Science of the Total Environment 896 (2023) 165069

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Increased rat-borne zoonotic disease hazard in greener urban areas

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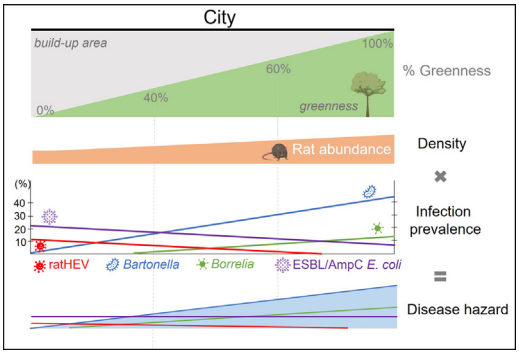
HIGHLIGHTS

- 13 zoonotic pathogens were detected, of which *Bartonella* spp. was the most prevalent.
- The prevalence of *Bartonella* spp. and *Borrelia* spp. increased with greenness.
- The prevalence of ESBL/AmpC-producing *E. coli* and ratHEV decreased with greenness.
- Overall, rat-borne zoonotic disease hazard increased in greener urban areas.
- Zoonotic infections should be taken into account when designing urban green spaces.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Shuqing Zhao

Keywords:
Disease ecology
Rodents
Richness
Co-infection
Epidemiology
Ectoparasites

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

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http://dx.doi.org/10.1016/j.scitotenv.2023.165069
Received 24 March 2023; Received in revised form 19 June 2023; Accepted 20 June 2023
Available online 29 June 2023

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.

1. 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 (Coutts and Hahn, 2015; Green et al., 2016; Lõhmus and Balbus, 2015). 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 (Livesley et al., 2016).

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 (Jones et al., 2013), 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 (Dobigny and Morand, 2022). 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 (Himsworth et al., 2013b). 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 (de Cock et al., under review; Traweger et al., 2006; van Adrichem et al., 2013), 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 (Ostfeld et al., 2006). For example, high rat abundance can increase density-dependent pathogen transmission, resulting in higher numbers of infected rats (Anderson and May, 1979). However, the strength of the relationship between rat density and pathogen prevalence may vary depending on the location or pathogen considered (Ayral et al., 2015b; Murray et al., 2020).

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.

2. Materials & methods

2.1. Ethics statement

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

2.2. 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 (Verner-Carlsson et al., 2015). 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.

2.3. 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 (Atama et al., 2022). 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).

2.4. 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

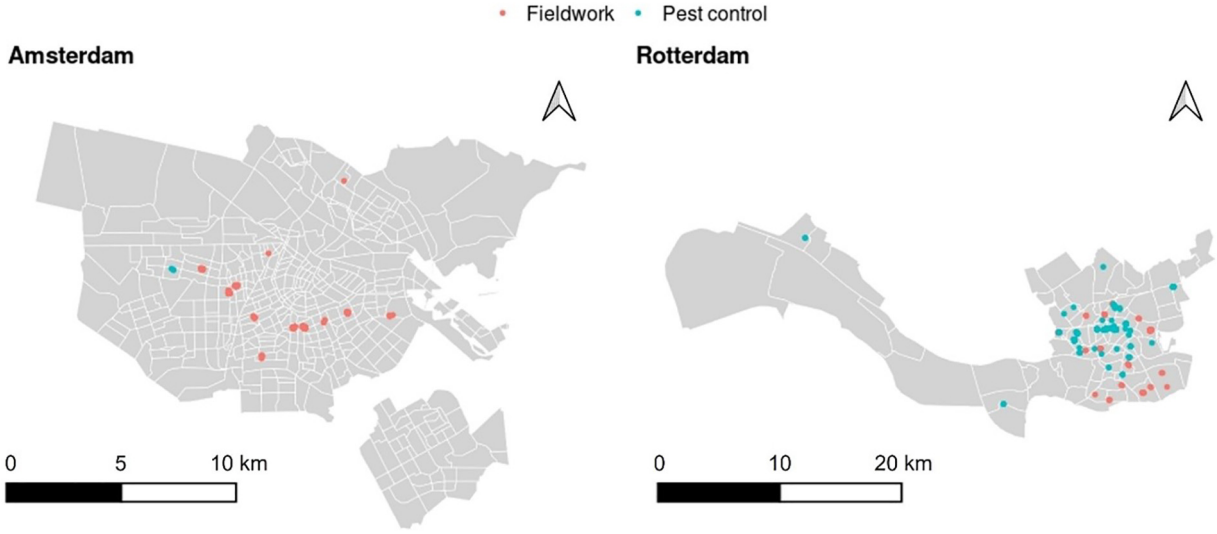

Fig. 1. Rat trapping locations in Amsterdam and Rotterdam, collected during fieldwork or by municipality pest controllers.

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 (Meijs et al., 2020).

2.5. 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 (Groenmonitor, 2022). 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 (Badi et al., 1992), in QGIS version 3.16 (GIS Development Team, 2022). 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 (pdok, 2022). A proxy for rat age was used based on the body length to mass ratio, body length (cm)/weight (g) ∗ 10 (Costa et al., 2015). 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.

2.6. Data analysis

All statistical analyses were conducted in R studio version 4.0.3 (RStudio team, 2015). All numerical variables were standardized using a z-transformation with two standard deviations (Gelman, 2008). We created separate binomial GLMMs per pathogen using the *glmmTMB* package (Brooks et al., 2017). 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 (Hartig and Hartig, 2017). Tukey HSD post-hoc tests were performed using the *Emmeans* package (Lenth et al., 2019). 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 χ^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 (de Cock, under review; Ostfeld et al., 2006).

3. Results

3.1. 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).

3.2. 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*) (Purdy et al., 2022) in 15 animals, but no antihepatitis 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).

3.3. 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 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 (τ = 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

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.

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**
$\sigma^2 = 3.29$	Age	13.41	5.48–32.81	**< 0.001**
Marginal $R^2 = 0.337$	Distance to water	1.55	0.69–3.45	0.285
Cond $R^2 = 0.424$	Sex	0.83	0.47–1.47	0.515
ICC = 0.13	Flea infestation	1.84	0.95–3.56	0.071
	Tick infestation	0.89	0.17–4.61	0.889
	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
$\sigma^2 = 3.29$	Age	4.03	1.90–8.53	**< 0.001**
Marginal $R^2 = 0.153$	Distance to water	0.78	0.32–1.90	0.583
Cond $R^2 = 0.384$	Sex	1.51	0.83–2.76	0.178
ICC = 0.27	Flea infestation	0.43	0.19–0.99	**0.047**
	Tick infestation	1.31	0.21–8.25	0.770
	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
ESBL/AmpC-producing *E. coli*	Greenness	0.23	0.07–0.78	**0.018**
$\sigma^2 = 3.29$	Age	1.21	0.55–2.67	0.633
Marginal $R^2 = 0.179$	Distance to water	0.51	0.16–1.65	0.260
Cond $R^2 = 0.352$	Sex	1.33	0.66–2.70	0.427
ICC = 0.21	Flea infestation	0.47	0.16–1.37	0.164
	Tick infestation	1.73	0.16–19.11	0.655
	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**
$\sigma^2 = 3.29$	Age	172.22	5.94–4991.16	**0.003**
Marginal $R^2 = 0.874$	Distance to water	3.61	0.07–177.36	0.518
Cond $R^2 = 0.933$	Sex	1.59	0.33–7.61	0.564
ICC = 0.47	Flea infestation	1.85	0.29–11.71	0.515
	Tick infestation	1.78	0.10–32.36	0.697
	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**
$\sigma^2 = 3.29$	Age	14.05	2.27–86.95	**0.004**
Marginal $R^2 = 0.849$	Distance to water	1.97	0.68–5.64	0.209
Cond $R^2 = NA$	Sex	1.29	0.42–4.01	0.659
ICC = NA	Flea infestation	0.78	0.21–2.99	0.723
	Tick infestation	0.00	0.00–Inf	1.000
	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
$\sigma^2 = 3.29$	Age	3.37	0.83–13.68	0.089
Marginal $R^2 = 0.887$	Distance to water	2.20	0.48–10.09	0.310
Cond $R^2 = NA$	Sex	2.61	0.84–8.07	0.096
ICC = NA	Flea infestation	2.39	0.69–8.31	0.172
	Tick infestation	0.00	0.00–Inf	0.998
	Season *spring/summer/winter*	1.25/0.18/0.00	0.35–4.45/0.03–1.0/0.00–Inf	0.731/0.064/0.996
Pathogen diversity	Greenness	1.26	0.91–1.72	0.159
$\sigma^2 = 3.30$	Age	2.98	2.18–4.09	**< 0.001**
Marginal $R^2 = 0.092$	Distance to water	1.03	0.74–1.42	0.882
Cond $R^2 = 0.100$	Sex	1.08	0.84–1.37	0.557
ICC = 0.01	Flea infestation	1.01	0.75–1.37	0.935
	Tick infestation	1.12	0.56–2.24	0.741
	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

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).

3.4. 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).

3.5. 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).

3.6. 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 the

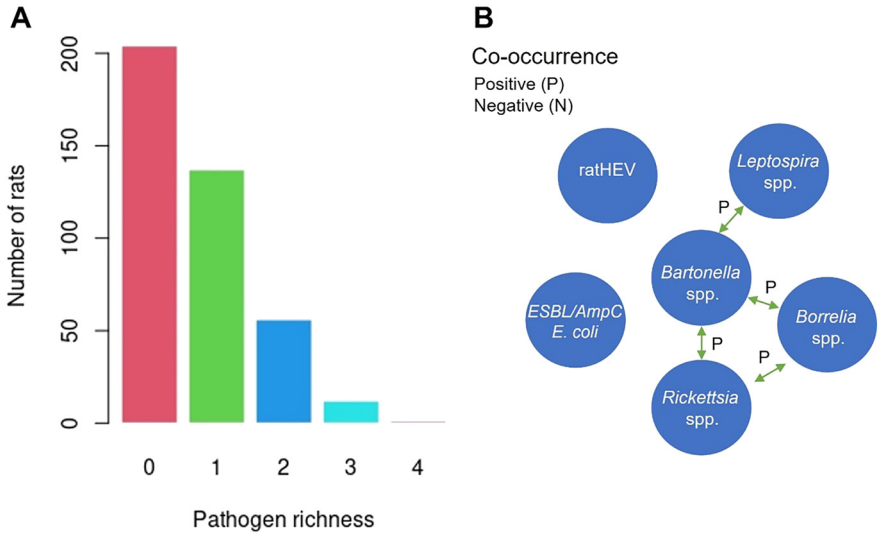

Fig. 2. A: Number of pathogens detected per rat. 2B: Coinfection patterns of the pathogens. Positive and negative associations in the coinfection patterns, which were significant in the 95 % CI (confidence interval) level are shown with green, respective red arrows.

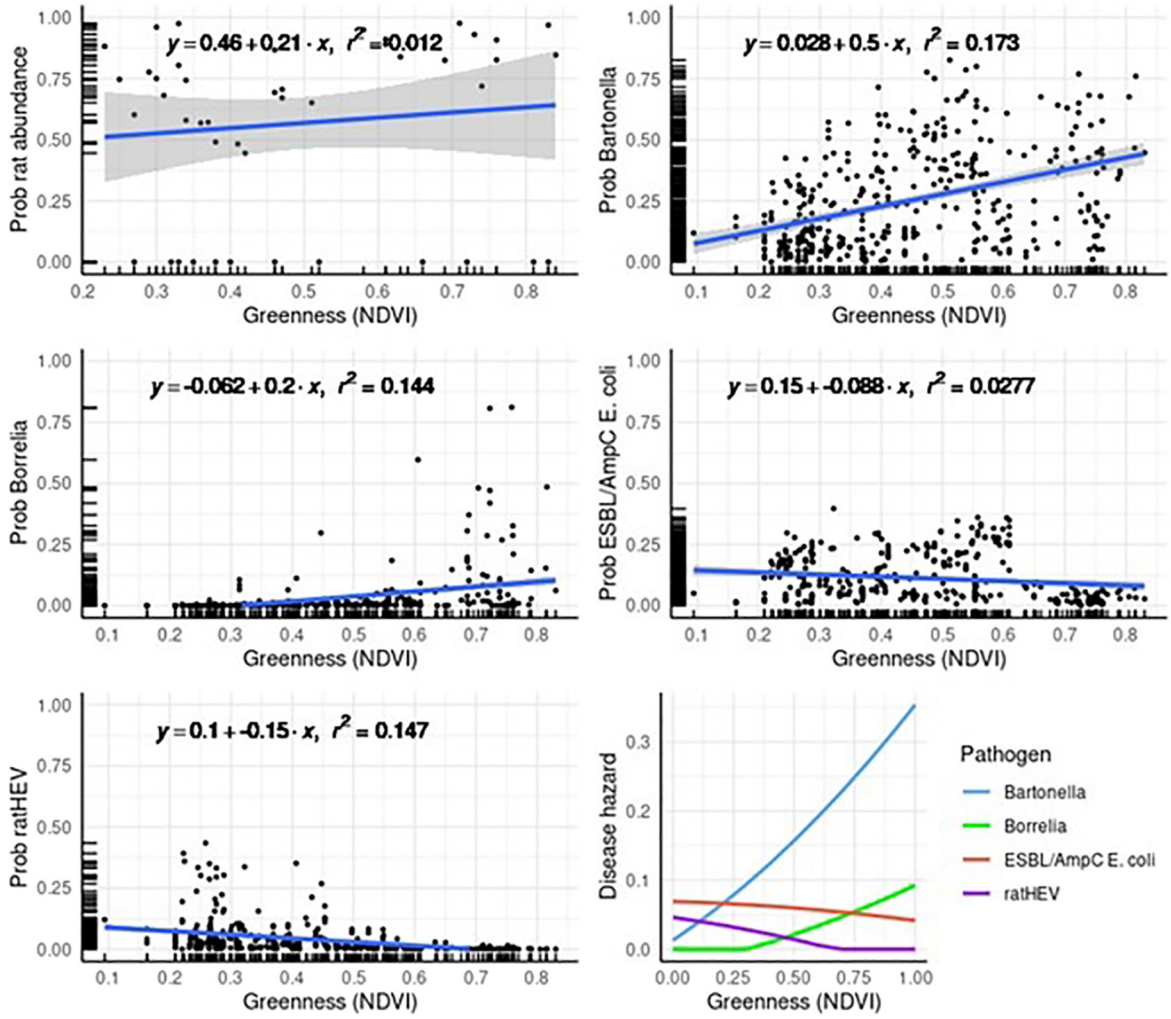

Fig. 3. Expected changes in rat-borne disease hazard with urban greenness, based on the probabilities (prob) of relative rat abundance and pathogen prevalence models (*Bartonella* spp., *Borrelia* spp., ESBL/AmpC-producing *E. coli* and ratHEV). Trendlines (blue) and equations are added in the plots.

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) (Meijs et al., 2020). 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), ST58 (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.

4. 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.

4.1. 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 (Murray et al., 2020), which may be more abundant in city centers, which are often less green areas. The transmission mode of ratHEV is still unknown (Reuter et al., 2020), 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 (Rothenburger et al., 2017). 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 (Rizzoli et al., 2014), 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 (de Cock, under review), 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 (Krasnov et al., 2001).

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 (Ayral et al., 2015b; Murray et al., 2020). 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.

4.2. 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 %) (Ayral et al., 2015a; Himsworth et al., 2013a; Richard et al., 2022; Strand et al., 2019), and within the range of previously observed prevalence in urban and non-urban areas in the Netherlands (3–57 %) (Krijger et al., 2020; Maas et al., 2018). While water bodies can act as an important source of *Leptospira* spp. infection (Mwachui et al., 2015), 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,

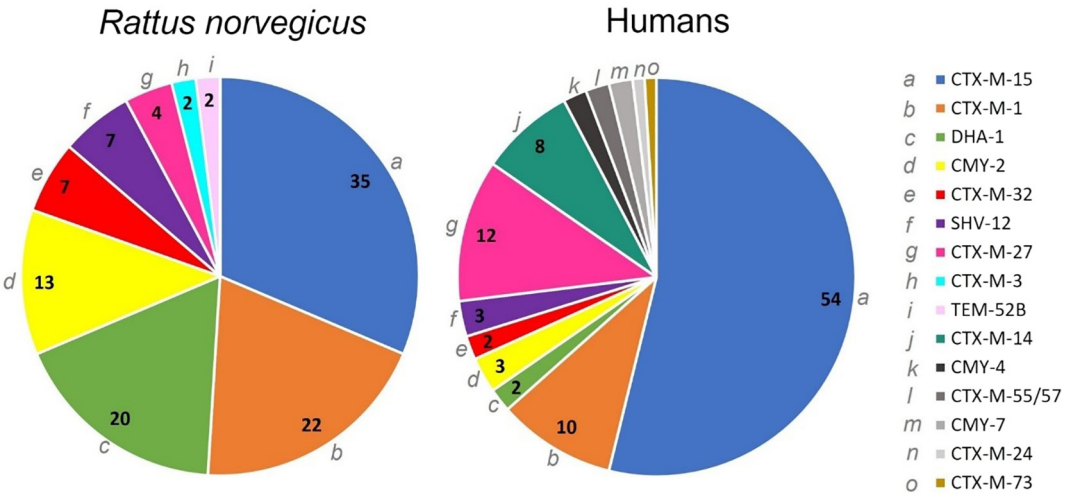

Fig. 4. Pie charts showing the percentage of ESBL and AmpC genes found in *Escherichia coli* from *Rattus norvegicus* (n = 45; left), and in *E. coli* and *Klebsiella pneumoniae* from the Dutch human population (n = 104; right) (Meijs et al., 2020). Data for humans were collected in 2015–2017. For the 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.

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 (De Oliveira et al., 2016; Ellis, 2015).

The observed *Bartonella* spp. prevalence (26 %) is comparable to a study in Belgium (37 %) (Krügel et al., 2020), in which the main *Bartonella* species was also found to be *Ba. tribocorum*. While *Bartonella* is considered predominantly a flea-transmitted pathogen (Billeter et al., 2008), 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 %) (Desvars-Larrive et al., 2017; Himsworth et al., 2020). 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 (Butler, 2012). Unexpectedly, we did find a negative relationship between *Leptospira* spp. and flea infestation. Whether this is related to the rat's swimming behavior (Tran et al., 2021), 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 (Antoniou et al., 2010; Desvars-Larrive et al., 2017; Frye et al., 2015; Hornok et al., 2015; Mihalca et al., 2012). As there are substantial numbers of ticks present in vegetation in urban areas (Heylen et al., 2019; Rizzoli et al., 2014), this might suggest that rats are not preferred tick hosts, which could be influenced by their swimming and grooming behavior (Lydecker et al., 2019). 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 %) (Desvars-Larrive et al., 2017; Hornok et al., 2015), *Rickettsia* spp. (0–1 %) (Desvars-Larrive et al., 2017; Heuser et al., 2017), *Neoehrlichia mikurensis* (< 1 %) (Obiegala et al., 2019), *Anaplasma* spp. (0–1 %) and *Babesia* spp. (0 %) (Desvars-Larrive et al., 2017; Obiegala et al., 2019).

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 (Kijlstra et al., 2008; Krijger et al., 2020). Moreover, a seroprevalence of 8–28 % was observed in France and Cyprus (Ayral et al., 2015b; Psaroulaki et al., 2010). 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 (Afonso et al., 2006; Gilot-Fromont et al., 2012), 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 %) (Ayral et al., 2015b; Johne et al., 2012; Murphy et al., 2019b; Obiegala et al., 2019; Ryll et al., 2017; Widén et al., 2014). 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 (Fawzy et al., 2022; Firth et al., 2014; Julius et al., 2021; Kimura et al., 2008). 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 (Fawzy et al., 2022). 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.

4.3. 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 % (Reusken et al., 2011; Runge et al., 2013). 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 %) (Desvars-Larrive et al., 2017; Heuser et al., 2017; Martina et al., 2006; Schmiedeknecht et al., 2010). While SEOV has been detected in wild rats from the Netherlands, France and the United Kingdom with a prevalence of 0–19 % (Ayral et al., 2015b; Desvars-Larrive et al., 2017; Maas et al., 2018; Murphy et al., 2019a; Verner-Carlsson et al., 2015), the absence of SEOV-positive wild rats in this study is supported by other studies (Heuser et al., 2023; Maas et al., 2018). Despite the concurrent COVID-19 pandemic, no SARS-CoV-2 was detected in our study nor in wild rats from two other European countries (Colombo et al., 2022; Wernike et al., 2022). This while infections with specific SARS-CoV-2 variants (Alpha and Beta) have been observed in rats in laboratory settings (Shuai et al., 2021; Zhang et al., 2022), and recently in wild rats from New York (Delta and Omicron variant) (Wang et al., 2022). 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).

4.4. 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. (de Cock et al., 2022; Heuser et al., 2017; Himsworth et al., 2013a; Krojgaard et al., 2009; Peterson et al., 2017; Ryll et al., 2017), and in other rodent species for *Rickettsia* spp., and *Bo. garinii* (Fischer et al., 2018; Taylor et al., 2013), 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.

4.5. 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. (Telfer et al., 2010). Negative interaction is thought to be caused by competition (e.g. for specific host resources) and positive interaction by increased host susceptibility (Eidelman et al., 2019). 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.

4.6. 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 (Radhouani et al., 2012; Strand and Lundkvist, 2019). In this study, the prevalence of *Salmonella* (*Sa.*) enterica serovar Typhimurium was 1 %, which is comparable with that found in Germany (4 %) (Runge et al., 2013), but not from Thailand (30 %) (Ribas et al., 2016). 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 (Jajere, 2019). Serovar Typhimurium is also one of the two main serovars found in the Dutch human population (Vlaanderen et al., 2021). 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 (Desvars-Larrive et al., 2019; Ge et al., 2019; Himsworth et al., 2014; Lee et al., 2019; Silva et al., 2021), and to the prevalence observed in humans from other European countries (Lozano et al., 2020). 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 (Silva et al., 2021). We also detected both genes. The *mecC* gene is considered to have a broad host range, including livestock, companion and wildlife animals (Becker et al., 2014) such as black and brown rats, rabbits, hares and hedgehogs (Bengtsson et al., 2017; Loncaric et al., 2013; Paterson et al., 2012). 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 (Bengtsson et al., 2017), 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 %) (Desvars-Larrive et al., 2019; Guenther et al., 2012; Guenther et al., 2013), and which is slightly higher compared to the prevalence of 7 % observed in the Dutch human population (Meijs et al., 2020). 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) (Palmeira et al., 2021), in cats and dogs (Ewers et al., 2012), 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 (Palmeira et al., 2021).

4.7. 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 (Vlaanderen et al., 2021).

5. 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.

Funding sources

This work was supported by funding from both the Dutch Ministry of Health, Welfare and Sport, DZIF Thematic Translational Unit (TTU) 'Emerging Infections' (RGU), and the European Union's Horizon 2020 Research and Innovation programme for the DESIRE project under grant agreement No 773830: One Health European Joint Programme (MPdC).

CRediT authorship contribution statement

Conceptualization and methodology: MPdC, MM, HS, WHMvdP and RGU. Investigation, resources and validation: MPdC, MM, AdV, MF, CM, MJ, DH, TE, KS, MH, HS and WHMvdP. Software, formal analyses and data curation: MPdC and HE. Writing original draft and visualization: MPdC. Review and editing: all authors. Supervision and project administration: MM, HS, WHMvdP and RGU. Funding acquisition: MPdC and RGU.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Peter Muijlwijk (municipality of Rotterdam) and his team for collecting additional rats for our study. We would also like to thank Suzanne Noorlander, Jens van der Post, Sara Wijburg, Clara Köhler, Marieta Braks and Tryntsje Cuperus for their help with the dissections. Furthermore, we would like to thank Paul Hengeveld for his great help and guidance with culturing the antimicrobial resistant bacteria, Renate Hakze-van der Honing and Arie Kant for their help with the SARS-CoV-2 analyses, Angela van Hoek for her help with the analyses of the ESBL/AmpC-producing *E. coli* isolates, Gereon Schares for his help with the *T. gondii* analyses, and Nnomzie Atama for extracting the brain tNA. Lastly, we would like to thank Sara Wijburg for her help with R coding the pathogen distribution maps. Data can be accessed via the Dryad repository.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.165069.

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