	1.22	0.51 – 2.92	0.650
	Habitat type [a] *natural*	0.00	0.00 – 0.02	**< 0.001**
$\sigma^2 = 3.29$	Weight	10.58	4.43 – 25.26	**< 0.001**
Marginal $R^2 = 0.611$	Sex [a] *F*	1.01	0.43 – 2.36	0.979
Cond $R^2 = 0.772$	Tick presence	0.63	0.20 – 2.00	0.434
***Rickettsia* spp.**	Greenness	1.31	0.71 – 2.39	0.387
	Habitat type [a] *natural*	8.74	0.32- 235.40	0.197
$\sigma^2 = 3.29$	Weight	0.70	0.30 – 1.68	0.429
Marginal $R^2 = 0.058$	Sex [a] *F*	0.77	0.33 – 1.82	0.557
Cond $R^2 = 0.645$	Tick presence	3.10	0.64 – 14.91	0.159
Pathogen diversity	Greenness	1.03	0.90 – 1.19	0.639
	Habitat type [a] *natural*	0.75	0.44 – 1.28	0.290
$\sigma^2 = 2.21$	Weight	1.49	1.27 – 1.74	**< 0.001**
Marginal $R^2 = 0.052$	Sex [a] *F*	1.21	1.00 – 1.46	0.050
Cond $R^2 = NA$	Tick presence	1.01	0.76 – 1.32	0.971

[a] Reference categories: natural (habitat type), female (sex). [b] Test results for *Borrelia burgdorferi* s.l. complex and *B. miyamotoi* were combined in this model

Table 4. *Overview of pathogen (diversity) statistical models for* Myodes glareolus*, including predictor variables, Odds Ratios (ORs), 95 % confidence intervals (CIs) and* p*-values. Significant values are given in bold.*

Outcome	Predictor variable	Odds Ratio (OR)	95 % CI	p-value
***Bartonella* spp.**	Greenness	0.80	0.37 – 1.76	0.582
$\sigma^2 = 3.29$	Habitat type [a] *natural*	2.11	0.31 – 14.49	0.447
Marginal $R^2 = 0.096$	Weight	1.18	0.49 – 2.83	0.709
Cond $R^2 = NA$	Sex [a] *F*	1.80	0.71 – 4.56	0.218
***Borrelia* spp.** [b]	Greenness	0.97	0.48 – 1.96	0.923
	Habitat type [a] *natural*	0.48	0.07 – 3.52	0.471
$\sigma^2 = 3.29$	Weight	0.88	0.40 – 1.95	0.756
Marginal $R^2 = 0.061$	Sex [a] *F*	0.75	0.25 – 2.29	0.613
Cond $R^2 = NA$	Tick presence	1.15	0.36 – 3.70	0.809
Neoehrlichia mikurensis	Greenness	0.79	0.25 – 2.53	0.691
	Habitat type [a] *natural*	0.00	0.00 – Inf	1.000
$\sigma^2 = 3.29$	Weight	5.05	1.63 – 15.60	**0.005**
Marginal $R^2 = 0.980$	Sex [a] *F*	0.77	0.25 – 2.35	0.650
Cond $R^2 = NA$	Tick presence	0.87	0.28 – 2.70	0.803
***Rickettsia* spp.**	Greenness	0.88	0.40 – 1.89	0.736
	Habitat type [a] *natural*	0.61	0.06 – 6.46	0.679
$\sigma^2 = 3.29$	Weight	1.02	0.34 – 3.11	0.966
Marginal $R^2 = 0.092$	Sex [a] *F*	2.73	0.96 – 7.75	0.059
Cond $R^2 = NA$	Tick presence	1.09	0.36 – 3.26	0.880
Pathogen diversity	Greenness	0.96	0.74 – 1.26	0.778
	Habitat type [a] *natural*	0.47	0.24 – 0.92	**0.028**
	Weight	0.97	0.74 – 1.26	0.806
$\sigma^2 = 1.49$	Sex [a] *F*	1.41	1.08 – 1.83	**0.011**
Marginal $R^2 = 0.089$	Tick presence	1.03	0.80 – 1.32	0.822
Cond $R^2 = NA$	Weight * Sex[a] *F*	1.59	1.17 – 2.15	**0.003**

[a] Reference categories: natural (habitat type), female (sex). [b] Test results for *Borrelia burgdorferi* s.l. complex and *B. miyamotoi* were combined in this model

Discussion

This study describes the prevalence and diversity of zoonotic pathogens in small mammals from urban and natural areas of the Netherlands. We found a significantly higher prevalence of tick-borne zoonotic pathogens (*Borrelia* spp. and *Neoehrlichia mikurensis)* in wood mice from natural areas, and a trend towards a higher prevalence of *Bartonella* spp. in wood mice from urban areas. Pathogen prevalence in bank voles did not differ significantly between urban and natural areas. However, pathogen diversity in bank voles was significantly higher in natural areas, a pattern that was not observed for wood mice.

Higher prevalence of tick-borne zoonotic pathogens in natural areas

We observed a significantly higher prevalence of *Borrelia* spp. and *N. mikurensis* in wood mice from natural areas compared to urban areas. These findings are likely explained by lower tick abundances in urban areas (including urban green spaces) than in natural areas

[26-28]. Ixodid ticks need vegetation (e.g., leaflitter for shelter) and suitable microclimatic conditions (e.g., temperature and humidity) for survival during the off-host part of their lifecycle [4, 29, 30]. Both natural areas and urban green spaces can provide these requirements to a certain extent. However, in general, larger mammalian wildlife (e.g., roe deer, foxes and wild boars), which function as propagation hosts of ticks [4, 28, 31], are more abundant in natural areas. Although other animals, such as domestic cats and dogs, can also function as propagation hosts in urban areas, their role and efficiency in completing the tick's lifecycle remains unclear [32, 33]. Moreover, a lower abundance of ticks in urban areas might also lead to less co-feeding, which could further decrease the transmission and prevalence of certain tick-borne pathogens [34]. Lastly, the virtual absence of *Ba. microti, A. phagocytophilum* and *N. mikurensis* in urban areas could be related to the absence or lower densities of competent wildlife reservoir hosts for these pathogens in urban areas [35-37].

Higher prevalence of *Bartonella* spp. in urban areas?

In contrast to the tested tick-borne zoonotic pathogens, we observed a trend towards a higher *Bartonella* spp. prevalence in urban areas compared to natural areas. A higher prevalence of *Bartonella* spp. in rodents from urban areas might be explained by a higher flea infestation, which was shown to increase with urbanization [11, 38, 39]. Moreover, rodent population densities might even be more important than vector abundance for the dynamics of flea-transmitted *Bartonella* spp. [10]. Unfortunately, due to the different trapping methods between urban and natural areas used in this study, we cannot compare rodent densities between these habitat types. For *Rickettsia* spp., we did not observe a difference in prevalence between urban and natural areas, which could be explained by the fact that *Rickettsia* spp. (including *R. helvetica*) can be transmitted by both fleas and ticks [40, 41].

Increased pathogen diversity related to weight and sex

For both wood mice and bank voles we observed that pathogen diversity was higher in males compared to females, although this difference was only significant for bank voles. Pathogen diversity also increased with host weight for both rodent species, but for bank voles this relationship was significant for males only. This is in line with previous research in which heavier (older) and male rodents more frequently carry pathogens, likely because of cumulative exposure combined with persistent infections in older individuals, more risk taking and competitive behaviour in males, and differences in immune responses between males and females that makes males more susceptible to infection [42-44].

No effect of urban greenness

We did not detect any significant relationships between urban greenness and pathogen prevalence and diversity in mice and voles, in contrast to what we found previously for wild rats [15]. As almost all urban rodents were captured in urban parks, we think that this absence of relationships is more related to the uneven distribution of rodents sampled from locations with low and high degrees of greenness than to an actual absence of relationships.

Zoonotic pathogens detected for the first time in rodents from urban areas in the Netherlands

Unexpectedly, we detected LCMV in a single house mouse from Rotterdam. House mice are the known reservoir host for LCMV [45] and, while it has been detected in neighbouring countries [46-48], this is the first time this virus is reported in house mice from the Netherlands. We also report the detection of MRSA in wood mice and a common vole for the first time in the Netherlands. All 13 isolates carried the *mecC* resistance gene, which has historically been associated with MRSA in livestock [49]. Recently, hedgehogs were found to be the natural reservoir host of *mecC*-carrying MRSA [50, 51]. The MRSA isolates in this study originated from two urban parks, one in Rotterdam and one in Eindhoven, which indicates potential MRSA clusters or local hotspots. We also detected ESBL/AmpC-producing *E. coli* in two wood mice and one house mouse for the first time in mice from the Netherlands [52]. The detected ESBL types correspond to frequently found ESBL types in humans [53].

Conclusions

This study shows that small mammals from urban areas carry a diverse array of zoonotic pathogens. Moreover, tick-borne pathogen prevalence (*Borrelia* spp. and *N. mikurensis*) was significantly higher in wood mice from natural areas than urban areas. This might be explained by lower densities of ticks and vertebrate host species that function as tick propagation hosts (e.g., roe deer) in urban areas. Monitoring zoonotic pathogens circulating in rodents in urban areas is important to assess the risks for public health, and for the early detection of emerging pathogens.

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Table S1. *Detection methods used per pathogen, e.g. (RT-) qPCR, (RT-) PCR, serology and culturing and typing, habitat type in which small mammals were trapped, and tissues used for pathogen detection.*

	Pathogen	Habitat	Tissue	(RT-) qPCR target(s)	(RT-) PCR target for typing	Serology (using heart fluid)	Culturing and typing
Bacteria	*Anaplasma phagocytophilum*		Spleen	msp2 [54]	groEL [55]	-	-
	Bartonella spp.		Spleen	ssrA [56]	gltA [57]	-	-
	Borrelia burgdorferi s.l. complex		Ear, spleen[1]	ospA and flaB [58]	IGS [59]	-	-
	Borrelia miyamotoi		Ear, spleen[1]	flaB [60]	-	-	-
	Coxiella burnetii		Spleen	COM1 and IS1111 [61]	-		-
	ESBL/AmpC-producing *E. coli*		Feces	-	-	-	Culture: [62][2,3] Typing: In-house sequencing for β-lactamase genes, serotype and sequence type[4]
	Francisella tularensis		Spleen	FopA [63]	-	-	-
	Leptospira spp.		Kidney	lipL32 [64]	-	-	-
	Methicillin-resistant *Staphylococcus aureus*		Throat swab	-	-	-	Culture: [65][2] Typing: Multiplex PCR targeting *mecA, mecC*, *lukF* and *spa* genes [66]
	Neoehrlichia mikurensis		Spleen	groEL [67]	-	-	-
	Rickettsia helvetica		Ear, spleen[1]	gltA [68]	gltA [69]	-	-
	Rickettsia spp.		Ear, spleen[1]	gltA [70]	gltA [69]	-	-
	Spiroplasma spp.		Ear, spleen[1]	rpoB [71]	-	-	-

Table S1. *continued*

	Pathogen	Habitat	Tissue	(RT-) qPCR target(s)	(RT-) PCR target for typing	Serology (using heart fluid)	Culturing and typing
Viruses	Cowpox virus		Nasal septum	14-kD [72]	-	Indirect fluorescent antibody test (IFA) [73]	-
	Hepatitis E virus		Liver	ORF1 [74][5]	ORF1 [75] [5,6]		-
	Old world Arenaviruses including Lymphocytic choriomeningitis virus		Liver	-	L gene [47, 76]	-	-
	Puumala orthohantavirus		Lung	S-segment [77][7]	-	-	-
	Tula orthohantavirus		Lung	S-segment [77][7]	-	-	-
	SARS-CoV-2		Lung	E gene [78]	-	Indirect ELISA (Supplement 1) and virus neutralization test (VNT) [79]	-
Protozoa	*Babesia microti*		Ear, spleen[1]	ITS [80]	-	-	-
	Babesia spp. (excl. *Bab. microti*)		Spleen	18S [81]	-	-	-
	Toxoplasma gondii		Brain	529 base pair (bp) repeat [82][8]	-	ELISA: ID Screen Toxoplasmosis Indirect Multi-species; ID.VET Innovative Diagnostics, France[9]	-

: urban areas. : natural areas. [1] For small mammals originating from natural areas, these tests were performed on spleen material. For small mammals from urban areas, these tests were performed on both spleen and ear pinna material. [2] The time from taking the sample during dissection to incubation was 3-5 days. [3] Suspicious colonies were typed using MALDI-TOF MS. [4] Sequenced on an Illumina NextSeq 550 system. Libraries (2 × 150 bp reads) were prepared using the Illumina Nextera XT DNA Library Prep kit (Illumina, Inc.). Reads were processed using an in-house developed pipeline "Juno assembly" consisting of trimming and *de novo* assembly as well as quality control of the raw reads, trimmed reads and assemblies [83]. The output of the "Juno assembly" pipeline was used in two in-house developed pipelines; "Juno typing" for *in silico E. coli* serotyping and multi-locus sequence typing (MLST) analysis [84], and "Juno AMR" for detection of antimicrobial resistance determinants based on the ResFinder and PointFinder tools created by the Center For Genomic Epidemiology [85]. [5] Initially pools of max. five samples were tested and thereafter positive pools were split and tested individually. [6] Nested conventional RT-PCR with primers HEV-cs, HEV-cas, HEV-csn and HEV-casn. [7] Using Taqman Fastvirus 1-step master mix (Life Technologies). [8] We used a final reaction volume of 25 µL, using a commercial master mix (PerfeCTa ToughMix, VWR International, Darmstadt, Germany), primers (800 nM) and probes (200 nM) (MWG- Biotech, Ebersberg, Germany) in a CFX96 instrument (Bio-Rad Laboratories). The cycling conditions were 50 °C for 2 min, followed by 95 °C for 10 min and then 55 amplification cycles of 95 °C for 15 s and 60 °C for 1 min. After each cycle, the light emission by the fluorophore was measured and results were analyzed using the CFX manager software Version 1.6 (Bio-Rad Laboratories)[82, 86, 87]. [9] All steps were carried out according to the manufacturer's instructions. Horseradish peroxidase labelled protein G (P21041) was used as conjugate (ThermoFisher, NL).

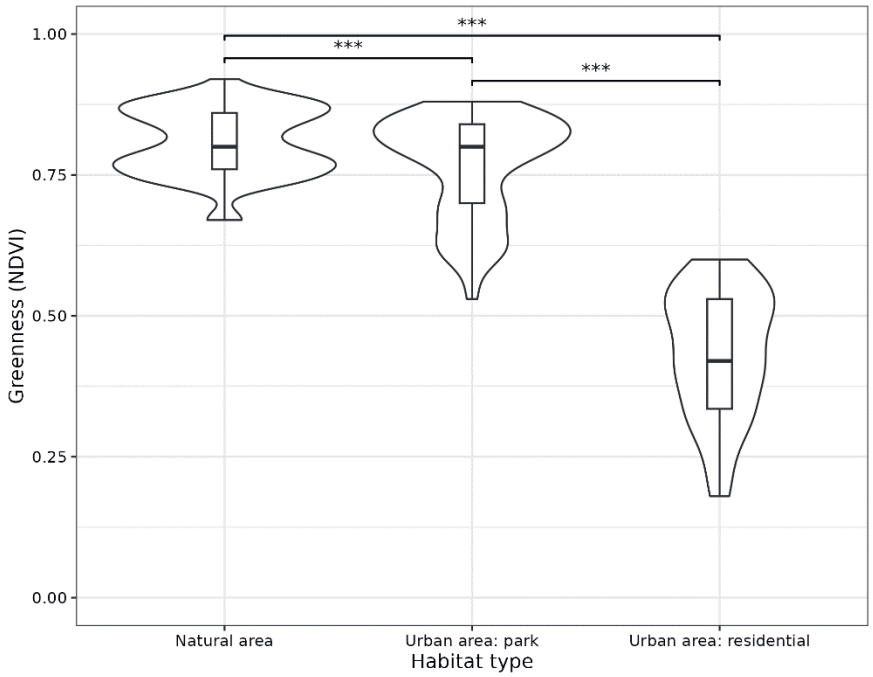

Figure S1. *Box plots showing the variation in the degree of greenness per habitat type. ***:* p-*value < 0.0001, Tukey post-hoc test. Natural area vs urban park: β = 0.04. Natural area vs residential area: β = 0.37. Urban park vs residential area: β = 0.33.*

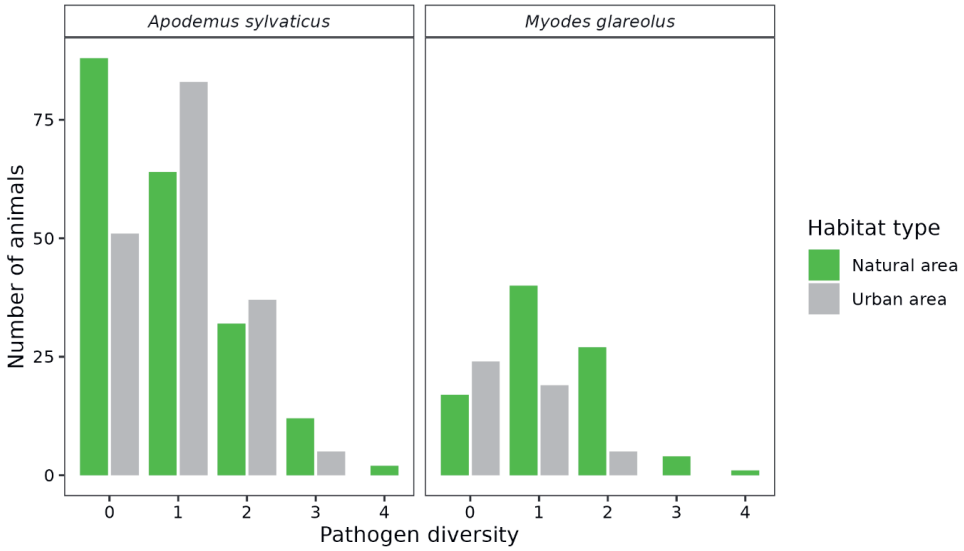

Figure S2. *The occurrence of coinfections for* Apodemus sylvaticus *and* Myodes glareolus.

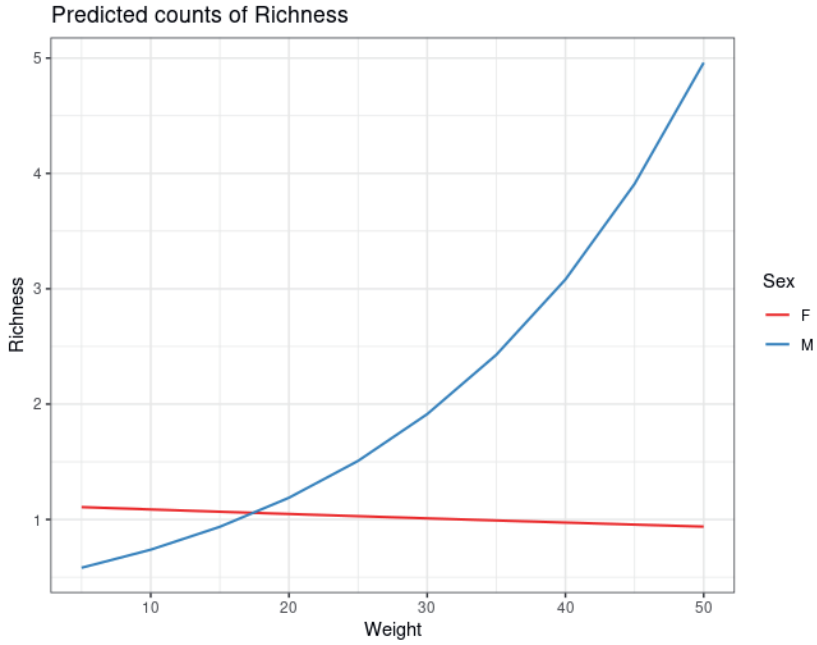

Figure S3. *The plotted significant interaction between sex and weight in bank voles.*

Supplement 1

We tested heart fluid in an indirect ELISA for the presence of antibodies directed against the nucleocapsid protein (NP) and the complete spike protein (S1-S2) of SARS-CoV-2. All heart fluid samples were incubated for 1 hour at 56 ˚C to inactivate complement prior to testing in the ELISA or virus neutralization assay (VNT). ELISAs with recombinant NP and S1-S2 proteins produced in insect cells (ECD-His tagged; both from Sino biological, Eschborn, Germany) were coated overnight in 50 mM $NaHCO_3$ (pH 9.6) at 4 ˚C in a concentration of 50 ng/100 µL. Thereafter, 96-well plates were washed in a washing machine with phosphate-buffered saline (PBS)-0.05 % v/v Tween 20 followed by incubation with 100 µL StabilBlockTM solution per well (SURMODICS: cat.nr ST-1-1000, USA) at 37˚ C for 1 hour. After washing as described before, plates were incubated for 1 hour at 37 ˚C with 100 µL/well of a 10- and 40-fold dilution of the heart fluid in PBS-0.05 % v/v Tween 20 containing 5 % v/v horse serum (PBS-Tw-HS). Plates were washed again and incubated for 1 hour at 37 ˚C with 100 µL/well of a 1:2000 dilution of Polyclonal Rabbit Anti-Mouse Immunoglobulins-horse-radish peroxidase (HRP) (Agilent Technologies Netherlands B.V. Abcoude, The Netherlands) in PBS-Tw-PS. After washing, the bound rat antibody-HRP anti-rat complexes were detected using 100 µL/well of 5'-Tetramethylbenzidine (TMB) substrate solution (1-StepTM Ultra TMB-ELISA Substrate Solution). Plates were stained for 5 minutes and 100 µL/well 0.5M H_2SO_4 was added to stop the reaction. Absorption per well was measured at 450 nM using a Molecular Devices SpectraMax® ABS Plus plate reader (San José, CA, USA). Samples were considered positive in case the absorbance at 450 nM was at least 2-fold higher than the absorbance of the 1:40 dilution of the negative control serum and significantly increased in the well with the 1:10 dilution of the sample. Samples scoring positive in one or both ELISAs (NP and S1-S2) were further tested in dilutions of 1:10, 1:20, 1:40 and 1:80 in the SARS-CoV-2 VNT as described by [88].

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Café

Chapter 8

General discussion

Rodent-borne zoonoses are a good example of a problem that requires a One Health approach, which includes humans, animals and the environment. In this thesis, we aimed to investigate the public health risks of rodent-borne zoonoses in urban environments by focusing on the surveillance of both the animal and environmental components of One Health. To assess these risks, both pathogen exposure and disease hazard need to be investigated. In this thesis, we only focused on disease hazard. Disease hazard can be investigated by monitoring both the hosts (i.e., rodent populations) and the zoonotic pathogens. In this current chapter, I will discuss the findings from my thesis chapters (Figure 1) in more detail, and the implications for urban greening, the urban ecosystem and the improvement of rodent-borne zoonotic disease surveillance.

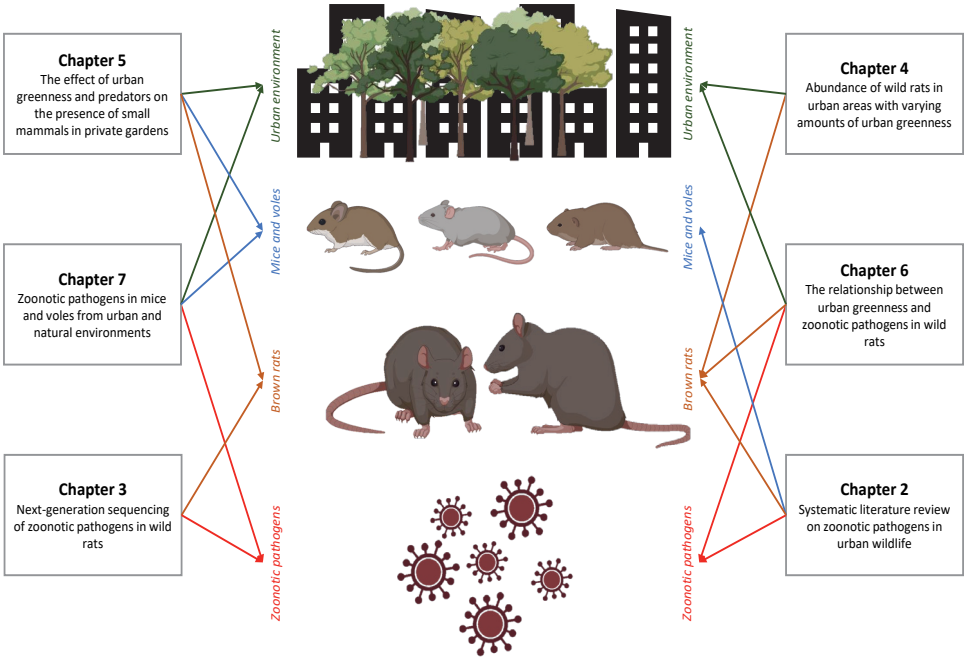

Figure 1. *Overview of the content of each research chapter and how they are related to each other.*

Zoonotic pathogens carried by urban rodents

There is relatively little research performed on zoonotic pathogens in rodents and other wildlife species in urban areas, although they can pose a high risk to public health due to close contact between humans and animals. To improve pathogen surveillance, we need to know what the current status is of zoonotic pathogen research in wildlife. Therefore, we created an overview of zoonotic pathogens studied in 10 common urban wildlife mammals in Europe based on a systematic literature review (**Chapter 2**). Most pathogens were studied in only a single animal species (54 %). Consequently, from all potential host-pathogen combinations, only 26 % has been studied in Europe so far. For brown rats, this

percentage is slightly higher (38 %). For some of those host-pathogen combinations the absence of research might be related to host specificity, or the current absence of the pathogen or its vector in a specific area due to environmental or climatic conditions [1, 2]. However, since the majority of pathogens can infect more than one host species [3], most of these pathogens likely have not been tested in multiple animal species yet, while they could infect multiple hosts. Thus, still many knowledge gaps remain. Filling these knowledge gaps can increase our knowledge about potential reservoir hosts for zoonotic pathogens, and enhances our understanding of pathogen transmission cycles and spillover. This can help to prioritize surveillance efforts and to raise awareness amongst health care professionals, which may result in better identification of human disease cases [4]. This review highlights host-pathogen combinations that need extra attention, and provides a starting point for future European-wide collaborations to improve and expand wildlife disease surveillance.

Before capturing and testing wild rodents for zoonotic pathogens, we assessed how pathogen detection can be best performed. We investigated the added value of using next-generation sequencing (NGS), specifically 16S rRNA gene amplicon sequencing and virome-enriched sequencing, to the more traditional molecular methods (i.e., (q)PCR) that are still often being used, using wild rat samples from the RIVM biobank (**Chapter 3**). We observed that 16S rRNA gene amplicon sequencing was limited in its capacity to identify potentially zoonotic bacteria to species level. As pathogen genera can include multiple species, including some zoonotic and non-zoonotic species, identification to species level is essential to determine the risk for public health. Therefore, in its current form, we would not recommend to rely on only NGS for pathogen detection, but it may be a useful tool for periodic screening of trends in (potentially zoonotic) pathogens circulating in wild rats.

After having established that zoonotic pathogens in rodents from urban areas need to be investigated further, and that currently the best method to investigate that is by using the more conventional methods, we trapped rodents in three Dutch cities. All animals were tested for various zoonotic pathogens, including bacteria, viruses and protozoan parasites, to investigate the potential disease risk of these pathogens for public health. Overall, the highest diversity of zoonotic pathogens were detected in brown rats (13/18 pathogens; **Chapter 6**), followed by mice (10/19) and voles (6/19); **Chapter 7**). Despite the high diversity of pathogens in rodents from urban areas, the prevalence was rather low (< 10 %), except for *Bartonella* spp. (26 %), *Leptospira* spp. (20 %) and ESBL/AmpC-producing *E. coli* (13 %) in rats, *Bartonella* spp. (56 %) and *Rickettsia* spp. (14 %) in mice, and *Bartonella* spp. (44 %) and *Rickettsia* spp. (13 %) in voles. The prevalence of *Rickettsia* spp. in wood mice and bank voles was much higher than in rats, which is assumed to be related to a higher infection susceptibility of these species [5], and higher tick infestation rates [6]. Our trapping methodology could have caused ticks to leave the dead hosts [7], which makes the detected tick densities prone to bias. However, we did observe higher tick infestation rates in mice and voles than in rats (**Chapter 6 and 7**). These infestation rates could be related to rat's swimming and grooming behavior [8], making them less preferred tick hosts compared to mice and voles, which correlates with the lower prevalence of tick-borne pathogens detected in rats compared to mice and voles. Also, rats' swimming behavior might be related to the higher prevalence of *Leptospira* spp. observed in rats

(**Chapter 3 and 6**) compared to mice and voles (**Chapter 7**), as *Leptospira* spp. can be transmitted via contaminated water. Overall, the most prevalent pathogen was *Bartonella* spp., similar to what we observed in rats in our NGS study (**Chapter 3**), followed by *Leptospira* spp. and various tick-borne zoonotic pathogens. These rodent-borne zoonotic pathogens are likely causing a higher risk for public health compared to the other tested pathogens that were detected with low prevalence.

The effect of urban greening on rodents and rodent-borne zoonotic pathogens

In the previous section, we observed that urban rodents are able to carry various zoonotic pathogens. Urban greening may increase the abundance of rodents and could, via increased density-dependent transmission, also increase the prevalence of rodent-borne zoonotic pathogens, which may lead to a higher disease risk in greener urban areas. As there is very limited information on the potential effects of urban greening on rodents and zoonotic pathogens, we investigated this by analyzing the relationships between urban greenness and both rodent abundance and rodent-borne zoonotic pathogen prevalence and diversity. We measured urban greenness using the Normalized Difference Vegetation Index (NDVI) [9], which quantifies vegetation greenness in a satellite image by measuring the difference between near-infrared (reflected by vegetation) and red light (absorbed by vegetation) in a range from 0 to 1. Although we did not measure the process of urban greening, we believe that the observed relationships with urban greenness give a good indication of what can be expected when cities will become greener.

The relationship between urban greenness and rodent abundance

To investigate the potential effect of urban greening on rodent abundance, we looked at the relationships between urban greenness and rodent presence and abundance. For brown rats, we observed a positive relationship between urban greenness and rat abundance, measured by systematically trapping rats using snap traps (**Chapter 4**). However, we observed no significant relationship between urban greenness and the presence of rats, measured using camera traps (**Chapter 5**). Similarly, for mice, voles and shrews, we observed a trend, but no significant relationship, between urban greenness and their presence (**Chapter 5**), but we did observe a significant relationship between urban greenness and the occupancy of mice, voles and shrews, which resembles their abundance. This suggests that urban greenness does not determine the mere presence or absence of small mammals, but does increase their abundance. Greener urban areas could provide both food (e.g., natural food resources and human food waste) and shelter resources (e.g., natural environment for digging burrows) for rodents, which could gradually influence their abundance.

We also included other environmental and socio-economic variables in our models to correct for their explanatory power. In addition to urban greenness, we observed that food availability and the presence of predators were also important determinants for rodent abundance (**Chapter 4 and 5**). For rat abundance, this included various variables

that could be proxies for the availability of food resources (i.e., the number of restaurants and the presence of petting zoos) and the absence of domestic cats. For mice, voles and shrews, domestic cats and red foxes were negatively related with their abundance. The relationship between the abundance of mice, voles and shrews and food availability could not be investigated. For all small mammals, the effect of domestic and/or natural predators was much larger than the effect of urban greenness (**Chapter 5**). This raises the question which factors are most important because we were not able to include information on all three predictor variables (i.e., food resources, predators and urban greenness) in each model. It seems that both urban greenness and food resources are approximately equally important, but that predators are more important than urban greenness. As previous research found that, in general, rodents are more affected by the presence of predators than by the availability of food resources (top-down control) [10], we expect to find a similar result for rodents from this study if we would have been able to include all three predictor variables in the models (**Chapter 5**).

However, for rats this finding is contradicting, as rats are controlled bottom-up, in which food resources are more important than predation [11]. Though, rats may become bolder when predator pressure is lower or when food resources are scarcer [12], which may differ between urbanized and more natural environments [13]. We found a positive interaction effect of domestic cats and urban greenness on the presence of rats, which implies that the negative effect of cats on rats is larger in greener areas and smaller in less green areas (**Chapter 5**). This might be related to a higher food availability in greener urban areas, which could make food resources not a limiting factor, whereby predation may become more important. Thus, in contrast to other rodent species, the effect of food resources and predators on the abundance of rats might vary with urban greenness. Limiting food availability, especially in greener urban areas, could therefore be very important to control the population size of rodents.

Each method of measuring rodent abundance (e.g., using snap traps or camera traps) has its own advantages and disadvantages that could influence the results [14]. Camera traps can measure occupancy, which is related to actual abundance [15]. However, without marking the animals, it is unclear whether you have photographed 10 different animals, or the same animal 10 times, which could lead to false associations or the absence of associations. In contrast, an animal cannot be trapped multiple times using snap traps. However, due to their neophobic behaviour [16, 17], brown rats can be more cautious to enter such traps, which could result in lower abundance estimates. Likewise, when ample food resources are available or when rats have previously encountered such traps (especially in residential areas) [16, 18], rats could be less likely to enter the traps, which could result in lower or biased abundance estimates. Supporting this, in the locations where most rats were trapped, we occasionally encountered half-eaten dead rats in the traps, which were probably eaten by other rats [19]. This might indicate that there was a shortage of food in those locations, which resulted in rats being more likely to enter the traps, and in additional cannibalistic behaviour. While this could create a difference in the trapping of rats in parks versus residential areas, we did not observe any differences in the response of rats to the traps when looking at trap success over time in parks versus residential areas (**Chapter 4**), which might indicate that their behaviour is not that

different, and thus did not largely influence the number of trapped rats.

The relationship between urban greenness and rodent-borne zoonotic pathogen prevalence and diversity

To investigate the potential effects of urban greening on rodent-borne zoonotic pathogens, we looked at the relationships between urban greenness and pathogen prevalence and diversity. We expected to find a higher prevalence and diversity of zoonotic pathogens in rodents from greener urban areas because urban greening could increase pathogen transmission and pathogen spillover between animals, and could enhance the survival of vectors and pathogens in the environment. For brown rats, we observed a higher prevalence of flea-borne *Bartonella* spp. and tick-borne *Borrelia* spp. in greener urban areas (**Chapter 6**). These two vector-borne pathogens seem to be particularly sensitive to urban greening. As we did not observe an increase in the prevalence of other tested pathogens, this increase is likely caused by a positive effect of greenness on the survival and abundance of fleas and ticks [20-22], which increases the chance of rats getting infected with vector-borne zoonotic pathogens, resulting in a higher prevalence of these vector-borne zoonotic pathogens in rats. This coincides with the higher infestation rate of ticks on rats in greener urban areas (**Chapter 6**). In addition, the abundance of rats might also play a role, as fleas are permanent ectoparasites and depend on the availability of hosts. Since rat density increases with greenness (**Chapter 4**), this could lead to an increased number of fleas and an increased prevalence of flea-borne *Bartonella* spp. via density-dependent transmission.

On the other hand, we observed a decrease in prevalence for rat hepatitis E virus and ESBL/AmpC-producing *E. coli* in greener urban areas (**Chapter 6**). For ESBL/AmpC-producing *E. coli* this relationship could reflect its relationship with the presence of food vendors [23], which may be more abundant in (less green) city centers, or it may be related to human population density (which is higher close to city centers), since antibiotic-resistant bacteria found in wildlife are often a reflection of those found in humans [24]. As the transmission mode of rat hepatitis E virus is still unknown [25], it is hard to explain this relationship. Possibly, rats in less green areas have a lower availability of food and shelter resources, forcing those rats to make use of the same resources, which could increase contact rates or fighting behavior between rats, which might lead to increased frequency-dependent pathogen transmission.

For the other pathogens we did not observe any significant differences in prevalence with urban greenness (**Chapter 6**). We expected more pathogens to have an increased prevalence in greener urban areas due to the increased abundance of rats, and thereby density-dependent pathogen transmission [26, 27]. Our results suggest that there is no clear effect of density-dependent pathogen transmission between rats. While there might still be density-dependent transmission, we might have not detect it if the increase in rat abundance in greener urban areas was not large enough, especially when density-dependent transmission is not linear but sigmoidal [28]. Thus, urban greening does not seem to influence (increase or decrease) the prevalence of every zoonotic pathogen, which is likely related to the pathogen's transmission mode.

In contrast to the rats, we did not observe any significant relationships between urban greenness and pathogen prevalence in mice and voles (**Chapter 7**). As for the rats, we expected a higher abundance of zoonotic tick-borne pathogens in mice and voles in greener urban areas [21, 22]. As almost all mice and voles were captured in urban parks, i.e., very green areas, we think that this absence of relationships is related more to the uneven distribution of rodents from locations with different degrees of greenness, than to an actual absence of relationships. Preferably, the relationships between urban greenness and pathogen prevalence in mice and voles should be further investigated using a more equal distribution of mice and voles from urban areas with different degrees of urban greenness.

We did not observe a significant relationship between pathogen diversity and urban greenness for either rats, mice or voles (**Chapter 6 and 7**), when we expected to find a positive relationship. We did observe a positive relationship between pathogen diversity and age/weight (for brown rats and wood mice), and a higher pathogen diversity in males than females (for wood mice and bank voles). There was no correlation between urban greenness and rodent age or weight. In general, older and male rodents more frequently carry pathogens, likely because of cumulative exposure combined with persistent infections, more risk taking and competitive behavior in males, and differences in immune responses between males and females [29-31]. These differences in behavior and immune status seem to increase pathogen diversity more than the environment (e.g., the degree of urban greenness). However, we did not measure the diversity of animal hosts per trapping location in relation to the degree of urban greenness, which could be an important underlying factor influencing the rate of pathogen spillover between animals, and thus pathogen diversity. Especially when the differences in animal host diversity between locations are not that large yet.

We tried to identify potential indications of pathogen spillover between rodent species, which could give an indication of pathogen transmission dynamics in urban environments, and which could be used for targeted surveillance. We found both rat and mouse fleas on the tested rats, mice and voles, and we found similar flea-transmitted *Bartonella* species on mice and voles, which were different from the flea-transmitted *Bartonella* species detected in rats. Thus, while there may be occasional spillover of *Bartonella* species between rodent species via fleas, *Bartonella* species may be less prone to spillover because they acquire host-specific virulence factors that are involved in host infection, leading to pathogen-host specificity [32]. For *Borrelia* species, there seems to be less host specificity or more pathogen spillover, as *B. afzelii* was found in both brown rats and wood mice (**Chapter 6 and 7**). We also detected *Ixodes ricinus* ticks on both rats, mice and voles. Since these ticks have a wide host range of > 300 vertebrate species [33], they can serve as vectors for pathogen transmission between animal species. Likewise, *Rickettsia helvetica* was detected in brown rats, wood mice, house mice and bank voles. Thus, for these tick-borne zoonotic pathogens, the detection of similar pathogen species in different animal species could indicate pathogen spillover between animal species, most likely via ticks. As tick infestation rates on rats increased with urban greenness (**Chapter 6**), this could increase the chance of tick-borne pathogen spillover in greener urban areas. Future research and surveillance should focus more on the community of urban wildlife species as a whole,

and at pathogen spillover, especially of vector-borne pathogens.

In addition, it is important to keep in mind that an animal's susceptibility to infection can also be related to an animal's immune status and the amount of stress. Stress and a decreased immunity can be caused by food scarcity or high predator pressure, but also by social stress arising from high population densities or unstable social structures [34-38]. Since we did not measure immune status and stress in rodents in our studies, we cannot draw any conclusions about its effects on our results, but it would be interesting for future research to investigate how immune status, stress, pathogen infection and urban greenness are related to each other.

The relationship between urban greenness and rodent-borne disease hazard ans risk

Rodent-borne disease hazard

We discussed how urban greening might affect rodent abundance and rodent-borne zoonotic pathogen prevalence. Disease hazard posed by rodents is the product of rodent population density and pathogen prevalence [39]. We observed that the abundance of rodents increased in greener urban areas (**Chapter 4 and 5**) and that the prevalence of most zoonotic pathogens did not significantly change with urban greenness (**Chapter 6 and 7**). Only for rats, there was an increase in the prevalence of two vector-borne zoonotic pathogens, *Bartonella* spp. and *Borrelia* spp, and a decrease in the prevalence of ESBL/AmpC-producing *E. coli* and rat Hepatitis E virus in greener urban areas (**Chapter 6**). Thus in general, rodent-borne zoonotic disease hazard increases with urban greenness, except for ESBL/AmpC-producing *E. coli* and rat Hepatitis E virus in rats. For pathogens without a significant increase in prevalence in greener urban areas, the increased disease hazard is due to the increase in rat abundance. For *Bartonella* spp. infection in rats, a doubling of the amount of urban green would result in almost a doubling of the disease hazard (**Chapter 6**), mainly caused by the strong increase in *Bartonella* spp. prevalence in greener urban areas. For the other pathogens, the increase in disease hazard is lower.

Rodent-borne disease risk

Whether this increased disease hazard also leads to an increase in human disease risk depends on human exposure. As we did not measure exposure in this study, we can only speculate how disease risk will be influenced because exposure can vary depending on human activities, which can again vary seasonally [40]. We expect exposure to rodent-borne zoonotic pathogens to be higher in spring and summer, due to people being more outdoors and engaging in activities such as gardening and swimming, that could increase the risk of pathogen transmission [41]. These activities are often linked to greener urban areas, which also have a higher rodent-borne zoonotic disease hazard and may thus form areas of increased disease risk for humans. On the other hand, rodents could move indoors (e.g., in houses and sheds) in the colder months, being closer to humans, which may increase the exposure to rodent-borne zoonotic pathogens as well.

The exposure to vector-borne zoonotic pathogens transmitted by rodents largely depends on the exposure to the vectors (e.g., ticks and fleas). While *Bartonella* spp. was

the most prevalent pathogen found in rodents, human disease cases are rarely reported [42]. This could be caused by a lack of awareness amongst health care professionals, low pathogenicity in humans, or a low exposure to this pathogen. Rodent-borne *Bartonella* species are mainly transmitted via fleas, that are often host specific and do not (regularly) infest humans [42]. However, the zoonotic transmission cycle of rodent-borne *Bartonella* species to humans is not fully understood yet, and needs further investigation, especially since this is one of the most prevalent zoonotic pathogens in urban rodents. Human exposure to tick-borne zoonotic pathogens such as *Borrelia* spp., mainly depends on the number of ticks present and thereby the frequency with which humans get bitten by ticks. Since the prevalence of both *Bartonella* spp. and *Borrelia* spp. is higher in greener urban areas, the abundance of vectors in greener urban areas is likely higher as well, which could lead to an increased exposure to these vectors, and thus an increased disease risk in greener urban areas. Thus, by preventing human exposure to both fleas and ticks, we could limit the disease risk of vector-borne zoonotic pathogens, including *Bartonella* spp. and *Borrelia* spp.

Implications for urban greening, the urban ecosystem and disease surveillance

In these next sections we will discuss the implications of our findings on urban greening, the urban ecosystem and rodent-borne disease surveillance, and what this means for public health, society, policy, and future research.

Urban greening: how to reduce rodent-borne zoonotic disease risk?

After reading the results from this thesis, one might wonder whether it is actually a good idea to perform urban greening, as it will likely increase the abundance of rodent populations and of rodent-borne zoonotic pathogens transmitted by vectors (**Chapter 2 and 4-7**). However, urban greening also has a lot of beneficial effects, such as its positive effects on mental health, water retention and biodiversity, and the reduction of urban heat-island effects and stress [43-46]. The positive effects of urban greening might increase human health and could thereby make humans more resilient to infection with zoonotic pathogens. Many of the costs and benefits of urban greening have been quantified in terms of money, showing that the benefits of urban greening are more likely to outweigh the costs in locations that currently have very low levels of urban greenness or a high population density [46]. Although the benefits of urban greening outweigh its negative effects, and urban greening will probably always be associated with wildlife-borne zoonotic risks, we should try to keep the risks as low as possible by also taking into account and quantifying the negative effects of zoonoses, and trying to limit the size of rodent populations and human exposure to zoonotic pathogens. This provides opportunity to further improve urban greening by investigating how we can maximize its beneficial effects, while minimizing its negative effects.

Reducing the availability of food resources

The increased hazard of most rat-borne zoonotic diseases in greener urban areas was

mainly caused by the increase in rat abundance. Thus by reducing the size of rodent populations, we could reduce the hazard of rodent-borne zoonotic diseases. Since rodent population densities are linked to the availability of food resources (**Chapter 4**), which is often created or sustained by human behavior, governments should work together with the public to tackle this problem. Preventive measures that limit food availability could help to control rodent population sizes. Some practical measures could be increasing the number of waste bins or the frequency of garbage collection, placing bread bins (in Dutch: 'broodcontainers') for the natural disposal of leftover bread as is currently a success in Amsterdam, storing food in rodent-proof containers, and increasing human awareness towards food waste [47, 48].

Altering urban green spaces

Another way to reduce rodent population densities might be altering urban green spaces to limit the availability of food and shelter resources. Food waste could get stuck or hidden more easily in dense vegetation, and this type of vegetation might also provide shelter from predators. However, for governing bodies such as municipalities that have to make decisions regarding urban greening, it can still be difficult to decide what measures to take because some measures can have opposing effects for different goals. For example, if two of their goals are to 1) reduce rat nuisance, and to 2) increase insect biodiversity, mowing the grass could help to reduce rat nuisance by reducing coverage, but at the same time it decreases the availability of flowers, which negatively affects insect biodiversity. The best decision will depend on the local context and priorities.

In this thesis, we only measured greenness using NDVI, while greenness encompasses a wide range of plant species and structural variations, which could have varying impacts on rodent abundance. Certain vegetation types may provide rats with more coverage to hide, as rats avoid open space [49]. Traweger et al. (2006) studied the relationship between rat abundance and vegetation types, and observed significant positive relationships between rat abundance and the presence of bushes, trees, ruderal vegetation, vegetation with fruits, riverbank vegetation, conifers, and deciduous trees, and a significant negative relationship between rat abundance and the presence of evergreens. We also investigated the influence of vegetation types on rat abundance, and we found that the category 'mixed shrub forest' (in Dutch: bosplantsoen) had a positive effect, and 'ground-covering plants' (in Dutch: bodembedekkers) had a negative effect on rat abundance (unpublished; Table 1). However, we could not correct for other important factors in this analysis, such as the amount of waste or the degree of greenness, due to limited data and model convergence problems. Therefore, these outcomes should be interpreted with caution. Future studies should include more specified measures of greenness to account for such differences in composition to investigate its effects on the abundance of not only rodents, but also vectors such as ticks [21, 22], in order to give practical advise on how to improve or adjust urban greening.

Using natural predators

If we can attract more natural predators of rodents by making cities greener, this might help to adjust the current disbalance between rodents and their predators. While this

Table 1. *Results from the multivariate zero-inflated negative binomial relative rat abundance model with only vegetation types (n = 48 trapping locations). No variables were included in the zero-inflated part of the model. Results are not corrected for other important variables, thus they should be interpreted with caution.*

Vegetation types (0-1)	Estimate	P-value
Ground-covering plants	-2.90	**< 0.001**
Mixed shrub forest	1.27	**0.014**
Roses	-0.61	0.273
Perennial plants	0.68	0.347
Fruit and nut-bearing plants and trees	-1.28	0.350
Grass and herbaceous plants	-0.68	0.379
Shrubs	0.50	0.409
Hedges	-0.15	0.780

might help to reduce the population sizes of mice, voles and shrews, the effect of wild predators on wild rats seems to be very low currently (**Chapter 5**). This can be due to the easy access to food resources in urban areas, which does not require wild predators to show their natural hunting behavior and thereby may limit predation [13]. Therefore, limiting the availability of food resources could not only help to directly limit rodent population sizes, but also to indirectly reduce their population sizes by enhancing the hunting behavior of their predators.

Lethal rodent control methods

While we should aim to first focus on preventive measures to control rodent populations, those measures can sometimes be insufficient. In those cases, non-chemical control methods, such as snap traps, could be used. However, these methods have some negative side-effects such as the killing of non-target species [47]. In this study, we also had a lot of by-catch of non-target species, especially in greener urban areas such as parks, when using rat snap traps (Figure 2). In total, we even trapped more other animals than rats. As we did not choose the trapping locations based on rat nuisance, this might have increased the chance of trapping non-target species compared to professional pest controllers. Most non-target species were trapped in locations where only few rats (< 5) were trapped (Figure 3). Thus, to minimize the trapping of non-target species, it is very important to first establish whether there are indeed rats present in the location you want to start trapping. Additionally, such lethal rodent control measures can have adverse effects on reducing zoonotic disease risks because they could even lead to an increase in the prevalence of zoonotic pathogens, such as *Leptospira interrogans*, by disrupting the social structures, which promotes new interactions and thereby facilitates pathogen transmission among the remaining rodents [50].

Reducing human exposure

Furthermore, the risk of humans getting infected with rodent-borne diseases can be reduced by lowering the exposure to rodents and their zoonotic pathogens, for example by creating extra awareness to increase the use of hygiene measures after visiting urban green spaces. Contact with potentially contaminated surfaces could also be reduced, such as preventing swimming in freshwater bodies that are likely contaminated with zoonotic

pathogens such as *Leptospira* spp. To lower the exposure to rodent-borne zoonotic pathogens that are transmitted by vectors such as ticks and fleas, the chance of getting bitten by these vectors should be reduced, for example by wearing protective clothing or insect repellents, or by altering the vegetation (e.g., mowing the grass) [51, 52].

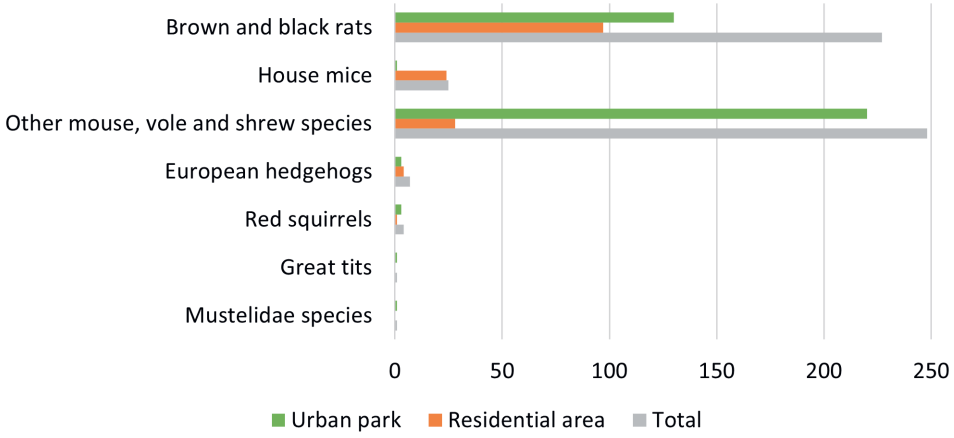

Figure 2. *Number of wild rats (brown and black rats) and other animal species trapped as by-catch while trapping wild rats. Divided over urban parks and residential areas.*

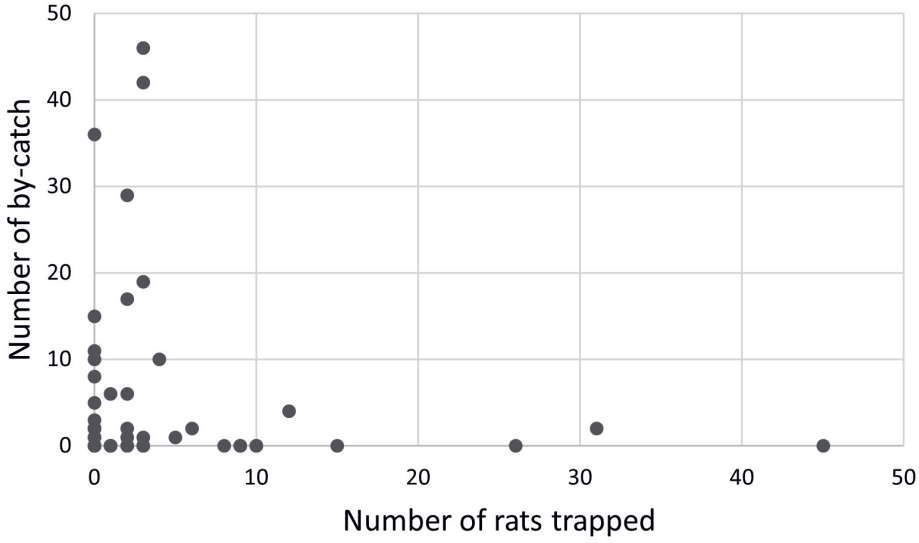

Figure 3. *Number of rats trapped versus the number of other animal species (by-catch) trapped per location. Most by-catch was observed when the number of trapped rats was < 5.*

The urban ecosystem

In this thesis we mainly focused on rodents and rodent-borne zoonotic pathogens in urban areas. However, they are part of a more complex urban ecosystem, with close contacts between humans and wild and domestic animal species, that could increase zoonotic disease risk for humans. Urban greening enhances the diversity and abundance of urban wildlife, for which we already observed several indications (i.e., an increased abundance of rats, mice, voles, red foxes and martens, in greener urban areas; **Chapter 4 and 5**). This could lead to an increase in the prevalence and diversity of zoonotic pathogens, for example via spillover of pathogens between animals (**Chapter 2, 6 and 7**). The lack of research investigating the complex interactions between urban wildlife species and zoonotic pathogens emphasizes the need for future research to further investigate interactions between different animal species, and how this affects wildlife-borne zoonotic disease dynamics. It is particularly important to perform such research in urban areas, where there is a higher chance of contact between humans and animals, and thus potentially higher exposure to zoonotic pathogens compared to natural areas [53]. This can be done in the form of studying contact rates and pathogen spillover between animals (e.g., by testing multiple animal species from the same locations), and studying pathogen transmission cycles. Ideally, this should be investigated using a longitudinal study in which animal and vector species composition and abundance can be related to the prevalence and diversity of zoonotic pathogens circulating in urban wildlife species.

How to improve rodent-borne disease surveillance in changing urban environments?

In this thesis, we looked at different monitoring parts of rodent-borne disease surveillance, namely the monitoring of rodent populations, of zoonotic pathogens, and the influence of the environment on these two.

Monitoring rodent populations

To get more insight into rodent-borne disease hazard and disease risk, we also need to monitor rodent populations. The increase we observed in rodent abundance in greener urban areas highlights the need for better monitoring of these populations, as this increase in abundance is for most rodent-borne zoonotic pathogens the main cause of their increase in disease hazard in greener urban areas (**Chapter 6**). However, there is still a lack of reliable nation-wide abundance data of rodents (and other wildlife species) in urban areas (personal communication with various Dutch urban ecologists), which hampers effective surveillance of rodent populations to monitor population trends and link this to changes in their environment. The 'Rattenmonitor', launched in 2020, aims to provide nation-wide surveillance of wild rat populations in the Netherlands. Although the monitoring coverage has increased in these past years, there are still many locations without data. For better insight into rodent-borne disease hazard and risk, we should aim to drastically increase the coverage of this monitoring platform. While voluntary commitment to contribute would be preferred, policy-enforced rules might in the end be necessary to involve all contributing parties.

Another form of monitoring rodent populations is by using rodent complaint data.

However, this source of data may over- or underestimate actual rat abundance [54]. For example, we observed that the number of rat complaints in urban parks was much lower than in residential areas, while the number of trapped rats was much higher in parks (**Chapter 4**). It is important to be aware of this reporting bias, because in this way rats in parks might reproduce uncontrolled, and may function as source populations from which rats could disperse to neighboring areas. Since it is quite hard to get reliable abundance data from rodents, we recommend to use at least two different methods (i.e., trapping data, rodent complaint data, or camera trapping data) to estimate abundance to get more reliable results. Other, previously mentioned, alternative methods could be chew cards or deep learning-based systems to detect and analyse ultrasonic vocalizations, such as DeepSqueak [14, 55, 56].

Monitoring zoonotic pathogens

The second aspect of getting more insight into rodent-borne disease hazard and disease risk, is by monitoring zoonotic pathogens in rodent populations. We observed that especially the prevalence of rodent-borne zoonotic pathogens transmitted by vectors increased in greener urban areas. Therefore, surveillance of zoonotic pathogens should focus more on pathogens (e.g., vector-borne pathogens) that are more likely to be affected by future changes of cities such as urban greening. In that way, we can better anticipate the expected changes in the prevalence of zoonotic pathogens, and thus disease hazard. We could start testing wildlife for zoonotic pathogens that are transmitted by vectors, or for which indications of spillover between animal species have already been found. For example, by collaborating with the Dutch Wildlife Health Centre (DWHC), a variety of wildlife species could be tested for such zoonotic pathogens, which will enhance our knowledge of host-pathogen specificity and potential pathogen spillover. This is especially valuable for wildlife species from urban areas or which are likely to be(come) present in urban areas.

In an ideal situation in which we would not be limited by money or other resources, we could start testing rodents for the whole list of zoonotic pathogens described in **Chapter 2**. However, in reality we will have to choose which pathogens to focus on. We could decide to focus on pathogens that pose the greatest risk to human health by ranking them based on a risk assessment [57]. However, in this way unexpected pathogens or pathogens occurring in low prevalence will be disregarded. One way to overcome missing unexpected pathogens is by using NGS methods, although these methods will need to be improved to reliably identify pathogens to species level (**Chapter 3**). Alternatively, we could investigate which pathogens are circulating in the environment, for example by using environmental DNA/RNA [58], and focus pathogen detection in wildlife on those pathogens that are circulating in the environment. In addition, public health would benefit from more European-wide collaboration to increase our common knowledge about wildlife-borne diseases to improve wildlife-borne disease surveillance, which could start with a common European database for wildlife-borne zoonotic pathogens, that could be extended with a European biobank for sharing samples, and an increased collaboration between European research institutes.

Monitoring the influence of the environment

As we observed in this thesis, environmental changes such as urban greening can increase the hazard of rodent-borne zoonotic diseases. Therefore, it is important to study the effects of such environmental changes on the hazard of zoonotic diseases in other urban wildlife species as well, in order to anticipate to the potential disease risks. We should further investigate the effects of different vegetation types because some vegetation types might enhance or decrease rodent abundance [49, 59]. Furthermore, we should also investigate the effects of environmental changes on disease vectors (e.g., ticks and fleas) because they also seem to increase with urban greenness, and in that way they could enhance zoonotic disease risk for humans. If that is the case, the disease risk could be reduced by implementing measures that also target these vectors.

Monitoring human disease cases

In this thesis, we did not focus on the surveillance of human disease cases with rodent-borne zoonotic pathogens. However, since the number of rodent-borne disease cases reported in humans in the Netherlands in the past years is relatively low, this raises the question whether there are only few human disease cases or whether human cases are not recognized. A lack of awareness of the circulation of these pathogens in rodents could lead to misidentification or underreporting of human disease cases [4]. Therefore, a next step would be to increase surveillance of rodent-borne diseases (e.g., *Bartonella* spp.) in humans to investigate the reasons behind these low numbers of human disease cases, and to better quantify human exposure to rodent-borne zoonotic pathogens to get a better indication of the disease risks.

Recommendations for future research, surveillance and policy

- Incorporate zoonotic disease risks in future urban greening plans and policies.
- Start with preventive measures to control rodent populations that focus on targeting food availability, the design of urban green spaces or both, especially in greener urban areas.
- Further investigate how structural variations within greenness influence the abundance of rats, vectors and pathogens. Ideally by using a before-after urban greening situation in which the effects of various types of urban greening on the abundance of rodents, vectors and pathogens can be studied.
- Improve NGS methods to enable reliable identification of pathogens to species level.
- Improve the surveillance of rodent populations by enhancing the coverage of a nation-wide monitoring platform.
- Increase the overall monitoring of urban wildlife species for a higher diversity of zoonotic pathogens to investigate pathogen spillover and the conditions that favor such events.
- Increase the surveillance of pathogens that are likely to be most affected by urban greening: vector-borne zoonotic pathogens.
- Invest in cross-country collaborations to improve rodent-borne and wildlife-borne disease surveillance on a European level.

- Start the surveillance of human disease cases with rodent-borne zoonotic pathogens to better quantify human exposure and thereby disease risk.

Conclusions

Rodents can carry a high diversity of zoonotic pathogens, which can form a risk for public health. Urban greening seems to increase both the abundance of rodents and the prevalence of specifically rodent-borne zoonotic pathogens transmitted by vectors, which could lead to an increase in rodent-borne disease hazard and disease risk. Measures should be taken to reduce this risk in greener urban areas by controlling rodent populations, reducing human exposure, and improving rodent-borne disease surveillance. Furthermore, we need more research on the urban ecosystem as a whole, and on the interactions between wildlife species that facilitate the transmission and spillover of zoonotic pathogens. This thesis underlines the importance of performing more One Health focused research on the effects of urban greening on wildlife-borne and vector-borne zoonotic diseases, and it provides a solid starting point to further improve rodent-borne and wildlife-borne zoonotic disease surveillance in urban environments.

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Summary

Zoonoses are infectious diseases caused by pathogens transmitted from animals to humans. Depending on the type of zoonosis, infections in humans can lead to severe health issues. It is estimated that more than 60% of all emerging infectious diseases are zoonotic, of which about 75% originates from wildlife. Wild rodents can host a great diversity of zoonotic pathogens, which can be transmitted to humans via direct or indirect contact, or via vectors such as ticks and fleas. Rodents such as rats and house mice prefer to live close to humans and are abundantly present in urban areas, which increases the likelihood of humans getting exposed to these rodents and their pathogens. Altogether, rodents likely pose a higher human disease risk in urban areas compared to other wildlife species.

Changes in the environment, such as urban greening, can influence the transmission of pathogens between animals. While urban greening is increasingly being implemented to counteract the negative effects of urbanization, little is known about the effects urban greening might have on rodent-borne and wildlife-borne zoonotic disease risk. To prevent increased disease transmission to humans in urban areas, it is important to get more insight in rodent-borne zoonotic disease risks, and how this may be affected by urban greening. In this thesis, we aimed to answer the question: "What are the public health risks of rodent-borne zoonoses in urban environments?". To assess these risks, both pathogen exposure and disease hazard need to be investigated. In this thesis, we only focused on disease hazard. We investigated disease hazard by monitoring both the hosts (i.e., rodent populations) and the zoonotic pathogens, in relation to urban greenness. We discuss our results and their implications for urban greening, the urban ecosystem and the improvement of rodent-borne zoonotic disease surveillance.

To assess the public health risks and to improve rodent-borne disease surveillance in urban areas, we need to know what the current situation is. While urban greening increases wildlife biodiversity in cities, which could increase the risk of zoonotic disease transmission to humans, there is relatively little research performed on zoonotic pathogens in wildlife from urban areas. In **Chapter 2**, we created an overview of zoonotic pathogens studied in 10 common urban wildlife species (including rodents such as brown rats, house mice, wood mice and common voles) based on a systematic literature review. This overview can help to identify current knowledge gaps and to prioritize surveillance efforts. We observed a high diversity of studied zoonotic pathogens, of which most have been studied in only one single animal species. Despite certain host-pathogen specificity, this still results in many knowledge gaps and opportunities for surveillance to improve both rodent-borne and wildlife-borne zoonotic disease surveillance, both on a national and a European level.

Before capturing and testing wild rodents for zoonotic pathogens, we investigated the use of next-generation sequencing (NGS) methods to detect zoonotic pathogens present in wild rats in **Chapter 3**. We noticed that NGS cannot always reliably identify pathogens to species level, which is often critical to determine a pathogen's zoonotic potential. Therefore, NGS might be useful for periodic screening of trends in the circulating of pathogens in wild rats, but it does not seem to be a suitable tool yet for the detection of zoonotic pathogens.

Changes in the urban environment, such as urban greening, could affect disease hazard by altering rodent densities and zoonotic pathogen transmission. Therefore, we investigated the relationships between urban greenness, rodent abundance and the prevalence and diversity of rodent-borne zoonotic pathogens. In **Chapter 4**, we conducted a field study in the cities of Amsterdam, Rotterdam and Eindhoven, in which we systematically trapped wild rats using snap traps in locations with varying degrees of urban greenness. We observed that both the degree of urban greenness and food-related variables were positively associated with the abundance of wild rats. These results imply that urban greening likely increases the abundance of wild rats. Since the use of snap traps for estimating rat abundance also has some disadvantages, we also investigated the relationship between urban greenness and small mammals in a different way to verify our results from Chapter 4. Therefore, we investigated the presence and occupancy (i.e., abundance) of small mammals (i.e., rats, mice, voles and shrews) in private gardens in the Netherlands using camera trapping data in **Chapter 5**. In this study, we also included small mammal predators (i.e., domestic cats, dogs, mustelids and red foxes) as predictor variables in the models. While we observed that for the abundance of small mammals, the absence of predators was more important than the degree of greenness, still urban greenness significantly enhanced the abundance of these small mammals.

An increase in the abundance of rodents might lead to an increase in pathogen prevalence via an increased density-dependent transmission of zoonotic pathogens between rodents. This may result in higher numbers of infected rodents and thus a higher zoonotic disease hazard. In **Chapter 6**, we studied the effect of urban greenness on the prevalence and diversity of rat-borne zoonotic pathogens. We observed that specifically vector-borne zoonotic pathogens (i.e., *Bartonella* spp. and *Borrelia* spp.) were positively associated with an increase in urban greenness. This can be caused by an increased abundance and survival of vectors (e.g., ticks and fleas) in greener urban areas. Furthermore, we also examined this same relationship for mice and voles in **Chapter 7**. We found no significant effect of urban greenness on pathogen prevalence in mice and voles, which is likely due to the low number of mice and voles collected in less green urban areas. Combining the results from Chapter 4 and 6, we found that urban greening will likely increase the abundance of wild rats in cities, and that it will also increase the prevalence of vector-borne zoonotic pathogens in wild rats. In this way, urban greening could lead to a higher disease hazard of rat-borne zoonotic pathogens. Whether this also leads to a higher disease risk depends on the exposure, which should be further investigated.

In **Chapter 8**, I discuss the findings of this thesis and the implications for urban greening, the urban ecosystem, and the improvement of rodent-borne disease surveillance in the Netherlands in more detail. This thesis provides new insights in the potential effects of urban greening on rodents and rodent-borne zoonotic diseases. Based on these new insights, we provide recommendations for practical measures, future research and policy to limit rodent-borne and wildlife-borne zoonotic disease risks.

Samenvatting

Zoönosen zijn infectieziekten die worden overgedragen door ziekteverwekkers van dier op mens. Een humane infectie met een zoönose kan, afhankelijk van het type ziekteverwekker, ernstige gezondheidseffecten hebben. Geschat wordt dat meer dan 60% van alle opkomende infectieziekten zoönotisch zijn, waarvan ongeveer 75% afkomstig is uit wilde dieren. Wilde knaagdieren kunnen een grote diversiteit aan zoönotische ziekteverwekkers bij zich dragen, die naar de mens overgedragen kunnen worden via direct en indirect contact, of via vectoren zoals teken en vlooien. Knaagdieren zoals ratten en huismuizen leven bij voorkeur in de buurt van mensen en zijn in grote getale aanwezig in steden, wat de kans op menselijke blootstelling aan knaagdieren en aan de ziektes die ze overdragen vergroot. Deze kenmerken zorgen ervoor dat knaagdieren waarschijnlijk een groter ziekterisico vormen voor de mens in vergelijking tot andere wilde dieren.

Veranderingen in de omgeving, zoals stedelijke vergroening, kunnen de overdracht van ziekteverwekkers tussen dieren beïnvloeden. Ondanks dat steden steeds vaker worden vergroend om de negatieve effecten van verstedelijking tegen te gaan, is er nog weinig bekend over de mogelijke effecten van stedelijke vergroening op het risico van zoönosen die worden overgedragen door knaagdieren en andere wilde dieren. Om een verhoogde overdracht van zoönotische ziekteverwekkers naar mensen in steden te voorkomen, is het belangrijk om meer inzicht te krijgen in de ziekterisico's van knaagdier-overdraagbare zoönosen, en hoe die beïnvloed worden door stedelijke vergroening. In dit proefschrift proberen we de volgende vraag te beantwoorden: "Wat zijn de risico's van knaagdier-overdraagbare zoönosen in stedelijke gebieden voor volksgezondheid?". Om deze risico's in kaart te brengen moeten zowel het aantal knaagdieren, als de prevalentie van ziekteverwekkers, als de blootstelling aan deze ziekteverwekkers onderzocht worden. In dit proefschrift focussen we op de gastheren (in dit geval knaagdier populaties) en op de zoönotische ziekteverwekkers, in relatie tot de mate van stedelijk groen. We bespreken onze resultaten en hun implicaties voor stedelijke vergroening, het urbane ecosysteem en de verbetering van de surveillance van knaagdier-overdraagbare zoönosen.

Om de ziekterisico's in kaart te brengen en om de surveillance van knaagdier-overdraagbare zoönosen in stedelijke gebieden te verbeteren, moeten we eerst weten wat de huidige stand van zaken is. Hoewel stedelijke vergroening de biodiversiteit van wilde dieren in steden verhoogt, wat het risico op overdracht van zoönosen naar mensen zou kunnen vergroten, is er relatief weinig onderzoek gedaan naar zoönotische ziekteverwekkers in wilde dieren in de stad. In **Hoofdstuk 2** hebben we een overzicht gemaakt van zoönotische ziekteverwekkers die bestudeerd zijn in 10 wilde zoogdieren die algemeen in de stad voorkomen (inclusief knaagdieren zoals bruine ratten, huismuizen, bosmuizen en veldmuizen), gebaseerd op een systematisch literatuuronderzoek. Dit overzicht kan helpen om nog ontbrekende kennis te identificeren en om surveillance activiteiten te prioriteren. We vonden een hoge diversiteit aan bestudeerde zoönotische ziekteverwekkers, waarvan de meeste maar in één enkele diersoort waren bestudeerd. Ondanks enige specificiteit tussen gastheren en ziekteverwekkers, ontbreekt er nog veel kennis en zijn er mogelijkheden om de surveillance te verbeteren van zowel knaagdier-overdraagbare zoönosen als zoönosen die door andere wilde zoogdieren in de stad

worden overgedragen, zowel op nationaal als op Europees niveau.

Voordat we knaagdieren gingen vangen en testen op zoönotische ziekteverwekkers, hebben we eerst de mogelijkheden onderzocht om next-generation sequencing (NGS) methoden te gebruiken voor het detecteren van zoönotische ziekteverwekkers in wilde ratten in **Hoofdstuk 3**. NGS kon niet altijd ziekteverwekkers tot soortniveau identificeren met een hoge betrouwbaarheid, wat vaak wel noodzakelijk is om te bepalen of een ziekteverwekker daadwerkelijk zoönotisch is. NGS zou dus wel nuttig kunnen zijn voor periodieke screening van trends in de circulatie van ziekteverwekkers in ratten, maar niet voor de detectie van zoönotische ziekteverwekkers.

Veranderingen in de stedelijke omgeving, zoals stedelijke vergroening, kunnen het risico op zoönosen beïnvloeden door veranderingen in de dichtheden van knaagdieren en in de overdracht van zoönotische ziekteverwekkers. Daarom hebben we de relaties onderzocht tussen de mate van stedelijk groen, de dichtheid van knaagdieren en de prevalentie en diversiteit van knaagdier-overdraagbare zoönosen. In **Hoofdstuk 4** hebben we een veldonderzoek uitgevoerd in de steden Amsterdam, Rotterdam en Eindhoven, waarbij we systematisch wilde ratten hebben gevangen met behulp van klapvallen op locaties met verschillende mate van stedelijk groen. We zagen dat zowel de mate van stedelijk groen als voedsel-gerelateerde variabelen positief geassocieerd waren met de dichtheid van wilde ratten. Deze resultaten impliceren dat stedelijke vergroening waarschijnlijk zorgt voor een hogere dichtheid van ratten. Omdat het gebruik van klapvallen voor het meten van de dichtheid van ratten ook enkele nadelen heeft, hebben we de relatie tussen de mate van stedelijk groen en knaagdieren tevens op een andere manier onderzocht om onze resultaten uit hoofdstuk 4 te verifiëren. In **Hoofdstuk 5** onderzochten we de aanwezigheid en dichtheid van zowel bruine ratten als andere kleine zoogdieren (d.w.z. muizen, woelmuizen en spitsmuizen) in privétuinen in Nederland met behulp van cameravallen. In deze studie hebben we ook predatoren van knaagdieren (d.w.z. huiskatten, honden, marterachtigen en vossen) meegenomen als variabelen in de modellen. Hoewel we zagen dat voor de dichtheid van deze kleine zoogdieren de afwezigheid van predatoren belangrijker was dan de mate van groen, had de mate van groen toch een significant positief effect op de dichtheid van deze kleine zoogdieren.

Een toename van de dichtheid van knaagdieren kan leiden tot een toename in de prevalentie van zoönotische ziekteverwekkers via een verhoogde dichtheids-afhankelijke overdracht van ziekteverwekkers tussen knaagdieren. Dit kan resulteren in hogere aantallen geïnfecteerde knaagdieren en dus een hoger risico op zoönosen. In **Hoofdstuk 6** bestudeerden we het effect van de mate van stedelijk groen op de prevalentie en diversiteit van zoönotische ziekteverwekkers die door ratten worden overgedragen. We zagen dat specifiek de zoönotische ziekteverwekkers die door vectoren worden overgedragen (d.w.z. *Bartonella* bacteriën en *Borrelia* bacteriën) positief geassocieerd waren met een toename in de mate van stedelijk groen. Dit kan worden veroorzaakt door een verhoogde dichtheid en overleving van vectoren, zoals teken en vlooien, in groenere gebieden. Daarnaast onderzochten we dezelfde relaties ook voor muizen en woelmuizen in **Hoofdstuk 7**. We vonden geen significant effect van de mate van stedelijk groen op de prevalentie van zoönotische ziekteverwekkers in muizen en woelmuizen, wat

waarschijnlijk komt door het lage aantal (woel)muizen uit minder groene locaties. Door de resultaten van hoofdstuk 4 en 6 te combineren, vonden we dat stedelijke vergroening waarschijnlijk zorgt voor een toename in de dichtheid van ratten in steden, en voor een toename in de prevalentie van zoönotische ziekteverwekkers die worden overgedragen door vectoren. Op deze manier kan stedelijke vergroening zorgen voor een hoger risico op knaagdier-overdraagbare zoönosen. Of dit ook leidt tot meer humane infecties hangt af van de blootstelling, wat verder onderzocht moet worden.

In **Hoofdstuk 8** bespreek ik de bevindingen van dit proefschrift en de implicaties voor stedelijke vergroening, voor het urbane ecosysteem en voor de verbetering van de surveillance van knaagdier-overdraagbare zoönosen in Nederland in meer detail. Dit proefschrift biedt nieuwe inzichten in de mogelijke effecten van stedelijke vergroening op knaagdieren en knaagdier-overdraagbare zoönosen. Op basis van deze nieuwe inzichten geven we aanbevelingen voor praktische maatregelen, toekomstig onderzoek en beleid. Hiermee kan het risico op zoönotische ziekteverwekkers die worden overgedragen door knaagdieren en andere wilde dieren worden beperkt.

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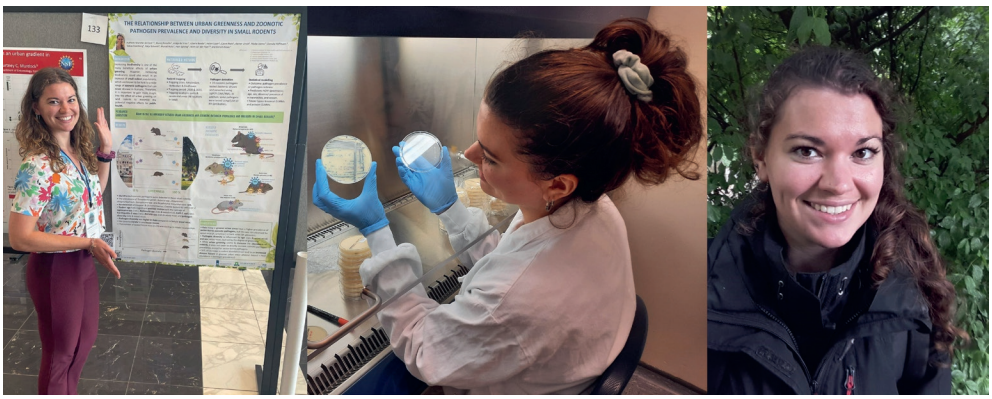
About the author

Marieke Pauline de Cock was born on the 8th of February 1994 in Terneuzen, Zeeland, the Netherlands. After completing Gymnasium at the Reynaertcollege in Hulst, she started her Bachelor's degree in Biology at Wageningen University, which she obtained in 2015 *cum laude*. She had a broad interest for research topics including Ecology, Immunology, Nematology, Entomology and Epidemiology. She continued her Master's degree in Biology, with specialization 'Health and disease' at Wageningen University, which she completed in 2018. Her interest in the complexity of vector-borne and zoonotic diseases resulted in a MSc thesis about optimizing the trapping efficiency of a mosquito trap for *Aedes aegypti* mosquitoes, followed by a MSc internship at the National Institute for Public Health and the Environment (RIVM) to perform risk mapping of *Aedes aegypti* mosquitoes in Curacao, for which she went to the Dutch Caribbean islands for 2,5 months.

After her studies, she first started working at the department of Antimicrobial resistance at the RIVM for one year. In 2019, she started her PhD at the department of Zoonoses and Environmental Microbiology (Z&O) at RIVM and at the Quantitative Veterinary Epidemiology (QVE) group at Wageningen University. During her PhD, she studied wild rodents and rodent-borne zoonotic pathogens in changing urban environments. The results of her research can be found in this thesis.

Marieke received a MedVetNet grant to present her research at the Rodens et Spatium conference in Valladolid, Spain, in 2023, and a One Health EJP grant to perform a short-term mission to the laboratory of the Friedrich-Loeffler Institut in Germany in March and April 2022. The results of that short-term mission are incorporated in Chapter 6 and 7 of this thesis. During the annual One Health EJP scientific meetings, she won the prize for best 3 minute pitch two times, in 2021 and 2022. As her PhD has now come to an end, she would like to continue working in the field of zoonoses.



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Publications

Published

de Cock, M. P., de Vries, A., Fonville, M., Esser, H. J., Mehl, C., Ulrich, R. G., Joeres, M., Hoffmann, D., Eisenberg, T., Schmidt, K., Hulst, M., van der Poel, W. H. M., Sprong, H., & Maas, M. (2023). Increased rat-borne zoonotic disease hazard in greener urban areas. Science of the Total Environment, 896, 165069.

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de Cock, M., Fonville, M., de Vries, A., Bossers, A., van den Bogert, B., Hakze-van der Honing, R., Koets, A., Sprong, H., van der Poel, W., & Maas, M. (2022). Screen the unforeseen: Microbiome-profiling for detection of zoonotic pathogens in wild rats. *Transboundary and Emerging Diseases*, *69,* 3881–3895.

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Accepted

de Cock, M. P., Esser, H. J., van der Poel, W. H. M., Sprong, H., & Maas, M. Higher rat abundance in greener urban areas. *(Chapter 4 in this thesis)*

Submitted

de Cock, M. P., Baede, V. O., Burt, S. A., van Tiel, R. F. N. A., Wiskerke, K. K., van der Post, J., van der Poel, W. H. M., Sprong, H., & Maas, M. WILDbase: towards a common database to improve wildlife disease surveillance in Europe. *(Chapter 2 in this thesis)*

de Cock, M. P., Wijburg, S. R., Raaijmakers, E., van Belkom, J., Dijkhuis, L. R., La Haye, M., de Jager, M., Maas, M., Mol, R., van Norren, E., Sprong, H., Westra, S., & Jansen, P. A. Determinants of small mammal presence in private gardens in the Netherlands. *(Chapter 5 in this thesis)*

de Cock, M. P., Baede, V. O., Esser, H. J., Fonville, M., de Vries, A., de Boer, W. F., Mehl, C., Ulrich, R. G., Schares, G., Hakze-van der Honing, R. W., van der Poel, W. H. M., Sprong, H., & Maas, M. T(r)icky environments: higher prevalence of tick-borne zoonotic pathogens in rodents from natural areas compared to urban areas. *(Chapter 7 in this thesis)*

Training and Education Statement

The Basic Package (2 ECTS)	
WIAS Introduction Day	2020
WGS course Scientific Integrity	2023
WGS course Ethics in Animal Sciences	2022

Disciplinary Competences (18 ECTS)	
WIAS Research proposal writing	2020
WIAS/PE&RC course Design of experiments	2019
SENSE course Geostatistics	2019
Laboratory animal science course (Utrecht University)	2020
Microbiome data analysis workshop (Uhasselt, Belgium)	2021
PE&RC course Introduction to R	2021
SENSE course Ecological and Evolutionary genomics	2021
Laboratory research skills training (Friedrich-Loeffler Institut, Germany)	2022
EWDA workshop on Emerging infectious diseases in the age of One Health (Barcelona, Spain)	2023

Professional Competences (7 ECTS)	
WGS course Brain friendly working and writing	2020
WGS course Presenting with impact	2020
WGS course Supervising BSc & MSc thesis students	2021
WGS course Scientific writing	2021
WGS course Scientific publishing	2021
WGS course Effective behaviour in your professional surroundings	2022
WGS course Critical thinking and argumentation	2022
WIAS course The final touch	2023
Career assessment	2023

Societal Relevance (2 ECTS)	
WIAS course Societal impact of your research	2023

Presentation Skills (maximum of 4 ECTS)	
NAEM Netherlands annual ecology meeting, Lunteren (poster)	2022
BWDS/DSWH symposium 'Wildlife diseases going viral', Utrecht (oral)	2022
WIAS annual conference, Ede (oral)	2023
Rodens et Spatium conference, Valladolid, Spain (oral)	2023
Wildlife Disease Association annual conference, Athens, Georgia, USA (poster & oral)	2023

Teaching competences (maximum of 6 ECTS)	
Supervising MSc internship	2020-2023
Supervising MSc thesis	2020-2021

Total credits	**38**

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