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The effect of urbanization on house sparrows (*Passer domesticus*) and their enteropathogens

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TABLE OF CONTENTS

Table of Contents 1
List of Abbreviations 3
Introduction 5
1. Defining Urbanization 7
2. The urban climate and stressors 9
3. Urbanization and Biotic Homogenization 9
4. Urbanization and house sparrows 10
4.1. The house sparrow, a perfect urban exploiter 10
4.2. The decline 12
5. The impact of urbanization on avian health 15
6. The impact of urbanization on pathogens 16
6.1. *Salmonella* Typhimurium 17
6.2. *Yersinia* spp. 18
6.3. The role of other hosts 21
References 23
Scientific Aims 35
Chapter 1: Overarching methodology 39
Overarching methodology: spatial scale 41
References 46
Chapter 2: House sparrows do not constitute a significant *Salmonella* Typhimurium reservoir across urban gradients in Flanders, Belgium 47
Abstract 49
Introduction 50
Materials and methods 51
Results 55
Discussion 56
Conclusion 58
Acknowledgments 58
References 59
Chapter 3: Effects of urbanization on host- pathogen interactions, using *Yersinia* in house sparrows as a model 63
Abstract 65
Introduction 66
Materials and methods 67
Results. 72

Discussion 75
Acknowledgements 78
Supporting Information 78
References 80
Chapter 4: Low prevalence of human enteropathogenic *Yersinia* spp. in brown rats (*Rattus norvegicus*) in Flanders 87
Abstract 89
Introduction 90
Materials and methods 90
Results and Discussion 92
Acknowledgements 96
Supporting Information 96
References 102
General Discussion 105
1. Human disturbances 107
2. Urbanization: how to define the spatial scale? 108
3. Pathogen pressure and disease in relation to anthropogenic altered environments 110
4. Is there an interaction between host, environment and pathogen? 112
4.1. The effect of environment on morphological changes 113
4.2. Is there a correlation between pathogens and morphology? 115
4.3. Pathogen pressure in relation to environment 116
5. Main Conclusions 117
6. Future perspectives 118
References 120
Summary 127
Samenvatting 133
Curriculum Vitae 139
Bibliography 143
Scientific publications 145
Professional presentations 146
Oral presentations 146
Poster presentations 147
Dankwoord 149

LIST OF ABBREVIATIONS

AIC	Akaike's Information Criterium
Ail-gene	Attachment Invasion Locus-gene
ANOVA	Analysis of Variance
BGA	Brilliant Green Agar
BT	Biotype
BU-area	Built-Up area
CIN	Cefsulodin Irgasan Novobiocin
COLS	Columbia agar with sheep blood
CORINE	Coordination of Information on the Environment
DT	Phage Type
ELISA	Enzyme-Linked Immuno Sorbent Assay
GIS	Geographic Information Systems
GL(M)M	Generalized Linear (Mixed) Model
HCCA	α-cyano-4-hydroxycinnamic acid
Hm	*Haemorhous mexicanus*
Hp	*Haemorhous purpureus*
HRP	Horseradish peroxidase
Inv-gene	Invasin protein-gene
IUCN	International Union for the Conservation of Nature and Natural Resources
LDA	Linear Discriminant Analysis
LMM	Linear Mixed Model
LRD	Large-scale Reference Database
MALDI-TOF	Matrix Assisted Laser Desorption Ionization-Time Of Flight
Mm	*Melospiza melodia*
OD	Optic Density
OLS	Ordinary Least Squares
PCR	Polymerase Chain Reaction
Pd	*Passer domesticus*
PMB	Phosphate Buffered Saline supplemented with 0.5% Peptone, 1% Mannitol and 0.15% Bile Salts
pYV	Plasmid for *Yersinia* Virulence
SE	Standard Error
SMA	Standardized Major Axis
SMI	Scaled Mass Index
St	*Spinus tristis*
Sv	*Sturnus vulgaris*
Tm	*Turdus migratorius*
TMB	Tetramethylbenzidine
XLD	Xylose Lysine Deoxycholaat
YstA-gene	*Yersinia* heat stable enterotoxin YstA-gene
YstB-gene	*Yersinia* heat stable enterotoxin YstB-gene
VirF-/ LcrF-gene	Virulence Factor- /Low calcium response F-gene

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INTRODUCTION

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1. Defining Urbanization

With 7,4 billion world citizens, of which 54% are settled down in urban environments, and with a projected increase of up to 66% residing in urban areas by 2050 (UN, 2015a; UN, 2015b), cities are expanding rapidly and urbanization represents one of the most intense and long-lasting anthropogenic modifications of natural systems (Blair, 1996; McKinney M.L., 2002; Evans et al., 2011; Seress and Liker, 2015).

"Urbanization" however can be viewed in various ways. From a socioeconomic perspective, it can give an indication of the amount of people residing in urban areas, whereas the concept "urban" is based on available information for each country (population density, minimum population threshold, presence of infrastructure, education and health services) (UN, 2015a). Belgium, for example, belongs to one of the most urbanized countries in the world with approximately 97.8% of the civilians living in urban areas, and considers a commune to be "urban" when it accommodates minimally 5000 inhabitants (UN, 2015a). On the contrary, the ecological perspective primarily focuses on the "environmental urbanization", where the emphasis lies on the urban expansion in space and the subsequent effects on animals and plants. This urban expansion is characterized by the conversion of (semi)natural (e.g. forests, pastures, agricultural areas) into urban areas (i.e. a high amount of built-up area and roads and a decrease in vegetation-density), which are specifically created to satisfy the human needs (Blair, 1996; Marzluff et al., 2001; McKinney, 2006; Seress et al., 2014). However, besides the expansion of the urban environment in space, the ecologists are also interested in the colonization and adaptation of animal and plant species to urban environments, which can also be seen as a form of 'urbanization' (Evans et al., 2009a; Møller et al., 2014). As such, the response of animal and plant communities to various degrees of urbanization (i.e. along an urbanization gradient) has been extensively studied in conservational and ecological research (Bókony et al., 2012; Seress et al., 2012; Seress et al., 2014). Also in this thesis, this approach was used while studying the relationship between urbanization, avian health and pathogen occurrence.

When using urban gradients for ecological studies, the parameters applied for the quantification of landscape urbanization should be mentioned in order to facilitate the comparison of different studies, different cities and different countries (Marzluff et al., 2001; Bókony et al., 2012; Seress et al., 2014). These characteristic habitat parameters can be obtained by means of manual scoring or semi-automated scoring using satellite images, and by the use of geographic information systems (GIS) (Seress et al., 2014). Nevertheless, despite the use of definitions it is logistically not always possible to compare the effect of urbanization on

biodiversity and ecosystem health between different countries, within a country, and even within the same city due to differences in urban design (with respect to habitat fragmentation and patch size of green areas), in human activities, in the socioeconomic status of the inhabitants, in the surrounding region of the cities, latitudinal differences and presence or absence of nonnative (invasive) species (Shaw et al., 2008; Evans et al., 2009c; Ferenc et al., 2013; Snep et al., 2016).

With a population density of 475 inhabitants per km² in Flanders, which is on average four times higher than the density in the European Union (ENRD, 2015), Flanders is highly subjected to urban sprawl (Fig. 1) (EEA, 2006). Urban sprawl can be viewed in various ways, but has most commonly been defined as '*the physical pattern of low-density expansion of large urban areas,..., mainly into the surrounding agricultural areas. Sprawl is the leading edge of urban growth and implies little planning control of land subdivision. Development is patchy, scattered and strung out, with a tendency for discontinuity…'* (cited from EEA, 2006). In Flanders, urban sprawl intensified by the end of the 19th century and was highly influenced by economic growth, political decisions, expansion of the public transport and the common cultural idea "to possess a nonurban single-family house with a garden" (De Meulder et al., 1999; De Decker, 2011; Tempels et al., 2012). This patchwork of buildings and connecting roads contributes highly to fragmentation of landscapes and suburbanization in between the cities, the so-called suburban areas of intermediate urbanization, which makes it difficult to draw a clear-cut line between urban and rural areas (Kesteloot, 2003; De Decker, 2011; Tempels et al., 2012; De Coster et al., 2015).

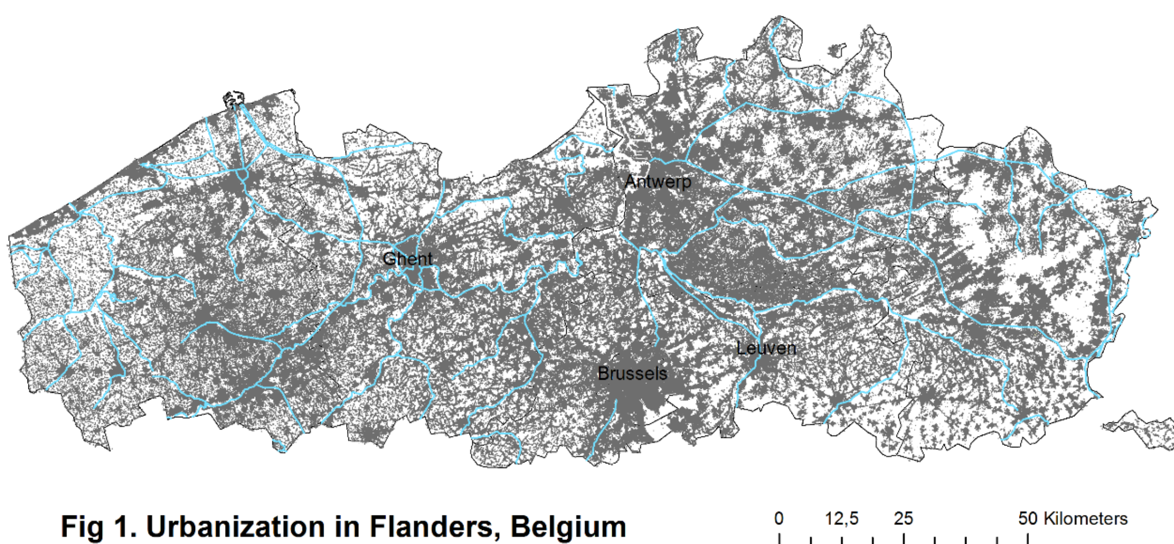

Fig 1. Urbanization in Flanders, Belgium

Grey background represents the level of build-up area in Flanders, based on the Large Scale Reference Database (LRD)

2. The urban climate and stressors

As a consequence of changes in landscape features, such as an increase in building density, roads and pavements and a decrease in greenery in urbanized areas, a specific "urban microclimate" is created within cities (Landsberg, 1981; Kuttler, 2008; Trusilova et al., 2008). The "urban heat island effect", an increase in urban environmental temperature, is formed through the combined action of several factors: the higher daytime storage of heat in e.g. buildings and the subsequent increased nocturnal heat release, the lower wind velocity, the increased pollution, and the lower evaporation efficacy due to presence of impermeable surfaces with increased water runoff and due to the removal of vegetation (Gartland, 2008; Kuttler, 2008; Trusilova et al., 2008). This urban climate could be favorable since the energetic demands of especially smaller birds decrease during winter and nighttime (Zuckerberg et al., 2011; Murthy et al., 2016). Nevertheless, besides the "heat island effect" stressors such as chemical, light and noise pollution can impact animal health, physiology and behavior (Peach et al., 2008; Bichet et al., 2013; Meillère et al., 2015a; Ouyang et al., 2017). In order to assess the impact of urbanization on animal and human wellbeing, using highly sedentary birds as a study object has many advantages: birds are relatively easy to observe (e.g. thanks to the many bird watchers, citizen science projects can be used more widely: De Coster et al., 2015) and capture (Pollack et al., 2017) and some of these sedentary species such as the house sparrow (*Passer domesticus*) are ubiquitous and have adapted to anthropogenic resources, i.e. they can be used to study interpopulation differences to compare the effect of urbanization worldwide. As such birds have been used as a biomonitoring tool (Pollack et al., 2017) for the presence of chemical contaminants and heavy metals (Roux and Marra, 2007; Bichet et al., 2013), the effect of light and noise pollution (Slabbekoorn, 2013; Ouyang et al., 2017), the risk and presence of infectious diseases including zoonoses (Niskanen et al., 2003; Giraudeau et al., 2014; Neiderud, 2015).

3. Urbanization and Biotic Homogenization

Urbanization highly influences species assemblages, communities and ecosystems (McKinney, 2002). In this perspective avian species have been classified as: 1) "urban avoiders", which are not able to tolerate the constraints of the urban environment and reach highest densities in the most natural areas, 2) "urban adapters", when they are able to adapt to urban constraints and maintain populations in urban environments and 3) "urban exploiters", which are more or less dependent on anthropogenic resources and reach highest densities in

urbanized areas (Blair, 1996; McKinney, 2002; Croci et al., 2008). Consequently, many studies have focused on identifying species specific traits that allow species to tolerate, or even flourish, in urban environments (Blair, 1996; Kühn and Klotz, 2006; Croci et al., 2008; Sol et al., 2014). For example, life history traits linked to habitat, diet, reproductive behavior, sociability and migratory status have been shown to influence urban bird communities (Croci et al., 2008; Sol et al., 2014). Such a filtering of species, based on their biological traits, reduces native biodiversity and ultimately results in a 'biotic homogenization' of urban communities (McKinney, 2002; McKinney, 2008; Evans et al., 2009c; Ferenc et al., 2013), where globally, urbanized areas are dominated by a set of successful urban exploiters, such as for example the house sparrow (Blair, 1996; McKinney, 2002; Evans et al., 2009c; Meillère et al., 2015b). Recent studies have paid more attention to the intermediate levels of urbanization, the suburban areas, which, due to the combination of additional resources supplied by humans and the proximity and connectivity of various vegetation types (Marzluff and Rodewald, 2008; Vangestel et al., 2010), could sustain an increased species richness (Blair, 1996; Chace and Walsh, 2006; Marzluff and Rodewald, 2008; McKinney, 2008; Seress and Liker, 2015), which mostly belong to the "suburban adapters". In addition to higher species richness, these suburban areas have the possibility to even sustain higher bird densities (Blair, 1996; Marzluff and Rodewald, 2008), compared to both rural and highly urbanized areas.

4. Urbanization and house sparrows

4.1. The house sparrow, a perfect urban exploiter

House sparrows belong to the order of the Passeriformes, family Passeridae (BirdLife International, 2016a). With an estimated >540.000.000 individuals globally, and an extremely wide distribution range (Fig. 2), house sparrows, although decreasing in numbers, are considered as "Least Concern" on the global Red List of the International Union for the Conservation of Nature and Natural Resources (IUCN) (BirdLife International, 2016a). The IUCN Red List provides an overview of the conservation status and estimated risk of extinction for a specific animal, plant and fungal species, which is based on data on population dynamics and size and the geographic distribution of the species. As such, when evaluated and not "data deficient", species can be listed as "Least Concern", "Near Threatened", "Vulnerable", "Endangered", "Critically Endangered", "Regionally Extinct", "Extinct in the Wild" and "Extinct" (IUCN, 2001; Devos et al., 2016).

House sparrows reside in flocks along the entire urbanization gradient, from rural over suburban to urban areas, where the close proximity to humans is striking (Summers-Smith, 1963; Heij and Moeliker, 1990). They have shown to exhibit an extremely sedentary lifestyle, mostly staying within two km of the breeding colony (Summers-Smith, 1963; Vangestel et al., 2010). Mainly due to their granivorous lifestyle, apart from the insectivorous nestling period, house sparrows were notorious for destroying crops in agricultural areas, sometimes flocking together in fields in populations of over 1000 birds (Summers-Smith, 1963; Heij and Moeiliker, 1990). To control their numbers, eradication based on pesticides and mechanical manners (traps) was used to chase the sparrows away or kill them (Heij, 1985), after which they were sometimes even served as a delicatesse for humans (Fig. 3) (Raffald, 1769).

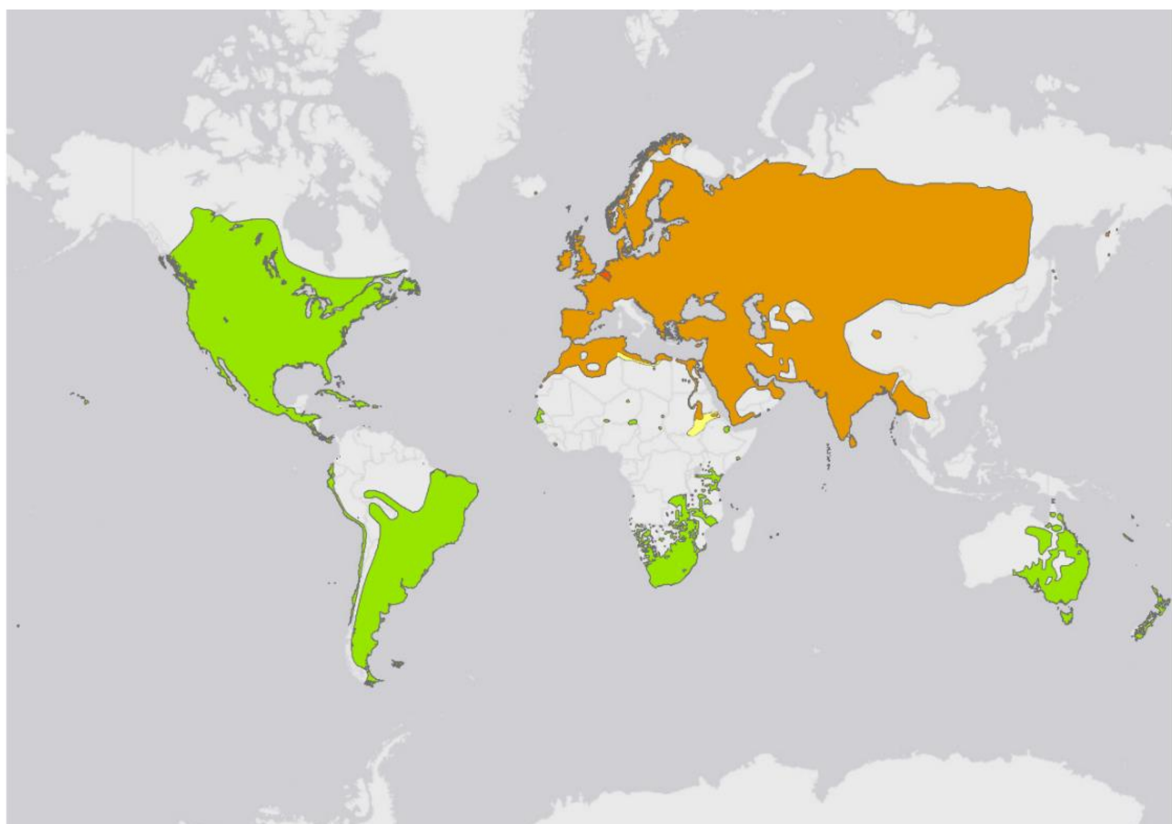

Fig 2. House sparrow distribution

Extant (resident)
Belgium (Extant: Resident)
Extant and Vagrant (seasonality uncertain)
Introduced and Extant (resident)

BirdLife International. 2017. *Passer domesticus*.
The IUCN Red List of Threatened Species 2017:
e.T103818789A111172035. Accessed on 31st of May 2017
Esri, HERE, DeLorme, MapmyIndia. © OpenStreetMap
contributors, and the GIS user community

To make a SPARROW DUMPLING.
MIX half a pint of good milk, with three eggs, a little ſalt, and as much flour as will make it a thick batter, put a lump of butter rolled in pepper and ſalt in every ſparrow, mix them in the batter, and tie them in a cloth, boil them one hour and a half, pour melted butter over them and ſerve them up.

Fig. 3 House sparrow-recipe (Elizabeth Raffald, 1769)

4.2. The decline

Although the future looked bright for the house sparrow in the first half of the 20th century –

"What of the future? We are steadily creating more built-up areas, the prime habitat for the house sparrow. Unless man exterminates himself in a nuclear war, the future looks bright for the bird" (Quote: Summers-Smith, 1963)

– this has changed rapidly during the forthcoming decennia (Summers-Smith, 2003; Robinson et al., 2005; De Laet and Summers-Smith, 2007; De Coster et al., 2015) and the bird has been listed as "Vulnerable" on the Red List from various European countries, such as the regional Red List of Flanders (Belgium) and on the national Red List of the Netherlands (Klok et al., 2006; Devos et al., 2016).

In northwestern European countries, as well as in southeast Asian Countries, house sparrow declines have been observed in both rural and urban areas (Crick et al., 2002; Summers-Smith, 2003; Robinson et al., 2005; De Laet and Summers-Smith, 2007; Kamath et al., 2014; De Coster et al., 2015; Modak, 2015). In contrast to the rural declines, which have stabilized at a lower population level, the urban declines have led to house sparrow free urban centers in some cities and are thought to be still in progress (Summers-Smith, 2003; De Laet and Summers-Smith, 2007; De Coster et al., 2015). This, in combination with the observation that the onset of the rural declines preceded the urban declines and the outcome of the declines differed between the two habitats (Crick et al., 2002; De Laet and Summers-Smith, 2007), have led to the hypothesis that, most likely, a combination of different underlying factors are responsible for the observed declines (Crick et al., 2002; Summers-Smith, 2003; Robinson et al., 2005).

4.2.1. Factors potentially contributing to the house sparrow declines

The rural declines have been linked to and co-occurred with the agricultural intensification, which involved the more intensive use of herbicides and insecticides, replacement of horses by the combustion engine, higher harvesting efficiency, better grain storage, the reduction of habitat diversity and deterioration of the habitat quality, which altogether affected many farmland birds (Crick et al., 2002; Hole et al., 2002; Robinson and Sutherland, 2002; Summers-Smith; 2003; Robinson et al., 2005; De Laet and Summers-Smith, 2007).

The reasons for the observed urban declines are much less clear and differences between cities complicate the situation even more. In London, Glasgow, Edinburg (Dott and Brown, 2000), Kortrijk (de Bethune, 2004), Ghent, Antwerp and Brussels (De Laet and Summers-Smith, 2007) declines, up to the brink of extinction, have occurred. This in contrast to populations in Paris and East Berlin, where no such declines have been observed (Summers-Smith, 2003; Robinson et al., 2005) and Wales, where populations even seem to have increased in urban and suburban areas (Crick et al., 2002).

Different factors have been suggested which could influence the urban house sparrow populations:

4.2.1.1. Predation

The predators most likely influencing house sparrows in multiple ways are the domestic cat *(Felis catus)* and the sparrowhawk *(Accipiter nisus)* (Barnard, 1980; Summers-Smith, 2003; Woods et al., 2003; MacLeod et al., 2006; Bell et al., 2010; Seress et al., 2011). The effect of the killing itself could potentially have an influence on the population numbers (Woods et al., 2003). Considering the domestic cat, different studies have tried to estimate the numbers of birds killed over a defined time period (Woods et al., 2003; Loss et al., 2013), which approximated 2.5 billion birds over the period of one year in the United Stated and 27.1 million birds over a 5 month timespan in Great Britain (Woods et al., 2003; Loss et al., 2013) and as such, cats are most likely participating in declines of specific bird species (Loss et al., 2013). With respect to the sparrowhawks, after a sharp decline in Europe in the 1950-1960s due to the widespread use of organochlorine pesticides, the predator bird has made a comeback (Bell et al., 2010; BirdLife International, 2016b). The timing of the recolonization events of rural and later urban areas preceded, and have been suggested to be correlated to, the observed house sparrow declines in the respective areas (Bell et al., 2010). Besides the effect of the actual kill, the presence or absence of a predator could potentially influence the house sparrows' behavior

and morphology (adaptive mass regulation related to predation risk) (Barnard CJ, 1980; MacLeod et al., 2006; Seress et al., 2011; Bókony et al., 2012). House sparrows living in urban areas with high densities of sparrowhawks, with higher levels of interfering background noise or with a lower availability of cover habitat were found to show higher antipredator response in comparison with their rural counterparts (Vangestel et al., 2010; Seress et al., 2011; Meillère et al., 2015a). This behavior could potentially constrain them from early morning feeding in the winter, rendering them more susceptible to variations in food predictability and thus starvation (MacLeod et al., 2006).

4.2.1.2. Habitat alterations

Although house sparrows have since long been associated with the presence of humans and are considered urban exploiters, recent urban developments have suggested to negatively impact populations, possibly aggravating or even causing the observed declines (Summers-Smith, 2003; Robinson et al., 2005; Chamberlain et al., 2007; Snep et al., 2016). In order to support viable house sparrow populations, foraging, nesting and hiding habitats need to be maintained and well connected (Snep et al., 2016) which is often problematic in urbanized areas (Robinson et al., 2005; Shaw et al., 2008; Snep et al., 2016). House sparrows preferentially nest in roof cavities or under tiles, however, these nesting locations are often sealed or removed while renovating the buildings (Wotton et al., 2002; Shaw et al., 2008). Supplementary food sources in suburban and urban areas (Shochat, 2004; Reynolds et al., 2017), could aid birds in harsh periods, although the mostly lower food quality of these supplements could adversely affect the body condition and lower the overall fitness of the birds (Shochat, 2004). In addition, the reduced availability of high qualitative invertebrate prey (such as insects, which have declined massively the past decades (Vogel, 2017)), is detrimental for the reproductive success of house sparrows (Peach et al., 2008; Seress et al., 2012). Peach et al. (2008) and Seress et al. (2012) compared the reproductive success of house sparrows along an urbanization gradient and clearly demonstrated a reduced number of fledglings (due to increased nestling mortality from starvation) and a reduced body condition of fledglings in the more urbanized areas (which is a proxy for post-fledging survival). They attributed this reproductive difference to the lower quality and lower abundance of certain insect prey (e.g. caterpillars, beetles). In addition, providing supplementary mealworms to house sparrows in more urbanized areas increased the reproductive output, demonstrated by an increased clutch size and fledging success (Peach et al., 2014). The presence and high abundance of insects (aphids, caterpillars, beetles,…) depends on the presence of native plants and the absence of insecticides or herbicides (Robinson et al.,

2005; Chamberlain et al., 2007; Peach et al., 2008; Seress et al., 2012), which can even vary within cities. Socioeconomically deprived areas mostly consist of a higher amount of native bushes and green spaces, providing cover and invertebrates, in contrast to gardens in affluent areas, with higher proportion of paved areas, nonnative plants and presence of pesticides and insecticides (Robinson et al., 2005; Chamberlain et al., 2007; Shaw et al., 2008). Traffic and related air pollution, which includes the use of unleaded petrol and heavy metals, have been shown to change hematologic values, the oxidative stress, reproductive success and prevalence of infectious disease in house sparrows (Roux and Marra, 2007; Peach et al., 2008; Bichet et al., 2013).

5. The impact of urbanization on avian health

Various parameters have been assessed in order to enhance our understanding regarding avian health and stress in urbanized regions. However, we need to keep in mind that most parameters are influenced by variables related to diet, reproductive status, season, diurnal variation, hydration and nutritional status, infectious and noninfectious diseases, which complicates their interpretation (Romero and Romero, 2002; Breuner et al., 2013; Salmón et al., 2016):

- Morphological parameters (as a proxy for the stress the animal has been subjected to in the past, although it can be related to specific biotic and abiotic characteristics):
  Stress has been shown to affect the animals' condition and appearance (Breuner et al., 2013; Maute et al., 2013), resulting in a decreased body mass and body condition due to the increased gluconeogenesis and lipolysis when exposed to chronic stress (Breuner et al., 2013). In this perspective, studies along urbanization gradients have attempted to assess the amount of stress birds are subjected to, based on morphological criteria such as the combination of body size (e.g. tarsal length, wing length, beak height, body weight) and body condition index (Liker et al., 2008; Vangestel and Lens, 2011; Bókony et al., 2012; Salleh Hudin et al., 2016; Meillère et al., 2017). The latter can be calculated in various ways (Peig and Green, 2009) and similarly to the weight, can be prone to behavioral, diurnal, seasonal and reproductive variation (Maute et al., 2013; Milenkaya et al., 2013; Salleh Hudin et al., 2016). In addition, ptilochronology as a measure for nutritional stress, and fluctuating asymmetry as a measure for developmental stability, which can be related to genetic or environmental stress, have received great attention (Vangestel et al., 2011; De Coster et al., 2013; Salleh Hudin et al., 2016; Meillère et al., 2017), however, also the interpretation of the

latter parameters can be difficult to interpret (Vangestel and Lens, 2011; De Coster et al., 2013).

- Physiological and genetic parameters (as a proxy for acute or chronic stress, although confounding factors should also be accounted for):
  Plasma, feather and faecal corticosterone, has been used in order to estimate the amount of stress individuals and populations are subjected to (Touma and Palme, 2005; Bortolotti et al., 2008; Chávez-Zichinelli, 2010; Meillère et al., 2015b). However we did not consider corticosterone in our research, since plasma corticosterone is subjected to great natural variation such as seasonal and circadian cycles, variation in life history stage (Lattin and Romero, 2015; Schwabl et al., 2017), interspecific variation (Sheriff et al., 2011), presence of infections (Dhont and Dobson, 2017), the chronicity of the stress where the animal is subjected to (Rich and Romero, 2005), capture induced variation (Romero and Reed, 2005) and since fundamental research to identify how corticosterone is deposited in feathers and faeces is still missing (Sheriff et al., 2011; Harris et al., 2017; Fischer et al., 2017). Besides the direct marker of stress, downstream changes in hematology and immunology (hematocrit, heterophil/lymphocyte ratio, immunoglobulin concentration) (Verbrugghe et al., 2012; Breuner et al., 2013; Milenkaya et al., 2013), blood chemistry (e.g. glucose, free fatty acids), reproductive hormones (Sheriff et al., 2011; Breuner et al., 2013) and genetic changes (telomere length, which has been shown to decrease when subjected to stress such related to urban environments) (Salmón et al., 2016; Ouyang et al., 2017) can be assessed to give a more complete image of the stress the animal is subjected to. However, species variations must be accounted for and the lack of reference values for blood chemistry and complete blood cell count (Harr et al., 2002; Geffré et al., 2009) hampers the use of these parameters in wild living animals (Bounous et al., 2000; Davis et al., 2008).

Ideally a combination of different (e.g. morphological, genetic and physiological) parameters should be assessed in order to have a more complete view regarding the stress animals are subjected to (Breuner et al., 2013).

6. The impact of urbanization on pathogens

An underexplored aspect of urban ecology that could help to unravel underlying ecological mechanisms driving population dynamics, is how urbanization impacts disease ecology, including its potential to alter wildlife-pathogen interactions (Daszak et al., 2000; Keesing et al., 2006; Bradley and Altizer, 2007; Evans et al., 2009b; Delgado-V and French, 2012; Hamer

et al., 2012). Birds are increasingly being recognized as important vectors or potentially even reservoirs for various diseases (Artois et al., 2001), nevertheless only few studies have focused on differential pathogen exposure between urban and rural areas and the effect on avian body condition consequently (Bichet et al., 2013; Delgado-V and French, 2012; Galbraith et al., 2017).

For frequency as well as density dependent and for generalist as well as specialist pathogens, a dilution or amplification effect can be observed in urbanized regions (Keesing et al., 2006; Bradley and Altizer, 2007). The following examples show how disease dynamics can be affected by urbanization:

1) An altered biodiversity and an increased density of several urban exploiters in cities (Blair, 1996; McKinney, 2002; Croci et al., 2008), could change the intra- as well as interspecific interactions and could affect the suitability of the host community to sustain a pathogen, hereby altering the disease transmission and stress, related to these interactions, which could change the disease outcome (Keesing et al., 2006; Becker et al., 2015).
2) An altered environmental survival of pathogens, or their vectors (when the pathogens are vectorborne), due to microclimate (heat island effect and precipitation) or habitat differences in between cities and natural habitats (Tashiro et al., 1991; Keesing et al., 2006; Trusilova et al., 2008; Krawiec et al., 2015) could increase or decrease the pathogen occurrence, persistency and transmission in the habitat (Keesing et al., 2006).
3) An increased level of stress, an increased exposure to pollutants or lower quality of food, can all affect host's immune function, host susceptibility, disease progression and pathogen excretion (Verbrugghe et al., 2012; Becker et al., 2015; Pollack et al., 2017).

Avian pathogens such as *Salmonella enterica* subspecies *enterica* and enteropathogenic *Yersinia* species, mainly transmitted through faeco-oral transmission routes (Brittingham and Temple, 1988; Refsum et al., 2003; Krawiec et al., 2015), which are believed to have the potential to establish a reservoir in birds (Tizard 2004; Benskin et al., 2009; Lawson et al., 2014; Mather et al., 2016) are of particular interest.

6.1. *Salmonella* Typhimurium

Salmonella enterica subspecies *enterica* serotype Typhimurium, a gram negative bacterium which belongs to the family of the *Enterobacteriaceae,* can cause disease outbreaks in endothermic animals, such as Passeriformes and humans (Alley et al., 2002; Lawson et al., 2014). In Britain, *Salmonella* Typhimurium definite phage types (DT)40, DT56(v) and DT160,

accounted for the majority of passerine salmonellosis incidents, most often recognized in greenfinches (*Chloris chloris*) and house sparrows (Pennycott et al., 2006; Lawson et al., 2010, Lawson et al., 2014). Despite the observation of some phage types being host adapted, DT2 and DT99 in pigeons (*Columba livia*) (Pasmans et al., 2003), DT40 and DT56(v) in passerines (Lawson et al., 2011), the latter two phage types have been isolated from captive birds and mammals and have been linked to disease in humans (Pennycott et al., 2006; Lawson et al., 2014; Horton et al., 2013). Infection with *Salmonella* Typhimurium in birds can result in one of several scenarios: 1) an asymptomatic intestinal carrier stage, 2) an acute, rapidly fatal septicemia with or without enteritis, 3) or chronic localized infections that may or may not be clinically apparent (Alley et al., 2002; Pennycott et al., 2002; Connolly et al., 2006; Hughes et al., 2008; Verbrugghe et al., 2012 and 2016). The chronic infection is most often related to host adapted *Salmonella* strains through their ability to spread systemically within macrophages and reach various internal organs (Rabsch et al., 2002; Pasmans et al., 2003). The best described scenario in passerines is the acute death during disease outbreaks, with the most obvious pathological lesions: spleno- and hepatomegaly and (caseous) necrotic lesions present in the crop and oesophagus, spleen, liver and brains (Alley et al., 2002; Refsum et al., 2003; Giovannini et al., 2013). Almost all *Salmonella* outbreaks in wild birds occur during stress periods, such as cold stress in winter (Alley et al., 2002; Refsum et al., 2002; Pennycott et al., 2006; Lawson et al., 2010), in areas marked by severe habitat alteration suggesting environmental contamination from human, or livestock activities and can be easily spread through the use of bird feeders (Fenlon, 1981; Brittingham and Temple, 1988; Cízek et al., 1994; Alley et al., 2002; Pennycott et al., 2002). However, not much is known about the ability of host adapted strains to cause chronic disease in passerines and potentially lower the body condition through trade off (e.g. continuous or intermittent stimulation of the immune system) (Lochmiller and Deerenberg, 2000; Wobeser, 2006).

6.2. *Yersinia* spp.

Yersinia spp. are gram negative bacteria of the family of the *Enterobacteriaceae,* of which three species are considered important human pathogens (Reuter et al., 2014). The vectorborne *Yersinia pestis,* etiologic agent of plague (Reuter et al., 2014) will not be further discussed. The enteropathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* are the etiologic agents of yersiniosis (Reuter et al., 2014; EFSA and ECDC, 2015) and, similar to *Salmonella* Typhimurium, are transmitted through faeco-oral contact (Brittingham and Temple, 1988). In humans, yersiniosis is most frequently caused by *Yersinia enterocolitica* biotype (BT) 1B and

2-5 and to a lesser extent by *Y. pseudotuberculosis* (Thoerner et al., 2003; EFSA and ECDC, 2015). In passerines, the facultative pathogen *Y. pseudotuberculosis* is the most probable etiologic agent of yersiniosis which often has an acute enteric disease progression (Clark and Locke, 1962; Mair, 1973; Cork, 1999), but has on several occasions been isolated from apparently healthy birds (Mackintosh and Henderson, 1984; Niskanen et al., 2003) (Table 1). The most remarkable pathological lesions in passerines (based on autopsy reports of canaries *(Serinus canaria)*, zebrafinch *(Taeniopygia guttata)* and common grackles *(Quiscalus quiscula)*) are a severe enteritis with granulomas in the caecal tonsils in combination with hepato- and splenomegaly with granulomatous lesions (Clark and Locke, 1962; Cork et al., 1999).

Apart from the known pathogenic *Yersinia* species, other *Yersinia* species such as *Y. aldovae, Y. aleksiciae, Y. bercovieri, Y. frederiksenii, Y. intermedia, Y. kristensenii, Y. mollaretii, Y. rohdei,* but also *Y. enterocolitica* biotype 1A, which are mostly regarded as nonpathogenic can be detected in the faeces of wild terrestrial animals (Sulakvelidze, 2000; Niskanen et al., 2003; Kisková et al., 2011; Oda et al., 2015) and should be differentiated from the known pathogenic species. The detection of a combination of chromosomal-borne (*ail*-attachment invasion locus-gene; *ystA*-heat stable enterotoxin YstA-gene, *ystB*-heat stable enterotoxin YstB-gene, *inv*-invasin protein-gene) and pYV-plasmid-borne (*virF*- and *LcrF*-gene for *Y. enterocolitica* and *Y. pseudotuberculosis* respectively) virulence genes (Thoerner et al., 2003), has been used extensively to distinguish pathogenic isolates from nonpathogenic isolates (Thoerner et al., 2003; Van Damme et al., 2015).

Bird species involved	**Pathogen prevalence (S = *Salmonella* Typhimurium; Yps = *Yersinia pseudotuberculosis*)**	**Country**	**Reference**
Black-capped chickadee *(Poecile atricapillus),* tufted titmouse (*Baeolophus bicolor),* white-breasted nuthatch *(Sitta carolinensis),* red-breasted nuthatch *(Sitta canadensis),* cardinal *(Cardinalis cardinalis),* purple finch *(Haemorhous purpureus),* pine siskin *(Spinus pinus),* American goldfinch *(Spinus tristis)*, dark-eyed junco *(Junco hyemalis)*	S: 0%	Winsconsin, USA	Brittingham et al. (1988)

Starling *(Sturnus vulgaris: Sv)*, house sparrow (*Pd)*, house finch *(Haemorhous mexicanus: Hm)*, purple finch *(Hp)*, song sparrow *(Melospiza melodia: Mm)*, American goldfinch *(St)*, American robin *(Turdus migratorius: Tm)*	S: 7,1% (*Sv*); 1,07% (*Pd*); 0% (*Hm, Hp, Mm, St, Tm*)	Ohio, USA	Morishita et al. (1999)
House sparrow (A,B), chaffinch *(Fringilla coelebs)* (A,B), greenfinch *(Chloris chloris)* (A), blackbird *(Turdus merula)* (A), blue tit (*Cyanistes caeruleus)* (A,B), starling (A), jackdaw *(Corvus monedula)* (A)	S: 48% (bird feeder A with occasional mortality reports), S: 2% (bird feeder B, no reports of mortality)	Scotland	Pennycott et al. (2002)
Migratory Corvidae, Turdidae, Sturnidae	S: 0%	Sweden	Hernandez et al. (2003)
House sparrow, greenfinch	S: 2% (64% during outbreak)	Norway	Refsum et al. (2003)
House sparrow, greenfinch, chaffinch, starling, blackbird	S: 4% (in proximity to passerines who died due to salmonellosis)	Scotland	Grant et al. (2007)
House sparrow, barn swallow (*Hirundo rustica)*, white wagtail *(Motacilla alba)*, starling, Eurasian blackcap *(Sylvia atricapilla)*, Cetti's warbler *(Cettia cetti)*, Rock pigeon *(Columba livia)*	S: Overall *Salmonella* spp. prevalence 1.85% (0.46% far from pig premises vs. 3.46 close to pig premises)	Spain	Andrès et al. (2013)
Eurasian siskins *(Carduelis spinus)*	S: 0%	Switzerland	Giovannini et al. (2013)
Song trush *(Turdus philomelos)*, redwing *(Turdus iliacus)* were found to be positive (in total 468 samples of 57 bird species examined mainly belonging to Anseriformes and Passeriformes)	Yps: 0,6% (bioserotype 1/O:2)	Sweden	Niskanen et al. (2003)
Dunnock *(Prunella modularis)*	Yps: 0%	Slovakia	Kisková et al. (2011)
House sparrow (*Pd)*, Starling *(Sv)*	Yps: 2,3% (*Pd*: O:1), 1.7% (*Sv*: O:1) (survey in the proximity of a red deer *(Cervus elaphus)* farm with known yersiniosis outbreak)	New Zealand	Mackintosh and Henderson (1984)

Table 1. Selected information regarding the prevalence of *Salmonella* Typhimurium and *Yersinia pseudotuberculosis* in apparently healthy (and predominantly) Passeriformes.

6.3. The role of other hosts

Bacterial enteropathogens such as *Salmonella* Typhimurium (although not all phage types), *Y. pseudotuberculosis* and *Y. enterocolitica* have a broad host range, with many animals (e.g. birds and mammals) potentially serving as carriers or even reservoir hosts (Hubbert 1972; Rabsch, 2002; Battersby et al., 2002; Niskanen et al., 2003; Traweger et al., 2006; Lawson et al., 2014). Due to their ubiquity and synantropic lifestyle, certain rodent and bird species have been the subject of different studies related to food safety, whereby these animals have been appointed as source of feed contamination of livestock (Mackintosh and Henderson, 1984; Daniels et al., 2003). When investigating the effect of urbanization on host-pathogen relationships, the comparison of pathogen occurrence in animal species, with small home ranges (e.g. some avian species and rodents (Daniels et al., 2003; Backhans et al., 2011; Nkogwe et al., 2011; Han et al., 2015; Pollack et al., 2017)), along urbanization gradients will be most informative.

The Brown rat (*Rattus norvegicus*), originally from Asia, is an opportunistic and omnivorous rodent species which has been able to colonize large parts of the world thanks to its synantropic lifestyle (Ruedas, 2016) and is widely distributed in Flanders (Fig. 4). The species has been shown to carry different (zoonotic) pathogens (Battersby et al., 2002; Himsworth et al., 2013; Firth et al., 2014) for which the prevalence can be highly variable between nearby located rat populations (Himsworth et al., 2013). Most likely the territorial and extremely sedentary lifestyle, whereby rats usually run over specific routes in between their foraging areas and harborages (Davis et al., 1948; Traweger et al., 2006; Brown, 2007; Himsworth et al., 2014), contributes to this population specific pathogen dynamics (Himsworth et al., 2013). In cities, roads have been demonstrated to form a barrier, which will (only seldom) be crossed when for example the rats are in search for new home ranges (e.g. dispersal of juvenile rats, due to resource limitation, as a result of habitat destruction) (Traweger et al., 2006; Feng and Himsworth, 2014). As such, home ranges, often expressed as the average distance a rat travels along a runway, are thought be smaller in urban areas, delineated by city-blocks, compared to more rural habitats (Traweger et al., 2006; Feng and Himsworth, 2014). Although conflicting results have been published regarding whether the home range depends on the sex of the rats (no home range difference between the sexes (Brown, 2007), versus males having a significant larger home range than females (Lambert, 2003)), also in rural areas the home ranges of rats have been demonstrated to be small (Davis et al., 1948; Lambert, 2003; Brown, 2007) and can depend on the proximity of a farm (home range average of 25,4m (female) - 35,2m (male) in

the proximity of a farm, versus 131.6m (female) - 160,8m (male) in the field margins (Lambert, 2003)).

Since these rats share similar feeding sites with birds (e.g. on supplementary feeding stations) (Banyas and Artigues, 2000; Sánchez-García et al., 2015) enhanced faeco-oral contact within and in between different species can be expected, which increases the chance of an infectious disease to spread easily in between and within species on these aggregation sites.

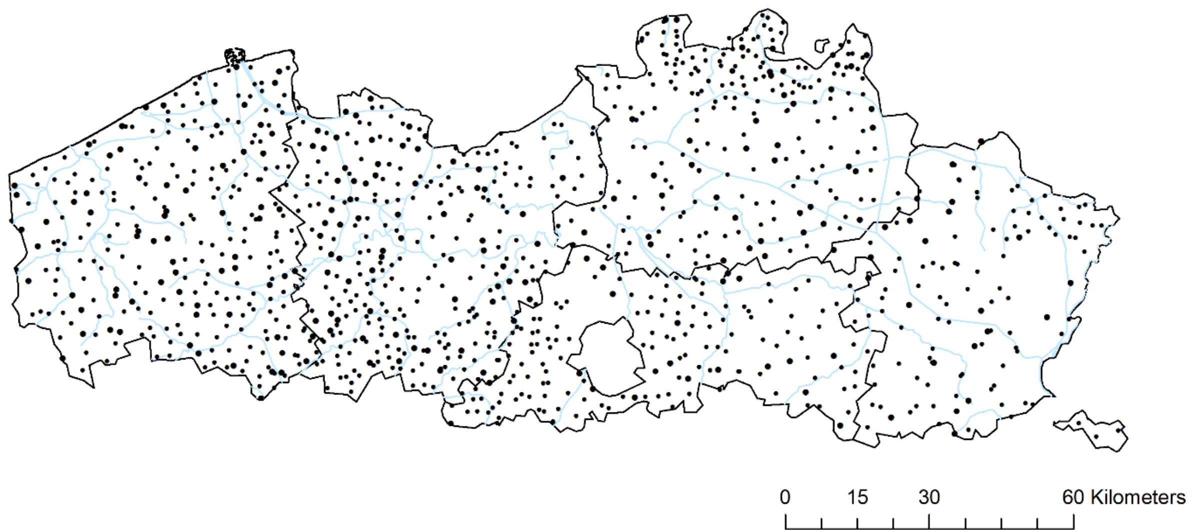

Fig 4. Distribution of brown rats in Flanders, based on the capture locations of the rats in this thesis.

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SCIENTIFIC AIMS

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Despite the general recognition that urbanization highly influences ecosystems and wildlife communities, how urbanization impacts urban disease ecology is still underexplored. This knowledge, however, is of great importance in order to better understand the effects of urbanization on animal health, including the mechanisms driving population dynamics.

The **general scientific aim** of this thesis was to gain insights into urban wildlife disease ecology by assessing the pathogen exposure along an urbanization gradient and the effect on the body condition of an urban exploiter consequently.

The **specific scientific aims** of this thesis were:

- To determine the differential **pathogen pressure** presented by the presence of ***Salmonella enterica*** **subspecies** ***enterica*** **serotype Typhimurium** (Chapter 2) and pathogenic ***Yersinia*** species (Chapter 3) in the faeces of urban, suburban and rural **house sparrow** ***(Passer domesticus)*** **populations**, clustered in three Flemish regions of Belgium.
- To **identify correlations** between **environmental variables** (granivore diversity, environmental temperature, urbanization level), **presence or absence of different** ***Yersinia*** **species** and variations in the house sparrow's **body condition (scaled mass index)** (Chapter 3).
- To assess the importance of other synantropic animal species such as the **brown rat (*Rattus norvegicus*)**, as a **potential reservoir host** from which *Yersinia* can spread to susceptible animals, in the ecology of **enteropathogenic** ***Yersinia*** species in Flanders (Chapter 4).

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CHAPTER 1: OVERARCHING METHODOLOGY

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Overarching methodology: spatial scale

The house sparrow represents a species which has shown to be highly sedentary (Summers-Smith, 1963) and, due to its ubiquity, has been widely used in studies. In order to assess the urbanization intensity or to classify different habitat types, previous research focusing on this species have used a 1 km² grid (Liker et al., 2008; Bókony et al., 2012; Meillère et al., 2017) or 1 km² circle surface (corresponding approximately to a circle with radius 565m) (Chávez-Zichinelli, 2010). Bichet et al. (2013) even used a circle with radius 10km to compare surrounding habitat structures.

In our research (Chapter 2 and 3) we have relied on studies performed by Vangestel et al. (2010) who used radio-telemetry to more accurately assess the home ranges of house sparrow populations along an urban gradient within the region of Ghent in Flanders. Vangestel et al. (2010) considered two spatial scales. The first scale combined the surface of habitat patches effectively used by the house sparrows, and consisted of clusters of different habitat patches, ranging between 0.0032-0.49ha. The second scale comprised the total surface area utilized by the sparrows, encompassing all the separate clusters and ranging between 0.028-2.86ha (Vangestel et al., 2010). Since home range sizes, at both spatial scales, varied significantly between populations inhabiting different urbanization levels (largest home ranges in rural populations) (Vangestel et al., 2010), we have agreed upon using the largest home range, being a spatial scale within a circle with 100m radius (3.14ha) and representing the most important foraging and thus transmission sites for enteropathogens (Chapter 3) (= "core home range"). Although, when including infrequently used foraging and roosting sites, referring to the maximum inter distance between point fixes of radiotagged house sparrows as determined by Vangestel et al. (2010), a home range scale with a circle of 400m radius can also be used as a proximate for the "maximum daily mobility range" (Chapter 2). Since house sparrows tend to live in close proximity to human settlements, thus inherently increasing the built-up (BU) density at the local home range scale, the addition of a larger landscape scale, following the suggestion of a multiscale approach, was indicated (Wiens, 1989; Melles et al., 2003; Litteral and Shochat, 2017). The landscape scale (within a circle of 1600m radius), based on the scale where different house sparrow populations can genetically be considered independent from each other (Vangestel et al., 2011), was chosen and used to differentiate the urbanization levels ("urban", "suburban", "rural") (Chapter 2 and 3).

Prior to the fieldwork (Chapter 2 and 3): A hierarchical (plot-subplot) stratified sampling design was used:

1) A stratified random sampling design was used to select 18 plots of 3x3km², differing in their level of urbanization (Fig. 5), within a 4655 km^2 polygon demarcated by the cities of Ghent, Antwerp, Leuven and Brussels (Flanders, Belgium). The stratification was performed at the urbanization ("high", "moderate" and "low" urbanization) as well as the regional level (Ghent, Antwerp and Leuven). These 18 plots were a subset of a larger set of 27 plots chosen for another project focussing on a wide array of taxa, including the house sparrows (SPatial and environmental determinants of Eco-Evolutionary DYnamics- anthropogenic environments as a model: SPEEDY). The level of urbanization was calculated based on the percentage of built-up structures within these defined areas. A built-up structure being defined as "a sustainable construction that encloses a space accessible for humans" (e.g. houses, garages, municipal buildings) (AGIV, 2013a). The cut-off points for the percentage of BU-areas were set at 0-3% for "rural" plots (lowest level of urbanization), 5-10% for "suburban" plots (intermediate level of urbanization), and >15% for "urban" plots (highest level of urbanization) and were calculated with ArcGIS v9.2 using the very high resolution (i.e. 0.15m pixels) 'Large-scale Reference Database' (LRD) (AGIV, 2013b). The urbanization levels in between the rural, suburban and urban plots (being 3-5% and 10-15% BU-area) were not considered when designating the plots in order to assure that house sparrow populations were searched for in the most contrasting plot levels. To ensure a more natural environment for the lowest urbanization class, only plots comprising >20% of ecologically valuable areas, as described by the 'Biologische Waarderingskaart' (Vriens et al., 2011) were chosen.
2) Every plot was subdivided in 200x200m² subplots (Fig. 6) in which 2 house sparrow populations were searched for (based on inventories collected during the winter of 2012-2013 and on citizen science projects), being located at least 1km apart from each other in most contrasting subplots, based on the levels of urbanization within each subplot (e.g. >15% BU-area and <3% BU-area).

In summary, this resulted in 36 paired house sparrow populations in 18 plots, following a hierarchical sampling design which was stratified at the urbanization as well as the regional level.

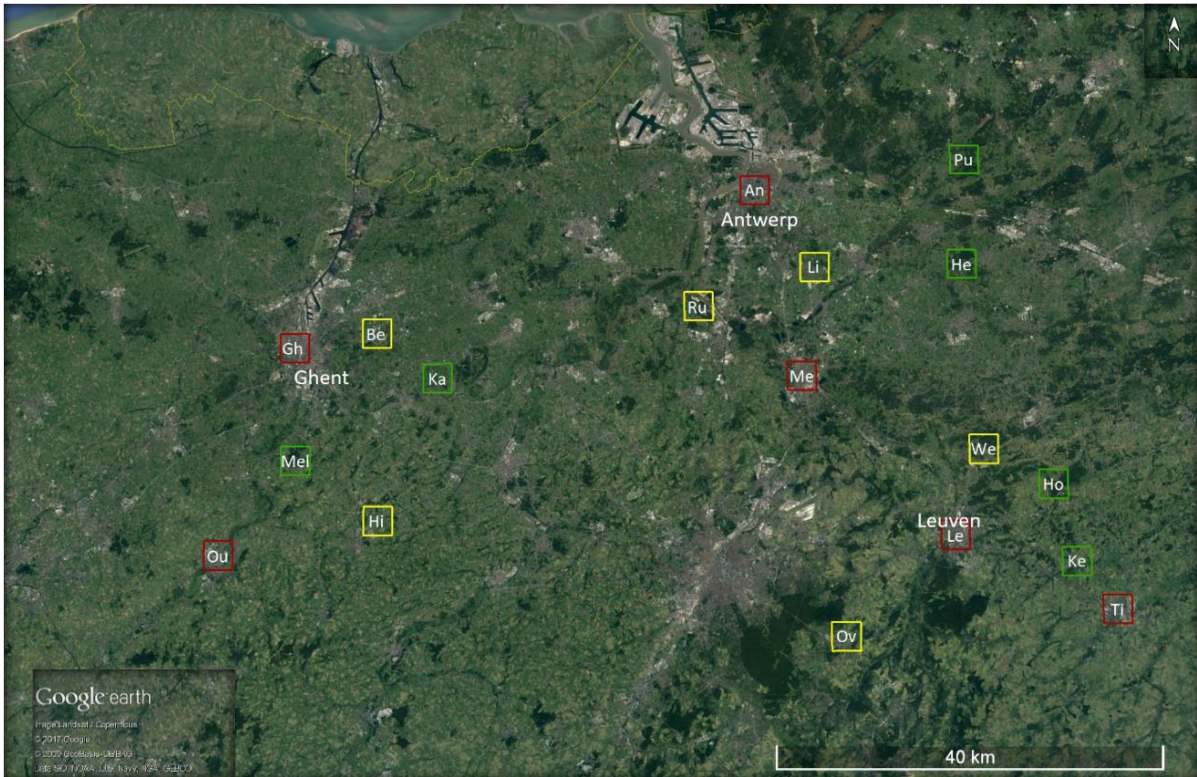

Fig 5. **Sampling plots of house sparrow populations:**
Color code: Red: Urban; Yellow: Suburban; Green: Rural.
Ghent region: Ghent (Gh),Oudenaarde (Ou), Beervelde (Be), Hillegem (Hi), Kalken (Ka), Melsen (Mel);
Antwerp region: Antwerp (An), Mechelen (Me), Ruisbroek (Ru), Lint (Li), Pulderbos (Pu), Herenthout (He);
Leuven region: Leuven (Le), Tienen (Ti), Overijse (Ov), Wezemaal (We), Houwaart (Ho), Kerkom (Ke)

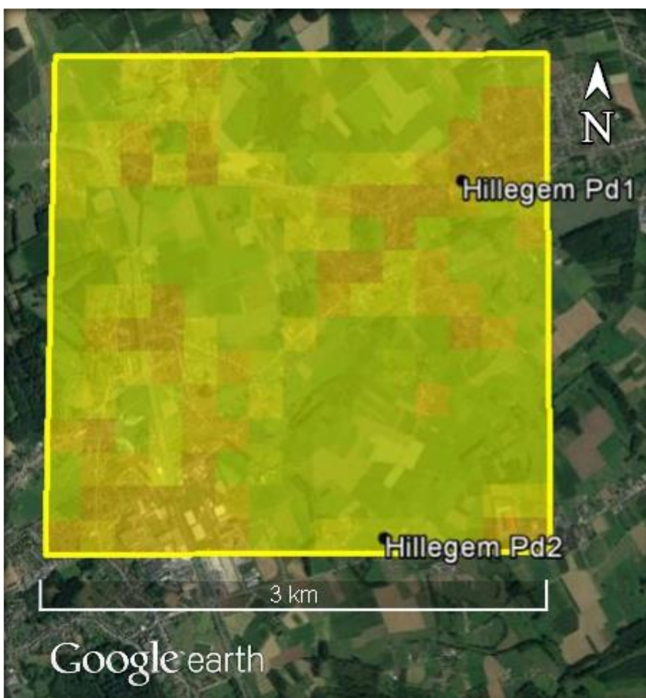

Fig 6. Subdivision of subplots (e.g. Hillegem plot)
Color-code: Red: >15% BU-area; Orange: 10-15% BU; Yellow: 5-10% BU; Light Green: 3-5% BU, Dark Green: <3% BU

<u>After the fieldwork:</u>

3) However, the urbanization level, which is representative for house sparrow populations that are found at the border of a plot or subplot, will vary from the *a priori* calculated urbanization level in the respective plot and subplot. Therefore, the level of urbanization was recalculated for all the populations, at two “local” scales (100m and 400m radius around the centre of the main capture site) and a “landscape” scale (1600m radius around the centre of the main capture site, thereby excluding the 100m and 400m radius of the local scale respectively) (Fig. 7). Since the urbanization level, after recalculation, also included plots and subplots with urbanization levels of 3-5% BU-area and 10-15% BU-area, these BU-levels were merged with the three urbanization levels (“Urban”, “Suburban”, “Rural”). Initially the recombination of the urbanization levels was performed in order to resemble the original SPEEDY design as closely as possible (which resulted in “urban” (>13%), “suburban” (5-13%), and “rural” (<5%)). However, since also by using these cut-off values the original level of stratification based on the urbanization level could not be guaranteed, this idea was abandoned and the recombination of the urbanization levels was changed into “urban” (red: >10%), “suburban” (yellow: 5-10%) and “rural” (green: <5%), based on close examination of the environment at landscape scale level.

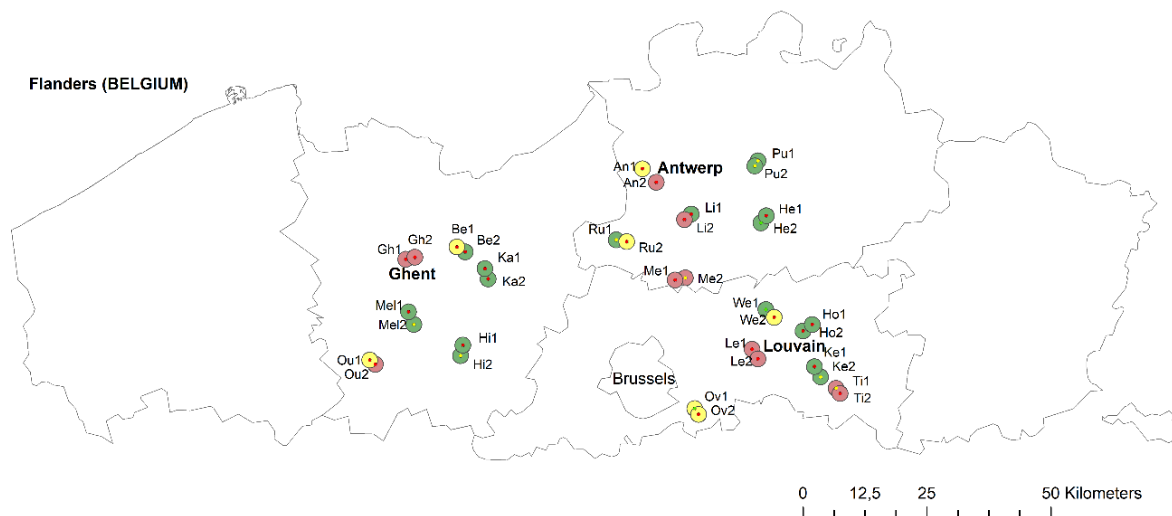

Fig 7. House sparrow population with circular BU-areas measured within the local scale (100m) and the landscape scale (1600m, excluding the BU-area within the 100m) radius:

Adapted color code: Urban-Red: >10% BU-area; Suburban-Yellow: 5-10% BU; Rural-Green: <5% BU

Table 2. Urbanization level calculated for the different house sparrow populations:

Overview of the level of Built Up (BU) area at different nested scales around the pairwise clustered house sparrow populations in the respective regions. (G) = least urbanized subplot; (R) = most urbanized subplot; Coordinates (LONG = Longitude; LAT = Latitude); outer = the percentage of BU-area within the outer shell, after extracting the BU-area of the respective inner shell; inner = the percentage of BU-area within the inner shell.

Region	Plot (Subplot)	LONG	LAT	1600m	1200m-outer	400m-inner	1500m-outer	100m-inner
GHENT	Ghent (G)	3,69	51,05	18,29	18,80	10,55	18,27	23,58
	Ghent (R)	3,72	51,06	38,99	38,09	52,51	38,98	41,39
	Oudenaarde (G)	3,59	50,87	9,68	10,16	2,47	9,68	10,03
	Oudenaarde (R)	3,61	50,86	17,17	17,16	17,23	17,17	14,63
	Beervelde (G)	3,87	51,07	4,64	4,57	5,71	4,62	10,52
	Beervelde (R)	3,84	51,08	7,33	6,81	15,10	7,30	15,80
	Hillegem (G)	3,85	50,88	4,66	4,81	2,35	4,65	5,97
	Hillegem (R)	3,86	50,90	4,69	4,16	12,62	4,62	22,17
	Kalken (G)	3,93	51,02	3,76	3,89	1,83	3,74	10,67
	Kalken (R)	3,92	51,04	4,68	4,23	11,38	4,62	20,88
	Melsen (G)	3,72	50,93	2,15	2,08	3,22	2,13	7,20
	Melsen (R)	3,70	50,96	3,85	3,46	9,78	3,81	13,97
ANTWERP	Antwerp (G)	4,38	51,22	7,58	6,99	16,29	7,51	23,51
	Antwerp (R)	4,42	51,19	24,64	24,25	30,42	24,52	55,03
	Mechelen (G)	4,50	51,02	16,54	16,89	11,42	16,58	6,28
	Mechelen (R)	4,47	51,02	22,16	21,66	29,62	22,14	27,37
	Lint (G)	4,52	51,14	4,35	4,36	4,10	4,30	16,91
	Lint (R)	4,50	51,13	11,87	11,27	20,85	11,79	31,61
	Ruisbroek (G)	4,30	51,09	3,10	3,24	0,98	3,09	5,95
	Ruisbroek (R)	4,33	51,09	6,14	5,71	12,63	6,09	19,97
	Pulderbos (G)	4,71	51,23	2,57	2,66	1,26	2,56	5,11
	Pulderbos (R)	4,70	51,22	3,70	3,34	9,00	3,68	7,04
	Herenthout (G)	4,72	51,12	1,91	1,98	0,76	1,91	1,72
	Herenthout (R)	4,73	51,13	2,82	2,59	6,24	2,72	27,83
LEUVEN	Leuven (G)	4,69	50,89	16,96	17,42	10,05	16,97	14,02
	Leuven (R)	4,71	50,87	27,92	26,91	43,08	27,90	33,75
	Tienen (G)	4,93	50,82	10,84	11,05	7,81	10,86	7,73
	Tienen (R)	4,94	50,81	17,88	16,56	37,73	17,85	27,20
	Wezemaal (G)	4,73	50,96	4,69	4,77	3,39	4,69	3,28
	Wezemaal (R)	4,75	50,95	5,41	4,78	14,87	5,31	30,83
	Overijse (G)	4,52	50,78	8,97	9,26	4,63	8,99	3,71
	Overijse (R)	4,54	50,77	8,36	7,55	20,48	8,23	39,41
	Houwaart (G)	4,84	50,92	1,53	1,57	0,96	1,50	10,12
	Houwaart (R)	4,86	50,93	1,72	1,45	5,78	1,66	17,13
	Kerkom (G)	4,89	50,84	1,84	1,82	2,07	1,82	7,16
	Kerkom (R)	4,87	50,86	2,03	1,75	6,34	1,99	14,32

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Chapter 2: House sparrows do not constitute a significant *Salmonella* Typhimurium reservoir across urban gradients in Flanders, Belgium

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House sparrows do not constitute a significant *Salmonella* Typhimurium reservoir across urban gradients in Flanders, Belgium

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Abstract

In recent decades major declines in urban house sparrow (*Passer domesticus*) populations have been observed in northwestern European cities, whereas suburban and rural house sparrow populations have remained rather stable or are recovering from previous declines. Differential exposure to avian pathogens known to cause epidemics in house sparrows may in part explain this spatial pattern of declines. Here we investigate the potential effect of urbanization on the development of a bacterial pathogen reservoir in free ranging house sparrows. This was achieved by comparing the prevalence of *Salmonella enterica* subspecies *enterica* serotype Typhimurium in 364 apparently healthy house sparrows captured in urban, suburban and rural regions across Flanders, Belgium between September 2013 and March 2014. In addition 12 dead birds, received from bird rescue centers, were necropsied. The apparent absence of *Salmonella* Typhimurium in fecal samples of healthy birds, and the identification of only one house sparrow seropositive for *Salmonella* spp., suggests that during the winter of 2013-2014 these birds did not represent any considerable *Salmonella* Typhimurium reservoir in Belgium and thus may be considered naïve hosts, susceptible to clinical infection. This susceptibility is demonstrated by the isolation of two different *Salmonella* Typhimurium strains from two of the deceased house sparrows: one DT99, typically associated with disease in pigeons, and one DT195, previously associated with a passerine decline. The apparent absence (prevalence: <1.3%) of a reservoir in healthy house sparrows and the association of infection with clinical disease suggests that the impact of *Salmonella* Typhimurium on house sparrows is largely driven by the risk of exogenous exposure to pathogenic *Salmonella* Typhimurium strains. However, no inference could be made on a causal relationship between *Salmonella* infection and the observed house sparrow population declines.

Introduction

Salmonella enterica subspecies *enterica* serotype Typhimurium has the potential to cause disease outbreaks in Passeriformes. In Britain, definite phage types (DT)40, DT56(v) and DT160, accounted for the majority of passerine salmonellosis incidents, most often recognized in greenfinches (*Chloris chloris*) and house sparrows (*Passer domesticus*) (Pennycott et al., 2006; Lawson et al., 2010; Lawon et al., 2014). Outbreaks of salmonellosis in Passeriformes occur mostly during the winter period (Alley et al., 2002; Refsum et al., 2002a; Pennycott et al., 2006; Lawson et al., 2010) with sometimes marked annual variation in salmonellosis incidents between winter periods of consecutive years (Refsum et al., 2002a; Lawson et al., 2010; Lawon et al., 2014). Harsh weather conditions (Daoust et al., 2000) and contaminated foraging areas (Cízek et al., 1994; Morishita et al., 1999; Andrés et al., 2013), sometimes related to supplemental feeding (Pennycott et al., 2002; Refsum et al., 2003) have been associated with a higher prevalence of *Salmonella* spp. (Daoust et al., 2000; Cízek et al., 1994; Morishita et al., 1999; Andrés et al., 2013; Pennycott et al., 2002; Refsum et al., 2003). Although some phage types of *Salmonella* Typhimurium are considered host adapted, DT2 and DT99 in pigeons (Pasmans et al., 2003), DT40 and DT56(v) in passerines (Lawson et al., 2011), the latter two phage types have been isolated from captive birds and mammals and have been linked to disease in humans (Refsum et al., 2002b; Pennycott et al., 2006; Giovannini et al., 2013; Horton et al., 2013; Lawon et al., 2014). In this perspective, most of the studies on prevalence and epidemiology of *Salmonella* spp. in free living birds have been performed in the surroundings of farms in order to evaluate food safety and human health risks areas (Cízek et al., 1994; Kirk et al., 2002; Andrés et al., 2013), or have been related to disease in animals or humans (Refsum et al., 2002b; Giovannini et al., 2013; Lawon et al., 2014). Other studies, none of which were conducted in Belgium, assessed the presence of pathogenic bacteria in moribund birds, or dead birds submitted for necropsy, whether or not related to epidemics in wild birds (Pennycott et al., 2006; Lawson et al., 2010; Refsum et al., 2002a; Refsum et al., 2003; Giovannini et al., 2013). While these studies provide important insights in the epidemiology and pathogenesis of these bacteria, they cannot be used to estimate the prevalence of long-term carrier birds. Few studies have been performed to assess the prevalence of *Salmonella* spp. in apparently healthy migrating and nonmigrating wild Passeriformes, not specifically related to ongoing disease outbreaks. A low prevalence ($\leq$ 2%) of *Salmonella* spp. was demonstrated in these studies (Brittingham et al., 1988; Morishita et al., 1999; Hernandez et al., 2003; Refsum et al., 2003; Andrés et al., 2013; Hamer et al., 2012). Since host adapted *Salmonella enterica* strains could

potentially reduce the reproduction success in their respective reservoir hosts (Faddoul and Fellows, 1965; Uzzau et al., 2000), it is important to understand to what extent passerines are indeed long term carriers of *Salmonella* Typhimurium, as birds in general have already been appointed as potential reservoirs for *Salmonella enterica* subspecies *enterica* (Morishita et al., 1999; Pasmans et al., 2004; Krawiec et al., 2015).

Little research has been performed to specifically assess the differences in prevalence of *Salmonella enterica* subspecies *enterica* in wild passerines inhabiting urban versus rural environments (Hamer et al., 2012). Previous studies have suggested that the prevalence of *Salmonella enterica* subspecies *enterica* may depend on microclimate differences between urban (heat island effect) and rural areas (Hamer et al., 2012; Krawiec et al., 2015). As such, this pathogen might be partly responsible for discrepant population dynamics in avian hosts from urban and rural areas, such as observed in house sparrows (*Passer domesticus*). In recent decades, urban populations of this species have indeed suffered dramatic declines throughout northwestern Europe and southeast Asia, whereas suburban and rural populations have remained rather stable or are recovering from previous declines (De Laet and Summers-Smith, 2007; Kamath et al., 2014). Understanding the role, if any, of house sparrows as *Salmonella* Typhimurium reservoirs is important for understanding infection and disease dynamics. This might help to explain the massive population declines observed, possibly related to disease outbreaks during the winter and lower reproduction successes in spring.

We here assess the prevalence of *Salmonella* Typhimurium in apparently healthy house sparrows along urban gradients, in order to reveal potential correlates with the ongoing population declines in urban areas. To achieve this goal, feces and blood samples of house sparrows, collected in urban, suburban and rural populations, were tested for the presence of *Salmonella* Typhimurium and anti-*Salmonella* antibodies respectively. In addition, a total of twelve deceased house sparrows, obtained from the bird rescue centers of Ostend and Merelbeke, and submitted for necropsy, were tested for the presence of *Salmonella* Typhimurium.

Materials and methods

Since *Salmonella* Typhimurium outbreaks in passerines are reported mostly during the winter period (Alley et al., 2002; Refsum et al., 2002a; Refsum et al., 2003; Pennycott et al., 2006, Lawson et al., 2010; Giovannini et al., 2013; Krawiec et al., 2015), feces and blood samples of 364 individual house sparrows were collected between September 11th and

December 20th, 2013 (first sampling) and between January 10th and March 28th 2014 (second sampling). Samples were collected in 36 house sparrow populations located in 9 urban, 9 suburban and 18 rural regions clustered pairwise around the Flemish cities of Ghent, Antwerp and Louvain, every population being sampled at least once per sampling period. House sparrows are treated as species of Least Concern on the IUCN Red List of Threatened Species (http://www.iucnredlist.org/), and all ringers involved in this study were holders of a scientific ringing certificate issued annually by the Agency for Nature and Forest. All sparrows were captured on private land for which oral permission was granted by the respective land owners. All trapping and sampling protocols were approved by the Ethical Committee VIB Ghent site (EC2013-027).

The level of built up area (BU) in circular plots around each trapping site was calculated from GIS layers at two nested scales, i.e. a local scale (radius of 400m) and a landscape scale (radius of 1600m) (Large-scale Reference Database (LRD)) the former corresponding to the average home-range size of Flemish house sparrows (De Laet and Summers-Smith, 2007; Vangestel et al., 2010). Built up values for the three urbanization classes were empirically set as "urban" >13% BU; "suburban" 5-13% BU; "rural" <5% BU, and neighboring populations were at least 1km apart. The landscape scale was used for the classification of the urbanization levels, whereas the local scale provides more detail regarding the urbanization of the center of each individual class, being the direct habitat of the house sparrows.

House sparrows were captured with standard mist nets after which each bird was individually put in an autoclaved cotton bag (approved by the Ethical Committee VIB Ghent site: EC2013-027). Feces were collected in sterile micro centrifuge tubes, 50 µl blood was collected in 200µl absolute ethanol and each individual was sexed, measured and equipped with a unique metal ring before being released at its original trapping site. The Scaled Mass Index (SMI) of the house sparrows was calculated using the equation of the linear regression of ln-body mass (measured with a digital balance: ±0.01g) on ln-tarsus length (measured with a digital caliper: ±0.01mm) estimated by type-II (standardized major axis; SMA) regression (Peig and Green, 2009). The regression slope and average tarsus length were fitted in the calculation [body mass x (average tarsus length/tarsus length)^regression slope] to measure the SMI in order to have an estimation of the body condition of the birds, and compared to the different urbanization levels at both scales (400m and 1600m radius) using ANOVA in R.

The ISO 6579:2002 method (ISO-6579, 2002), for the isolation of different *Salmonella* serotypes including *Salmonella* Typhimurium, was initiated within 24 hours of sampling. In

summary, the fecal samples were pre-enriched overnight at 37°C in nonselective Buffered peptone water (Oxoid, Hampshire, UK), after which the samples were simultaneously added to selective "Tetrathionate brilliant-green enrichment broth for Microbiology' (Merck, Belgium) and "Rappaport Vassiliadis medium with Soya Peptone Broth" (Oxoid, Hampshire, UK) for overnight enrichment at 37°C and 41°C respectively. Xylose Lysine Deoxycholate (XLD) agar (Oxoid, Hampshire, UK) and Brilliant Green agar (BGA) (Oxoid, Hampshire, UK), incubated overnight at 37°C, were used for plating out the samples after the enrichment procedures.

Indirect ELISA was performed on the blood samples. The preparation of ELISA plates was conducted according to Leyman et al. (2011) using a formol-inactivated *Salmonella* Typhimurium DAB69 (pigeon strain) for plate-coating. Before initiation of the indirect-ELISA the plates were washed with a 1% skim milk powder solution in distilled water. The blood samples, stored in ethanol were thoroughly vortexed, after which 100µl of a 1/100 dilution of the samples in Sample Diluent Buffer (0.6 g NaH2PO4·2H2O, 5.6 g NaH2PO4. 12H2O, 0.5 ml Tween 20 (Merck, Germany), 12.5 g NaCl, 22g skim milk powder, 1000ml distilled water) was added to the wells. The plate was incubated for 1 hour at 37°C after which the plate was washed three times with washing buffer (0.6 g NaH2PO4·2H2O, 5.6 g NaH2PO4. 12H2O, 0.5 ml Tween 20, 12.5 g NaCl, 1000ml distilled water). A 1/1000 dilution of Polyclonal Goat Anti-Bird IgG (H+L)-horseradish peroxidase (HRP) conjugate (Cat-number: 90520, Alpha Diagnostics Intl. Inc., San Antonio, Texas, USA), reactive against sparrow and dove antibodies, was added to the wells. The plates were incubated at 37°C for 1 hour and washed three times, after which 100µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma Aldrich Chemie Gmbh, Steinheim Germany) was added. After 15min incubation at room temperature in a dark environment, the reaction was stopped using stop reagent for TMB substrate (Sigma Aldrich Chemie Gmbh, Steinheim Germany), and the optical density was measured (450nm). Blood in ethanol of *Salmonella* infected pigeons (infected with the DAB69 strain) served as a positive control. The control blood was obtained from another study approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience Engineering, Ghent University (EC2014/96). The cut-off point for the optical density (OD) was calculated as the mean OD from three *Salmonella* free pigeons, calculated from an entire 96-well plate, plus three times the standard deviation (0.238). All measurements were performed in duplicate. The pigeons used for calculation of the cut-off value for the OD were ascertained *Salmonella* free, since they were retrieved at 4 weeks of age from a *Salmonella* negative colony, whereafter they were housed strictly separately from other pigeons following the biosecurity

measurements and were tested for several weeks by the use of ISO 6579:2002 method on mixed feces. In addition the individual pigeons were screened for the presence of *Salmonella* Typhimurium by performing bacteriology on cloacal swabs and a rapid slide agglutination test on serum. The positive control consisted of an experimentally *Salmonella* Typhimurium infected pigeon.

During 2013-2014, bird rescue centers were asked to transfer deceased house sparrows to the lab facilities of the Faculty of Veterinary Medicine (Ghent University), for necropsy. A total of twelve birds, received between May 2013 and December 2014 were tested for the presence of *Salmonella* Typhimurium. The entire intestinal tract, heart, lungs, liver, spleen, kidneys, reproductive organs and brains were checked for abnormalities. If obvious lesions were present, these were aseptically swabbed, prior to enrichment, and immediately plated out onto BGA and Columbia agar with sheep blood plus (COLS) (Oxoid, Wesel, Germany) for overnight incubation at 37°C and onto MacConkey agar (Oxoid, Hampshire, UK) for overnight incubation at 30°C. Direct microscopic investigation was conducted on the intestinal content. Unless postmortem decay was too advanced, cytology was performed on the liver, spleen, kidney and lungs and a separate enrichment according to the ISO 6579:2002 protocol was initiated for the intestinal content, liver, spleen, and organs with lesions.

If fecal or autopsy samples were positive for *Salmonella* spp., these *Salmonella* spp. were further analysed by serotyping at the 'Belgian Scientific Institute of Public Health (WIV-ISP)' and by phage typing at the 'Bacteriology Reference Department of the Public Health of England (BRD-PHE)'.

In order to estimate the probability of absence of *Salmonella* serotypes in our population, we applied the epi.detectsize function of the R library 'epiR' (Stevenson, 2015). This test determines the number of individuals that need to be randomly sampled to declare a population free from a pathogen at a certain confidence level. The test is based on the pathogen prevalence level we want to be able to reveal, the population size and test sensitivity and specificity. Based on literature regarding *Salmonella* prevalence among passerines, we can expect the between- and within-population prevalence to be lower than 2% (Brittingham et al., 1988; Morishita et al., 1999; Hernandez et al., 2003; Refsum et al., 2003; Hamer et al., 2012; Andrés et al., 2013). Average sparrow population size in our study area was estimated at about 25 birds. The analyses are based on the highly sensitive ISO 6579:2002 method outlined above. This test is characterised by a sensitivity of at least 0.90 and a specificity of at least 0.99 (Hyeon et al., 2012; Mainar-Jaime et al., 2013). ISO based analyses yield a conservative estimate of the power

and precision of our analyses. In addition, we used the truePrev function of the R library ‘prevalence’ (Devleesschauwer et al., 2015) to obtain a Bayesian estimate of true prevalence from apparent prevalence obtained by testing individual samples, using the sensitivity and specificity values mentioned above.

Results

In total, feces and blood of 364 house sparrows were screened for the presence of *Salmonella* Typhimurium and the presence of anti-*Salmonella* antibodies. The house sparrows consisted of 42.6% female birds, 57.1% male birds and 1 undefined young house sparrow, which belonged to urban house sparrow populations (28.3%), suburban populations (21.15%), and to rural populations (50.55%). Nineteen birds were recaptured within or between both sampling periods, from which 19 fecal and 11 blood samples were obtained, respectively. The recaptured birds all originated from the same house sparrow population as the one they were first captured from. Sparrow SMI (95%CI: mean=27.68g+/-3.86g) did not vary across urbanization gradients (1600m scale: ANOVA $F_{1,352}$= 2.19, P-value = 0.14; 400m scale: $F_{1,352}$ = 1.70, P-value: 0.19) and all trapped individuals appeared healthy, with the exception of one bird which was diagnosed with poxvirus based on the macroscopic cutaneous lesions and the detection of typical intracytoplasmic Bollinger bodies within the epidermal cells. *Salmonella* Typhimurium was not isolated from any of these fecal samples. One house sparrow (0.27%) trapped in the city of Ghent (Ghent: 51,052083 /3,694134: U), proved to be positive for anti-*Salmonella* antibodies (mean OD: 0.388).

Statistical analyses show that to be 95% certain that *Salmonella* Typhimurium is not present in the study area (i.e. prevalence < 1%), if all tests were to be negative, we would need to sample 12 sparrows from 18 populations (216 sparrows in total), which is close to our actual sampling (364 birds from 36 populations). As our sampling represents a stratified random sampling along urbanization gradients across Flanders, our results can be regarded as representative for the whole region. House sparrows are unlikely to number more than one million birds in Flanders (Vermeersch et al., 2004), and calculations show that a minimum of 331 sparrows need to be sampled to confirm the absence of *Salmonella* serotypes in Flanders. Bayesian analyses showed we can be 95% certain that the true prevalence in Flanders varies between 0 and 1.3%.

Twelve deceased house sparrows, received from the bird rescue centers of Merelbeke (7) and Ostend (5), were necropsied and screened for the presence of *Salmonella* Typhimurium. Two of these individuals, collected in the city of Ostend, showed macroscopically visible

granulomas (1.5mm and 3mm diameter) in the cerebrum, histologically consisting of an accumulation of heterophilic granulocytes and macrophages. Ziehl Nielsen, PAS and Gram staining of the granulomas yielded negative results for *Mycobacterium* spp., fungi and Gram-positive bacteria respectively. Both house sparrows however tested positive for *Salmonella* Typhimurium, which was isolated in pure culture from the granulomas in the brains. Serotyping and phage typing revealed the presence of *Salmonella* Typhimurium var. Copenhagen (O:1,4,12) DT195 and a pigeon specific phage type DT99. The former bird also showed a black intestinal content suggestive for hemorrhagic diathesis, while the latter house sparrow was found to be positive for cestodes using direct microscopic investigation of intestinal content. Because of the postmortem decay, the other organs, besides the brains, were not subjected to histology. Nevertheless cytology of the liver, spleen and lungs was performed and did not reveal any *Atoxoplasma* inclusions, whereas a slight infiltration of granulocytes and macrophages was present in the lungs of the house sparrow infected with DT99. No other major abnormalities were detected. Six house sparrows brought in for necropsy, died due to trauma (2 cases), coccidiosis (2 cases), predation (1 case) or predation with additional *Pasteurella multocida* infection (1 case) while four other individuals died due to unknown reasons. Unfortunately, no information regarding the habitat type nor the level of urbanization was available for the necropsied house sparrows.

Discussion

Since the onset of the severe population declines in rural and urban house sparrows, researchers have been searching for possible explanations (reviewed in 'De Laet and Summers-Smith, 2007'). Loss of nesting and foraging areas, changes in socioeconomic status, electromagnetic radiation, predation, depletion of food resources, pesticides, herbicides, the use of unleaded petrol and pathogens have all been suggested to cause these declines, either separately or in synergy (De Laet and Summers-Smith, 2007; Kamath et al., 2014; Summers-Smith, 2003; Everaert and Bauwens, 2007; Shaw et al., 2008). While the impact that pathogens have on the population health when present in sublethal doses or in carrier birds is not very well known, it could potentially depend on infection pressure, which has been suggested to be higher in urban environments (Benskin et al., 2009; Krawiec et al., 2015).

Our findings suggest that house sparrow populations across Flemish urban gradients, during the winter of 2013-2014, do not constitute a considerable *Salmonella* Typhimurium reservoir, such that birds could overall be considered naïve to infection. Not isolating *Salmonella*

Typhimurium from the feces and a seroprevalence for *Salmonella* spp. of 0.27% in the house sparrows screened during this study, indeed suggests a very low prevalence of *Salmonella* Typhimurium. Bayesian estimates confirm that true *Salmonella* Typhimurium prevalence is unlikely to be higher than 1.3%. Annual variation in salmonellosis incidents (Lawson et al., 2010; Lawson et al., 2014) should however be kept in mind when interpreting the results, since the study was limited to a single winter period (2013-2014). Based on our results, no patterns regarding *Salmonella* prevalence in house sparrow populations along an urban-rural gradient could be demonstrated and no inference could be made on a causal relationship between *Salmonella* and the house sparrow declines. Despite the lack of historical data regarding the prevalence of *Salmonella* in apparently healthy Passeridae in Belgium, our findings are consistent with those obtained from house sparrow populations in Northern Spain (Andrés et al., 2013), which investigated the difference in prevalence of *Salmonella* in house sparrows living close to or far from pig premises, and in Ohio (Morishita et al., 1999), which focused on house sparrows and other birds in the surrounding of human settlements. Both studies detected low prevalence of *Salmonella* spp. in these birds (Morishita et al., 1999; Andrés et al., 2013), especially in birds inhabiting areas far from pig premises (Andrés et al., 2013). Occasional detection of *Salmonella* in feces from apparently healthy house sparrows (Morishita et al., 1999; Refsum et al., 2003; Andrés et al., 2013), which were not corroborated by follow up data, may reflect mechanical or temporal carriage after foraging in contaminated areas, or could indicate the presence of *Salmonella* excreting birds still in the incubation period of the disease, rather than the demonstration of the presence of actual *Salmonella* carrier birds.

Anti-*Salmonella* antibodies were detected in one of 364 house sparrows. To the authors' knowledge, this is the first study to detect antibodies against *Salmonella* spp. in apparently healthy wild passerines. Serum-IgG-antibodies have proved to provide a good indication of *Salmonella* Typhimurium infection as they increase already 2 weeks after primary infection has taken place and as they can persist in the blood for several months (Hassan et al., 1991; Barrow, 1992; Proux et al., 1998). Despite this knowledge, the onset of the antibody response and the height of the antibody titer depends on the maturity of the immune system, on whether or not the infection is a primary infection or a reinfection and on the susceptibility of the bird to *Salmonella* Typhimurium (Hassan et al., 1991; Barrow, 1992; Proux et al., 1998). The main advantage of ELISA, when performed in conjunction with isolation methods and recapture of birds, is that ELISA could provide a better assessment of the prevalence of long term carriers

and survivors and could aid in the detection of intermittent shedders, however, more research is needed for the accurate interpretation of the results.

Two out of 12 deceased house sparrows sent for autopsy tested positive for *Salmonella* Typhimurium var. Copenhagen (O:1,4,12) DT99 and DT195, isolated from granulomatous brain lesions. Although the sole demonstration of these cerebral lesions, without concurrent hepatomegaly, splenomegaly and granulomatous lesions in the upper alimentary tract, is not typical for a *Salmonella* Typhimurium infection in passerines, histological evidence of encephalitis has previously been demonstrated in passerines and brain abscesses have been recognized in pigeons in the past (Faddoul and Fellows, 1965; Connolly et al., 2006; Giovannini et al., 2013). Phage type DT195 has been shown to be pathogenic for a variety of animals including humans (Palmgren et al., 2006; Ewen et al., 2007). DT99, on the contrary, is regarded a pigeon adapted variant of *Salmonella* Typhimurium (Pasmans et al., 2003) which has previously caused morbidity and mortality in mice (Pasmans et al., 2004) as well as in passerines (Refsum et al., 2002a). Since *Salmonella* Typhimurium DT99 circulates endemically in feral pigeons that can reach high local densities in urbanized areas (Pasmans et al., 2004), feral pigeons constitute a potential source for *Salmonella* Typhimurium DT99 associated disease in passerines in urbanized areas.

Conclusion

These results suggest the apparent absence (prevalence: <1.3%) of a *Salmonella* Typhimurium reservoir in apparently healthy house sparrows and an association of *Salmonella* Typhimurium infection with clinical disease which is most likely driven by the risk of exogenous exposure to pathogenic *Salmonella* Typhimurium strains. However, no inference could be made on a causal relationship between *Salmonella* and the house sparrow population declines.

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Chapter 3: Effects of urbanization on host-pathogen interactions, using *Yersinia* in house sparrows as a model

GHENT UNIVERSITY

EFFECTS OF URBANIZATION ON HOST-PATHOGEN INTERACTIONS, USING *YERSINIA* IN HOUSE SPARROWS AS A MODEL

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Abstract

Urbanization strongly affects biodiversity, altering natural communities and often leading to a reduced species richness. Yet, despite its increasingly recognized importance, how urbanization impacts on the health of individual animals, wildlife populations and on disease ecology remains poorly understood. To test whether, and how, urbanization driven ecosystem alterations influence pathogen dynamics and avian health, we use house sparrows (*Passer domesticus*) and *Yersinia* spp. (pathogenic for passerines) as a case study. Sparrows are granivorous urban exploiters, whose western European populations have declined over the past decades, especially in highly urbanized areas. We sampled 329 house sparrows originating from 36 populations along an urbanization gradient across Flanders (Belgium), and used isolation combined with 'matrix-assisted laser desorption ionization- time of flight mass spectrometry' (MALDI-TOF MS) and PCR methods for detecting the presence of different *Yersinia* species. *Yersinia* spp. were recovered from 57.43% of the sampled house sparrows, of which 4.06%, 53.30% and 69.54% were identified as *Y. pseudotuberculosis, Y. enterocolitica* and other *Yersinia* species, respectively. Presence of *Yersinia* was related to the degree of urbanization, average daily temperatures and the community of granivorous birds present at sparrow capture locations. Body condition of suburban house sparrows was found to be higher compared to urban and rural house sparrows, but no relationships between sparrows' body condition and presence of *Yersinia* spp. were found. We conclude that two determinants of pathogen infection dynamics, body condition and pathogen occurrence, vary along an urbanization gradient, potentially mediating the impact of urbanization on avian health.

Introduction

With growing human populations, cities are expanding rapidly and urbanization represents one of the most intense anthropogenic modifications of natural systems, strongly affecting species, communities and ecosystems (Grimm et al., 2000; Evans et al., 2009b). The direction and strength of responses of bird species to urbanization is function of their life history strategies (Sol et al., 2014). This has led to the 'biotic homogenization' of urban bird communities (McKinney, 2008), i.e. whereby the latter become gradually dominated by a limited number of 'urban exploiter' species, such as house sparrows *(Passer domesticus)* (McKinney, 2002). Studies focussing on how avian communities respond to urbanization find that bird species richness (Blair, 1996; Chace and Walsh, 2006; McKinney, 2008) and population densities (Blair, 1996) are often highest at intermediate levels of urbanization. However, although several authors have addressed the effects of urbanization on avian stress levels and body condition (e.g. Vangestel et al., 2010; Bókony et al., 2012; Salleh Hudin et al., 2016; Meillère et al., 2017), how individuals of urban exploiters successfully cope with urban environments, remains poorly understood.

How urbanization affects disease ecology, wildlife-pathogen interactions and animal health remains particularly underexplored, despite its potential effect on ecological and evolutionary mechanisms driving population dynamics (Keesing et al., 2006; Bradley and Altizer, 2007; Evans et al., 2009a; Delgado-V and French, 2012; Hamer et al., 2012). In addition, wildlife is increasingly being recognized as an important vector, or potentially even reservoir, for various human diseases (Artois et al., 2001), such as yersiniosis, the third most commonly reported bacterial zoonotic disease in Europe in 2013 (EFSA and ECDC, 2015) In humans, yersiniosis is most frequently caused by *Yersinia enterocolitica* biotype (BT) 1B and 2-5 and to a lesser extent by *Y. pseudotuberculosis* (Thoerner et al., 2003; EFSA and ECDC, 2015). In passerines, the facultative pathogen *Y. pseudotuberculosis* is the most probable etiologic agent of yersiniosis, which typically has an acute enteric disease progression (Clark and Locke, 1962; Mair, 1973; Cork, 1999), but has on several occasions been isolated from apparently healthy birds (Mackintosh and Henderson, 1984; Niskanen et al., 2003). Although it is possible that these birds were in the incubation phase of the disease, it has been speculated that wild ranging birds maintain the bacteria at low level, developing acute disease when subjected to stressful conditions (Niskanen et al., 2003). Yet, the potential existence of subclinical effects on avian health and body condition remains a gap in our knowledge.

So far only few studies have focused on the combination of differential pathogen exposure along urbanization gradients and the effects on the body condition of their avian hosts (e.g. Delgado-V and French, 2012; Bichet et al., 2013; Galbraith et al., 2017). With respect to *Yersinia*, their psychrotolerant nature (Tashiro et al., 1991) potentially renders these bacteria susceptible to microclimate differences (e.g. heat island effect) between urbanized and rural areas (Trusilova et al., 2008). In addition, the distinct metabolic flexibility of various *Yersinia* species (Reuter et al., 2014) may affect environmental survival and persistence, enhancing the survival of the less pathogenic environmental strains with higher metabolic capacity compared to the more pathogenic strains which are metabolically more constrained and are more dependent on the presence of suitable hosts. Depending on the pathogen suitability of the hosts, higher host diversity or density may both reduce or amplify the bacterium load in the environment (Keesing et al., 2006), and hence, the faeco-oral transmission of pathogenic *Yersinia* species. Not only can *Yersinia* affect birds' health, but vice versa, avian health, related to stress and estimated by body condition (Peig and Green, 2009), could affect the excretion of pathogens in the environment (Kisková et al., 2011; Verbrugghe et al., 2012).

In order to gain more insights into urban wildlife disease ecology, we assessed the prevalence of an important zoonotic and avian pathogen (i.e. *Yersinia* spp.) in house sparrows along an urbanization gradient. House sparrows constitute an adequate study species as they inhabit rural, suburban and urban areas, they are considered to be very sedentary, and they have experienced severe population declines over the last decades, especially in urban centres (De Laet and Summers-Smith, 2007; Everaert and Bauwens, 2007; Vangestel et al., 2011; De Coster et al., 2015). We evaluated how urbanization and the local community of granivorous birds impact on house sparrows' body condition and on the presence of *Yersinia* spp. in their faeces, in combination with the two-way host-pathogen interaction, taking into account temperature and time of sparrow capture during sampling.

Materials and methods

House sparrow sampling and environmental data

Since disease outbreaks most often occur during winter (Mair, 1972; Mackintosh and Henderson, 1984; Cork et al., 1995), faecal samples from 329 house sparrows were collected during two consecutive sampling periods, i.e. 3 October till 20 December 2013 ('autumn') and 10 January until 28 March 2014 ('winter-early spring'), respectively. Sampled house sparrows originated from 36 populations located in 11 'urban', 7 'suburban' and 18 'rural' regions

(details on urbanization levels are given in the supporting information: S1 Table) clustered pairwise around the Flemish cities of Ghent, Antwerp and Leuven (Fig. 8), every population being sampled at least once per sampling period.

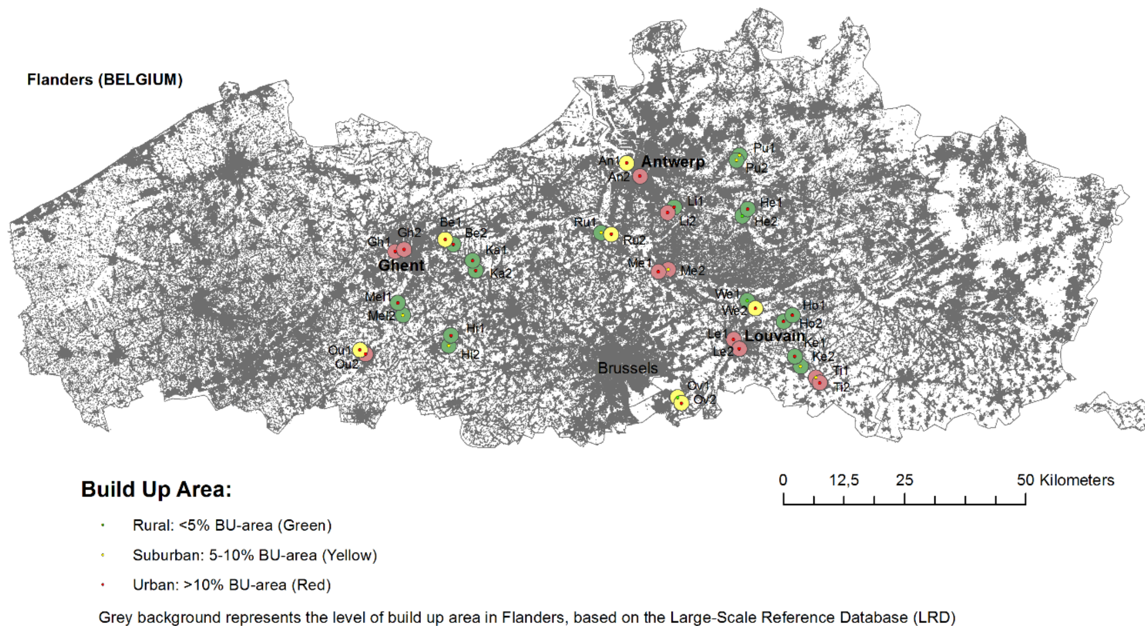

Fig 8. Sampled house sparrow populations clustered around three cities in Flanders (Ghent, Antwerp, Louvain)

The sampling protocol is as described in Rouffaer et al. (2016). Upon capture, each individual was ringed, sexed, weighed (±0.01g: digital balance) and their tarsus length was measured (±0.01mm: digital calliper). To quantify sparrow body condition, we applied the scaled-mass index (SMI), which adjusts the mass of all individuals to that which they would have obtained if they all had the same body size, using the equation of the linear regression of ln-body mass on ln-tarsus length estimated by type-II (standardized major axis; SMA) regression (Peig and Green, 2009). Two outliers were present in the data (i.e. |standardized residuals| > 3), these two observations were not considered for deriving the SMI relationship. The regression slope was 1.50 and average tarsus length was 18.8 mm. We thus calculated the SMI as body mass x (18.8/tarsus length)^1.50 (Peig and Green, 2009). House sparrows are considered species of Least Concern on the 'IUCN Red List of Threatened Species' (BirdLife International, 2016) and all people involved in the sampling were holders of a scientific ringing certificate issued annually by the Agency for Nature and Forest. The sparrows were captured on private land for which oral permission was granted by the respective land owners. All trapping and sampling protocols were approved by the Ethical Committee VIB Ghent site (EC2013-027).

As environmental predictors, we considered the degree of urbanization, the average air temperatures at the day of sampling and the presence of other granivorous birds. In order to quantify the degree of urbanization at sampling sites, the level of built-up area (BU) was calculated in circular plots around each trapping site based on the very high resolution (i.e. 0.15m pixels) 'Large-scale Reference Database' (LRD) GIS layers (AGIV, 2013a and 2013b), both at a local 'home range' scale (using a 100 m radius around the capture site) and at a 'landscape' scale (using a 1600 m radius around capture site, thereby excluding the 100 m radius of the home range scale) (Melles et al., 2003; Vangestel et al., 2011). The extent of the home range scale was based on radio-telemetric observations of habitat use by Flemish house sparrows (Vangestel et al., 2010) and represents the extent of daily foraging movements. The landscape scale was based on population genetic estimates (Vangestel et al., 2011) and reflects the average distance at which sparrow populations can genetically be considered independent from each other. To ensure a more natural environment for the lowest urbanization class, we only selected plots comprising >20% of ecologically valuable areas, as described by the Flemish Governments' Biological Valuation Map (Vriens et al., 2011). Urbanization at the home range scale was modelled as a continuous variable (range 1.72-55.04% BU area), while at the landscape scale, it was modeled as class variable, i.e. 'rural' (<5% built-up area), 'suburban' (5-10%) or 'urban' (>10%) (Teyssier et al., 2018). Average daily temperatures were derived from the nearest located weather station and were provided by the Belgian Royal Meteorological Institute (RMI). For every house sparrow population under study, a granivore-index was calculated, i.e. indicating the degree to which a local bird assemblage is dominated by granivorous species which could, through similar foraging strategies, have a higher potential of exchanging enteropathogenic bacteria through the faeco-oral transmission route (Brittingham and Temple, 1988; Pennycott et al., 2002; Refsum et al., 2003; Perkins et al., 2007). Since conducting bird surveys during sampling was not feasible because of logistic reasons, we relied on data collected during the most recent Flemish breeding bird atlas (Vermeersch et al., 2004a) whereby the Flemish region was divided in a grid of 5km x 5km. Within each of these squares, bird surveyors were instructed to carry out two one-hour long visits to sets of eight fixed 1km x 1km squares in order to arrive at a list of breeding bird species (see Vermeersch et al. 2004b for details). For each sparrow sampling site, we determined the closest (5x5 km) grid cell sampled by the breeding bird atlas (using Euclidean distance) and extracted the species list for that grid cell. Each bird species present was assigned a 'granivore score', varying from 0 to 1, based on bird diets as mentioned in Cramp and Perrins (1985). Following Sol et al. (2014), scoring was as follows: 0 = no grains, 0.1 = occasionally grains,

0.5 = frequently grains, 1 = almost exclusively grains. In order to obtain an overall 'granivore-index' for each sparrow sampling site, we summed the granivore-scores of all birds present in a grid cell and divided this sum by the total number of bird species present.

Yersinia *isolation and identification*

Faecal samples were subjected to a cold enrichment procedure in combination with an alkali (KOH) treatment as described in Rouffaer et al. (2017). This isolation method has previously been demonstrated to be the most successful method for the isolation of *Y. pseudotuberculosis* and *Y. enterocolitica,* even when only small numbers of bacteria are present in a sample (Niskanen et al., 2002; Niskanen et al., 2003). All the colonies suspicious for *Yersinia* were biochemically tested at 30°C using Kligler (Oxoid, Ltd), Aesculine (Oxoid, Ltd.) and Urea (Oxoid, Ltd), before performing MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry) at the Department of Clinical Microbiology, Laboratory Medicine, AZ Sint-Lucas in Ghent. Every MALDI-TOF assigned-*Y. enterocolitica* and *Y. pseudotuberculosis* was subjected to virulence PCR on chromosomal- (*ail, ystA, ystB, inv*) and plasmid-borne (*virF*) virulence genes, according to the PCR-protocol and primers used by Thoerner and others (2003). *Yersinia pseudotuberculosis* (22.36a), human pathogenic *Y. enterocolitica* 4/O:3 (75.55b) and *Y. enterocolitica* BT1A (FAVV208) were used as positive controls. If virulence genes were detected, *Y. pseudotuberculosis* isolates were serotyped at the National Reference Center *Yersinia* (IREC).

Although PCR on the combination of chromosomal- and plasmid-borne virulence genes and MALDI-TOF MS has previously been used for the identification of (enteropathogenic) *Y. enterocolitica* and *Y. pseudotuberculosis* (Thoerner et al., 2003; Singh and Virdi, 2004; Ayyadurai et al., 2010; Stephan et al., 2011; Stephan et al., 2013; Rouffaer et al., 2017), the accurate species identification of the latter technique is highly dependent on the validation of the reference library used to identify the bacterial isolates, resulting in high sensitivity and specificity for the validated species (Seng et al., 2009; Ayyadurai et al., 2010; Stephan et al., 2011). This validation was performed for *Y. pseudotuberculosis* and *Y. enterocolitica* on the Bruker Daltonik MALDI Biotyper at the Department of Clinical Microbiology (CLSI, 2015), but not for other *Yersinia* species. As such, the *Yersinia* species other than *Y. enterocolitica* and *Y. pseudotuberculosis* were not identified up to species level and are included in the statistics as "*Yersinia* species".

Statistical analyses

First, in order to test whether *Yersinia* spp. prevalence was related to the degree of urbanization and presence of possible host species (expressed by the granivore-index), we applied Generalized Linear Mixed Models (GLMM) (Bates et al., 2015; Bates et al., 2016) with a binomial error distribution, using the R 'lme4', 'lmerTest', 'Hmisc', 'plyr' and 'effects' packages (Bates et al., 2016; Fox et al., 2016; Harrell, 2016; Wickham, 2016). Degree of urbanization at home range and landscape scales (and the two-factor interaction), granivore-index, daily average temperature, sex and host SMI were modelled as fixed effects, while sampling period was modeled as a random effect using the glmer command. To account for possible spatial autocorrelation in *Yersinia* prevalence, latitude and longitude of sampling locations were included as fixed effects (Dormann et al., 2007). Separate models were run to identify factors influencing the distribution of "*Y. enterocolitica", "Y. pseudotuberculosis",* "*Yersinia* spp. other than *Y. enterocolitica* and *Y. pseudotuberculosis".* We applied a model selection procedure based on Akaike's Information Criterium AIC (Burnham and Anderson, 2002) and calculated AICc values for all possible models, using the R MuMIn package (Barton, 2015). Models were ranked based on their AICc values, and the relative importance of variables was assessed by summing the AICc weights of all models in which the variable under consideration was included. Important variables are characterized by a high AICc weight (i.e. >0.5) and model-averaged estimates that are higher than their standard errors (Anderson, 2008).

Second, to test whether host SMI was impacted by *Yersinia* spp. along the urbanization level, we applied a linear mixed model (LMM) using a Gaussian error distribution, including presence or absence of *Y. enterocolitica, Y. pseudotuberculosis* or other *Yersinia* spp., degree of urbanization at home range and landscape scales (and two-factor interaction), sex, granivore-index, daily average temperature and time (hour) of capture as fixed effects, and sampling period as random effect, using the same packages as for the GLMM, and the lmer-function. Model residuals were normally distributed (Shapiro-Wilk $W > 0.95$). Since the AIC-weight of the two-way interaction (see higher) was low (<0.5) for all the GLMM and LMM analyses, models were rerun without interaction to obtain final AIC-weights. All analyses were conducted in R (R Development Core Team)

Results

In total, 329 house sparrows (143 females, 186 males) were captured from rural (51%), suburban (14%) and urban habitats (35%) (S1 Table). All individuals, with the exception of one bird which was diagnosed with poxvirus (Rouffaer et al., 2016), were apparently healthy. *Yersinia* species were isolated from 59% (193/329) of the examined hosts with *Y. enterocolitica* being the most commonly isolated *Yersinia* species, isolated from 31% (103/329) of the individuals (S1 Table). Except for the *ystB*-gene*,* identified in 92 (89%) of the *Y. enterocolitica* isolates, none of the isolates harbored the examined virulence genes. *Y. pseudotuberculosis* was recovered from 2% (8/329) of the hosts (S1 Table). With four isolates, serotype I was the most encountered serotype. Two isolates were identified as serotype II and two as serotype III and V respectively. All the isolates, apart from both serotype II isolates, originated from different house sparrow populations. Except for serotype III and V, which did not possess the *virF* plasmid-borne virulence gene, both the *inv*- and *virF*-gene were detected in the different serotypes. *Yersinia* species, other than *Y. enterocolitica* and *Y. pseudotuberculosis* were isolated from 41% (134/329) of the house sparrows. In total 51 house sparrows harbored multiple *Yersinia* species in their faeces.

When testing for drivers of different *Yersinia* spp. presence in house sparrow' faeces, AIC-based model averaging appointed different variables as important explanatory variables, depending on the *Yersinia* species tested (Table 3). Presence of *Y. pseudotuberculosis* was best explained by the granivore-index, for which a positive relationship was observed (AIC-weight: 0.90, estimate ± standard error: 1.18±0.59; Table 4 and 5). In addition, landscape-level urbanization influences *Y. pseudotuberculosis* distribution: compared to rural habitats, this species tends to be most prevalent in suburban habitats, and to a lesser extent in urban habitats (AIC-weight: 0.61, estimate: 2.83±1.35 and 1.95±1.08 resp.; Table 4 and 5). No strong evidence for an effect of host SMI, sex, daily average temperature and home range level factor on presence of *Y. pseudotuberculosis* was evident (AIC-weights <0.5; Table 4). Variables best explaining the presence of *Y. enterocolitica* were, in order of importance, daily average temperature, the granivore-index, the percentage of built-up area at the home range scale and, to a lesser extent, at the landscape scale. *Yersinia enterocolitica* was negatively correlated to daily average temperatures (AIC-weight: 1.00, estimate: -0.68±0.17), to the granivore-index (AIC-weight: 0.92, estimate: -0.39±0.15) and to the percentage of built-up area at home range level (AIC-weight: 0.75, estimate: -0.32±0.16) (Table 4 and 5). At the landscape level, the prevalence of *Y. enterocolitica* tends to be lower in suburban house sparrows, compared to the

urban and (to a lesser degree) to the rural birds (AIC-weight: 0.59, estimate: 0.96±0.48 and 0.79±0.49 resp.; Table 5). Nor the SMI, nor the sex influenced *Y. enterocolitica* prevalence (AIC-weight: <0.5; Table 4). Presence of other *Yersinia* species was best explained by the average daily temperature (AIC-weight: 0.92, estimate: -0.31±0.12), to which it was negatively related, and by the home-range level (AIC-weight: 0.67, estimate: -0.21±0.12), as *Yersinia* species tended to be less prevalent in more urbanized core habitats (Table 4 and 5).

After accounting for the effect of time of capture (AIC weight: 0.76, 0.06±0.0.3), we found that sparrow body condition (i.e. SMI) was correlated to landscape level urbanization (AIC weight: 0.64) (Table 3-5). The SMI was generally higher for suburban house sparrows compared to either urban (estimate: -0.43±0.18) or rural house sparrows (estimate: -0.27±0.17) (Table 5). Specifically, suburban sparrows were on average 3% heavier than urban birds and 2% than rural sparrows. Presence of *Y. enterocolitica, Y. pseudotuberculosis* or other *Yersinia* species, average daily temperatures, sex, granivore-index or home range level urbanization did not affect hosts SMI (all variable AIC-weights <0.5; Table 4).

Response variable: explanatory variables	Log(L)	AIC	ΔAIC	weight
Y. pseudotuberculosis: Granivore-index, Urbanization (landscape level)	-32.21	78.76	0.00	0.64
Y. enterocolitica: Average temperature, Granivore-index, Urbanization (home range level), Urbanization (landscape level)	-185.47	389.50	0.00	0.42
Other *Yersinia* species: Average temperature, Urbanization (home range level)	-218.75	449.75	0.00	0.60
SMI: Time of capture, Urbanization (landscape level)	-465.15	946.74	0.00	0.55

Table 3. Best models using AIC-based model selection for *Y. pseudotuberculosis, Y. enterocolitica,* other *Yersinia* species and Scaled Mass Index as respective response variables

	Y. pseudotuberculosis	*Y. enterocolitica*	Other *Yersinia* species	SMI
Granivore-index	**0.90**	**0.92**	0.32	0.49
Urbanization (landscape level)	**0.61**	**0.59**	0.16	**0.64**
Urbanization (home range level)	0.38	**0.75**	**0.67**	0.48
Average temperature	0.39	**1.00**	**0.92**	0.27
Scaled Mass Index	0.26	0.30	0.35	NA
Seks	0.44	0.26	0.44	0.38
Time of Capture	NA	NA	NA	**0.76**
Y. pseudotuberculosis	NA	NA	NA	0.26
Y. enterocolitica	NA	NA	NA	0.39
Other *Yersinia* species	NA	NA	NA	0.38

Table 4. Variable Importance after model-averaging in order to explain the presence of *Y. pseudotuberculosis, Y. enterocolitica* and other *Yersinia* species and the SMI of the host. NA (not applicable)

Parameters for *Y. pseudotuberculosis*	Estimate ± SE
Granivore-index	1.18±0.59
Urbanization landscape (Suburban)[a]	2.83±1.35
Urbanization landscape (Urban)[a]	1.95±1.08
Parameters for *Y. enterocolitica*	
Average temperature	-0.68±0.17
Granivore-index	-0.39±0.15
Urbanization home range	-0.32±0.16
Urbanization landscape (Urban)[b]	0.96±0.48
Urbanization landscape (Rural)[b]	0.79±0.49
Parameters for other *Yersinia* species	
Average temperature	-0.31±0.12
Urbanization home range	-0.21±0.12
Parameters for SMI	
Time of capture	0.06±0.0.3
Urbanization landscape (Urban)[b]	-0.43±0.18
Urbanization landscape (Rural)[b]	-0.27±0.17

Table 5. Parameter estimates and standard deviation for response variables: *Y. pseudotuberculosis, Y. enterocolitica,* other *Yersinia* species and SMI (shown in Table 3)

[a] Urbanization within 1600m radius is compared to the Rural habitat

[b] Urbanization within 1600m radius is compared to the Suburban habitat

Discussion

A high prevalence of *Yersinia* was demonstrated in the faeces of the examined house sparrows, of which most isolates belonged to *Y. enterocolitica* and only a small percentage to *Y. pseudotuberculosis.* These results are in agreement with previous reports using cold enrichment methods (Cork et al., 1995; Niskanen et al., 2003; Kisková et al., 2011). Apart from the *ystB*-gene, which was demonstrated in most of the *Y. enterocolitica* isolates and is associated with biotype 1A (Thoerner et al., 2003; Singh and Virdi, 2004), no human pathogenic *Y. enterocolitica* biotype was recovered from our house sparrows. In humans, controversy exist regarding the pathogenicity of *Y. enterocolitica* BT1A (Tennant et al., 2003; Stephan et al., 2013), in birds however no case reports related to disease caused by BT1A were found. This could either be an indication that *Y. enterocolitica* BT1A does not tend to be pathogenic in birds, or that only limited research has been conducted on the pathogenicity of *Y. enterocolitica* BT1A in birds.

On the contrary, all recovered serotypes of *Y. pseudotuberculosis,* with serotype I being the most encountered serotype in Europe (Niskanen et al., 2003; EFSA, 2007; Niskanen et al., 2009), have been implicated in yersiniosis cases and outbreaks in birds and mammals, including humans (Bradley and Skinner, 1974; Nakano et al., 1989; Fukushima et al., 1989; Cork et al., 1995; Nuorti et al., 2004; EFSA, 2007; Niskanen et al., 2009), but have also been isolated from apparently healthy birds and mammals (Mackintosh and Henderson, 1984; Hamasaki et al., 1989; Fukushima et al., 1991; Cork et al., 1995; Niskanen et al., 2003). The absence of the *virF* plasmid-borne virulence gene in serotype III and V is potentially an indication of a decrease in virulence (Fukushima et al., 1991; Nagano et al., 1997), however, loss of the pYV virulence plasmid during the isolation, or purification procedure cannot be ruled out (Thoerner et al., 2003; Niskanen et al., 2009). Since none of the positive birds in our study were recaptured, no inference can be made whether these house sparrows were temporary carriers with the potential of eliminating the pathogen, whether the passerines were in the incubation phase of the disease or actually presented a wildlife reservoir of *Y. pseudotuberculosis*.

The dominant feeding strategy of the local bird assemblage affected the presence of *Y. pseudotuberculosis* and *Y. enterocolitica* in opposite ways. As for the pathogenic *Y. pseudotuberculosis*, higher prevalence of these bacteria was detected when the local bird populations were dominated by granivorous species, such as the highly susceptible Fringillidae (Cork et al., 1999; Sandmeier and Coutteel, 2005), which, by using similar foraging strategies could enhance faeco-oral transmission (Brittingham and Temple, 1988; Pennycott et al., 2002;

Refsum et al., 2003; Perkins et al., 2007). On the other hand, *Y. enterocolitica* BT1A was negatively influenced by the degree of granivory of local bird communities, which could be an indication that, at least for this *Yersinia* species, granivourous birds are less suitable hosts, or carriers than birds with other feeding patterns (Novotný et al., 2007; Benskin et al., 2009). With respect to the other *Yersinia* species, no relation with granivory was demonstrated, suggesting that other, potentially more abiotic factors drive the distribution and prevalence of these *Yersinia* species (Reuter et al., 2014). However, since the group "*Yersinia* species" most likely comprises various species, the effect of granivores on the species-group could be neutralized due to counteracting effects on the separate *Yersinia* species. We should also keep in mind that for all analyses, the density of the different bird species was not taken into account, nor were other animals that could potentially act as a reservoir, which could likewise alter disease ecology (Keesing et al., 2006).

The prevalence of *Yersinia enterocolitica* and other *Yersinia* species was highly affected by the average daily temperature, being more prevalent when temperature was lower. As was previously observed when comparing *Yersinia*-survival in soil and water at different temperatures (Tashiro et al., 1991), the increased survival at colder temperatures potentially increases the bacteria load in the environment and subsequently the prevalence in faeces. No such an effect was observed for *Y. pseudotuberculosis*, however the low prevalence likely decreased the power of the statistical analyses and potentially obscured potential relationships between temperature and prevalence.

The amount of built-up area had various effects on the presence of *Yersinia*. At the landscape scale, *Y. pseudotuberculosis* tended to be more prevalent in suburban hosts, and to a lesser extent in urban ones, compared to rural individuals. Although not investigated in our study, previous research has demonstrated higher densities of urban exploiters in suburban and urban regions (Blair, 1996; Evans et al., 2009b), which could enhance the pathogen transmission in these habitats. On the contrary, *Y. enterocolitica* BT1A tends to be less prevalent in suburban house sparrows. The higher prevalence observed in the more urban areas could, similarly as for *Y. pseudotuberculosis,* be related to the higher density of other urban exploiters (Blair, 1996; McKinney, 2006). In rural areas on the other hand, other animals such as rodents, hares and livestock (Frandölich et al., 2003; Vanantwerpen et al., 2014; Rouffaer et al., 2017), possibly contribute to an increased occurrence of *Y. enterocolitica* BT1A in the examined house sparrows. Nevertheless, further investigations are warranted, including different taxa, and taking densities of all potential host species into account.

At the scale of individual home ranges, *Y. enterocolitica* and other *Yersinia* species were shown to be less prevalent in more urbanized habitats. This could be explained by the lower permeability of the surfaces in the more urbanized habitats, from which water excess is lost through runoff and as such dry up relatively faster compared to actual soil substance (Tashiro et al., 1991; Trusilova et al., 2008). Since *Yersinia* species are known to have a higher survival in wet to damp soil (Tashiro et al., 1991) the prevalence will likely be higher in less urbanized local habitats. The SMI did not have an influence on the presence of *Y. pseudotuberculosis, Y. enterocolitica* or other *Yersinia* species, neither did these *Yersinia* isolates affect the SMI of the house sparrows. With respect to *Y. enterocolitica* BT1A and the environmental *Yersinia* spp. it has been suggested that these *Yersinia* species are part of the normal avian microbiota (Niskanen et al., 2003; Kisková et al. 2011), which could explain the lack of effect on house sparrows SMI. Nevertheless, only limited research has been performed on the pathogenicity of *Y. enterocolitica* BT1A and environmental *Yersinia* species in birds. *Yersinia pseudotuberculosis* on the other hand is known to be pathogenic for Passerines, and as such, a bidirectional effect of SMI and *Y. pseudotuberculosis* was expected. The lack of effect in either direction could be due to the low prevalence of *Y. pseudotuberculosis* in our house sparrow populations. However, as Niskanen et al. (2003) previously suggested, wild birds potentially are able to sustain *Y. pseudotuberculosis* at low levels, without clinical signs, developing acute disease when exposed to stressful conditions.

The SMI was observed to increase from the morning to the afternoon, probably related to overnight fasting (Milenkaya et al., 2013; Galbraith et al., 2017) although this observation is not always apparent (Meillère et al., 2015). Regarding the effect of urbanization on house sparrow body condition, most studies have compared strongly urbanized with rural habitats, disregarding the suburban areas (e.g. Bókony et al., 2012; Meillère et al., 2015; Salleh Hudin et al., 2016; Meillère et al., 2017). In this study, no significant differences were observed between populations from rural and strongly urbanized habitats, however, individuals from suburban populations had a higher SMI compared to urban populations (and to a lesser extent rural ones). Body condition has earlier been associated with stress response and overall health (Peig and Green, 2009; Bókony et al., 2012), though environmental factors such as habitat coverage (Vangestel et al., 2010), predictability of food supply and quality (Salleh Hudin et al., 2016), presence of predators (Vangestel et al., 2010) have been hypothesized to influence the body condition of the birds. Suburban habitats in Flanders are typically characterized by strongly connected hedges and bushes, which are generally considered good habitat for house

sparrows, allowing for a higher foraging efficiency compared to more fragmented highly urbanized or rural habitats. Indeed, Vangestel et al. (2010) found that suitable foraging and shelter sites are highly scattered in urban areas. In rural areas, shelter sites are more connected than in highly urbanized areas, but the presence of intensive agricultural fields forces sparrows to occupy larger home ranges, increasing the energy expenditure when patrolling the entire home range, and thus potentially decreasing the body condition (Vangestel et al. 2010).

In conclusion, we here show that the urbanization gradient affects body condition and pathogen occurrence, two determinants of pathogen infection dynamics, suggesting a potential impact of urbanization on avian health. When assessing the impact of urbanization on animal health and pathogen dynamics, information regarding the presence and absence and preferably also the density of other suitable hosts, the two-way interaction between pathogen and host, and various levels of urbanization including the suburban habitat is required in order to have a better understanding of how urbanization can have an impact on urban wildlife health and diseases.

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Supporting Information

Plot	Landscape %BU	Home-range %BU	*Y. pseudotuberculosis*	*Y. enterocolitica* BT1A: #positive/total
Ghent (Gh)1	18.27	23.58	Serotype I	0/9
Gh2	38.98	41.39	Serotype I	7/14
Beervelde (Be)1	7.30	15.80	-	1/5
Be2	4.62	10.52	-	9/13
Kalken (Ka)1	4.62	20.88	-	3/8
Ka2	3.74	10.67	-	8/14

Hillegem (Hi)1	4.62	22.17	-	1/6
Hi2	4.65	5.97	-	7/12
Melsen (Mel)1	3.81	13.97	-	0/8
Mel2	2.13	7.20	-	0/6
Oudenaarde (Ou)1	9.68	10.03	-	1/10
Ou2	17.17	14.63	Serotype I	1/6
Antwerp (An)1	7.51	23.51	-	1/7
An2	24.52	55.03	-	1/11
Pulderbos (Pu)1	2.56	5.11	-	4/10
Pu2	3.68	7.04	-	11/16
Herenthout (He)1	2.72	27.83	Serotype I	0/11
He2	1.91	1.72	-	0/5
Lint (Li)1	4.30	16.91	-	2/7
Li2	11.79	31.61	-	3/7
Mechelen (Me)1	22.14	27.37	-	4/11
Me2	16.58	6.28	-	2/10
Ruisbroek (Ru)1	3.09	5.95	-	0/9
Ru2	6.09	19.97	-	2/4
Leuven (Le)1	16.97	14.02	-	4/11
Le2	27.90	33.75	-	6/9
Wezemaal (We)1	4.69	3.28	-	0/6
We2	5.31	30.83	Serotype V	1/9
Houwaart (Ho)1	1.66	17.13	Serotype III	3/9
Ho2	1.50	10.12	-	2/8
Kerkom (Ke)1	1.99	14.32	-	2/11
Ke2	1.82	7.16	-	6/10
Tienen (Ti)1	10.86	7.73	Serotype II	6/12
Ti2	17.85	27.20	-	2/15
Overijse (Ov)1	8.99	3.71	-	2/5
Ov2	8.23	39.41	-	1/5

S1 Table. Sampled house sparrow populations, indicating the percentage of Built-Up-area in the local and landscape scale and providing information regarding presence or absence of *Y. pseudotuberculosis* and *Y. enterocolitica*. Abbreviations similar as in Fig 8.

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CHAPTER 4: LOW PREVALENCE OF HUMAN ENTEROPATHOGENIC *YERSINIA* SPP. IN BROWN RATS (*RATTUS NORVEGICUS*) IN FLANDERS

GHENT UNIVERSITY

LOW PREVALENCE OF HUMAN ENTEROPATHOGENIC *YERSINIA* SPP. IN BROWN RATS (*RATTUS NORVEGICUS*) IN FLANDERS

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Abstract

Brown rats (*Rattus norvegicus*) have been identified as potential carriers of *Yersinia enterocolitica* and *Y. pseudotuberculosis,* the etiological agents of yersiniosis, the third most reported bacterial zoonosis in Europe. Enteropathogenic *Yersinia* spp. are most often isolated from rats during yersiniosis cases in animals and humans, and from rats inhabiting farms and slaughterhouses. Information is however lacking regarding the extent to which rats act as carriers of these *Yersinia* spp.. In 2013, 1088 brown rats across Flanders, Belgium, were tested for the presence of *Yersinia* species by isolation method. Identification was performed using MALDI-TOF MS, PCR on chromosomal- and plasmid-borne virulence genes, biotyping and serotyping. *Yersinia* spp. were isolated from 38.4% of the rats. Of these, 53.4% were designated *Y. enterocolitica,* 0.7% *Y. pseudotuberculosis* and 49.0% other *Yersinia* species. Two *Y. enterocolitica* possessing the *virF-, ail-* and *ystA*-gene were isolated. Additionally, the *ystB*-gene was identified in 94.1% of the other *Y. enterocolitica* isolates, suggestive for biotype 1A. Three of these latter isolates simultaneously possessed the *ail*-virulence gene. Significantly more *Y. enterocolitica* were isolated during winter and spring compared to summer. Based on our findings we can conclude that brown rats are frequent carriers for various *Yersinia* spp., including *Y. pseudotuberculosis* and (human pathogenic) *Y. enterocolitica* which are more often isolated during winter and spring.

Introduction

Yersiniosis was the third most commonly reported bacterial zoonotic disease in Europe in 2013, causing illness in 1.92 out of 100 000 inhabitants (EFSA and ECDC, 2015). The etiologic agents are human pathogenic *Yersinia enterocolitica* biotype (BT) 1B and 2-5, which possess chromosomally encoded virulence genes and carry the pYV (plasmid for *Yersinia* virulence), and to a minor extent *Y. pseudotuberculosis* (Thoerner et al., 2003; EFSA and ECDC, 2015). *Y. enterocolitica* BT1A is most commonly regarded as nonpathogenic and often possesses the chromosomally encoded *ystB*-gene (Tennant et al., 2003). Wildlife has increasingly been recognized as reservoir, or vector for various zoonotic diseases (Artois et al., 2001). Especially rodents, such as the brown rat (*Rattus norvegicus*), have been appointed as potential carriers of pathogenic *Yersinia* spp.. Since brown rats are considered synanthropic rodents (Traweger et al., 2005) they can be a possible source of infection for humans and other animals (Mair, 1973; Cork et al., 1999; Battersby et al., 2002; Backhans et al., 2011). To evaluate food safety and human health risks, most of the studies on prevalence and epidemiology of *Yersinia* spp. in small mammals have therefore been conducted during yersiniosis outbreaks in animals and humans, in urban areas, in the surroundings of (pig) farms and in slaughterhouses (Kaneko et al., 1978; Mackintosh and Henderson, 1984; Battersby et al., 2002; Kangas et al., 2008; Backhans et al., 2011). Although epidemiologically important, information is lacking regarding the extent to which rats represent a potential reservoir of human pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis*.

In this study we assessed the prevalence of *Y. enterocolitica* and *Y. pseudotuberculosis* in brown rats across Flanders, Belgium, not specifically related to disease outbreaks or to cities, with the aim to evaluate the contribution of brown rats as carriers of these *Yersinia* spp..

Materials and methods

Within the framework of a rodenticide resistance study conducted by the Research Institute for Nature and Forest (INBO), in 2013, a total of 1088 brown rats were caught across Flanders, Belgium, by certified pest control operators of the Flanders Environment Agency (VMM) using wire mesh live traps measuring 50 length x 15 width x 13 height (cm). Most brown rats were captured on public land and occasionally on private land when oral permission was granted by the respective land owners. The brown rats were humanely killed by a percussive blow on the head (Directive 2010/63/EU; Annex IV) in the context of the pest control as stated by the Belgian legislation concerning animal protection and welfare (KB 14/08/86 art.15). According

to the same legislation (KB 14/08/86 art.3.15) the killing of animals, only for the use of their organs and tissues, is not considered as an animal experiment. Therefore an approval of an ethical committee, as foreseen by the Belgian legislation concerning the protection of laboratory animals (KB 29/05/13), was not required. The brown rat is considered a major pest species which is legally controlled, for the trapping and killing of the rats no legal permits were required. Most individuals (71%) were captured in rural areas. The capture occasions were predominantly during the spring period (76.8%), while 18.1% of the rats were caught during summer, 2.7% during fall and 2.5% during winter. Capture dates were missing for 37 rats.

All the trapped individuals were kept frozen (-20°C) until April 2014, after which 0.5g of colon content was collected for the isolation of *Yersinia* spp.. Cold (4°C) enrichment was performed for three weeks using a 1/10 dilution of colon content in Phosphate Buffered Saline supplemented with 0.5% Peptone, 1% Mannitol and 0.15% Bile Salts (PMB). Before plating out onto cefsulodin–irgasan-novobiocin (CIN)-agar (Bio-rad, UK), an alkali treatment was performed using a 1/10 dilution of PMB-sample in KOH-solution (0.25%KOH, 0.75%NaCl) which was vortexed for 20 seconds. The CIN plates were incubated for 24 hours at 30°C, and reassessed after being kept at room temperature for 24 hours. Suspicious colonies were purified onto MacConkey agar (Oxoid, Hampshire, UK). MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization- Time-of-Flight Mass Spectrometry), was performed using the Bruker Daltonik MALDI Biotyper, at the Department of Clinical Microbiology, Laboratory Medicine, AZ Sint-Lucas in Ghent. The samples were cultured for 24 hours at 30°C on Columbia agar with sheep blood (Oxoid, Wesel, Germany). One colony per sample was smeared upon a MALDI steel target plate, covered with 1 µl α-cyano-4-hydroxycinnamic acid (HCCA) matrix and, after air drying, loaded into the MALDI-Biotyper. Mass Spectrometry detections were carried out with Maldi biotyper 3.0 RTC software in standard IVD settings, using the 5627 reference strains library. Every MALDI-TOF assigned-*Y. enterocolitica* and *Y. pseudotuberculosis* was tested for the presence of chromosomal- (*ail, inv, ystA, ystB*) and pYV-plasmid-borne (*virF*) virulence genes (Thoerner et al., 2003). Positive controls, *Y. enterocolitica* BT1A (FAVV208), human pathogenic *Y. enterocolitica* 4/O:3 (75.55b), and *Y. pseudotuberculosis* (22.36a), were provided by the Department of Veterinary Public Health and Food Safety of Ghent University. When *Y. enterocolitica* harboured the *ystB*-gene in combination with the *ail*-gene*,* the latter was sequenced and analyzed using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). *Ail*-positive *Y. enterocolitica* isolates were bioserotyped and *Y. pseudotuberculosis* isolates were serotyped at

the National Reference Center *Yersinia* (IREC) and at the Department of Veterinary Public Health and Food Safety of Ghent University. The MALDI-TOF profiles were compared against the biotypes with linear discriminant analysis (LDA) to try to identify discriminating peaks (Wu et al., 2003).

To test whether *Y. enterocolitica* prevalence differed between seasons, we applied a Generalized Linear Model (GLM) with *Y. enterocolitica* presence or absence as binary dependent variable and season as independent predictor variable, using a binomial error distribution. To account for possible spatial autocorrelation in *Y. enterocolitica* prevalence, latitude and longitude of capture locations were forced into the model as fixed effects, fixed predictor variables (Dormann et al., 2007). To test for differences in prevalence between seasons, contrasts were set up using the general linear hypothesis test (glht)- function of the R library 'multcomp' (multiple comparisons), resulting in Bonferroni-corrected p-values adjusted for multiple testing (Hothorn et al., 2016). All analyses were conducted in R (R Development Core Team, version 3.2.3. "Wooden Christmas-Tree").

Results and Discussion

In Flanders, *Yersinia* spp. were isolated from 418 out of 1088 (38.4%) brown rats tested (Fig. 9), which, in 13 individuals, harbored more than one *Yersinia* spp.. Of these *Yersinia* spp., 53.4% (223/418) were designated *Y. enterocolitica,* 0.7% (3/418) *Y. pseudotuberculosis*, and 49.0% (205/418) (Table 6) other *Yersinia* species.

	Spring	Summer	Fall	Winter	Total number of *Yersinia* isolates
Y. enterocolitica	196[a]	12[b]	2	7	217*
Y. pseudotuberculosis	3	0	0	0	3
Yersinia species	163	25	6	5	199*
Total number of brown rats examined	807	190	28	26	

Table 6. Total number of rats testing positive for *Yersinia,* in the respective seasons

[a] Including *Y. enterocolitica* BT 3/O:1,2,3 and one *ail* positive *Y. enterocolitica* BT 1A

[b] Including *Y. enterocolitica* BT 2/O:5,27 and one *ail* positive *Y. enterocolitica* BT 1A

*Dates of 37 brown rats were missing, six of which were identified as *Y. enterocolitica* (incl. one *ail* positive *Y. enterocolitica* BT 1A) and six environmental *Yersinia* spp..

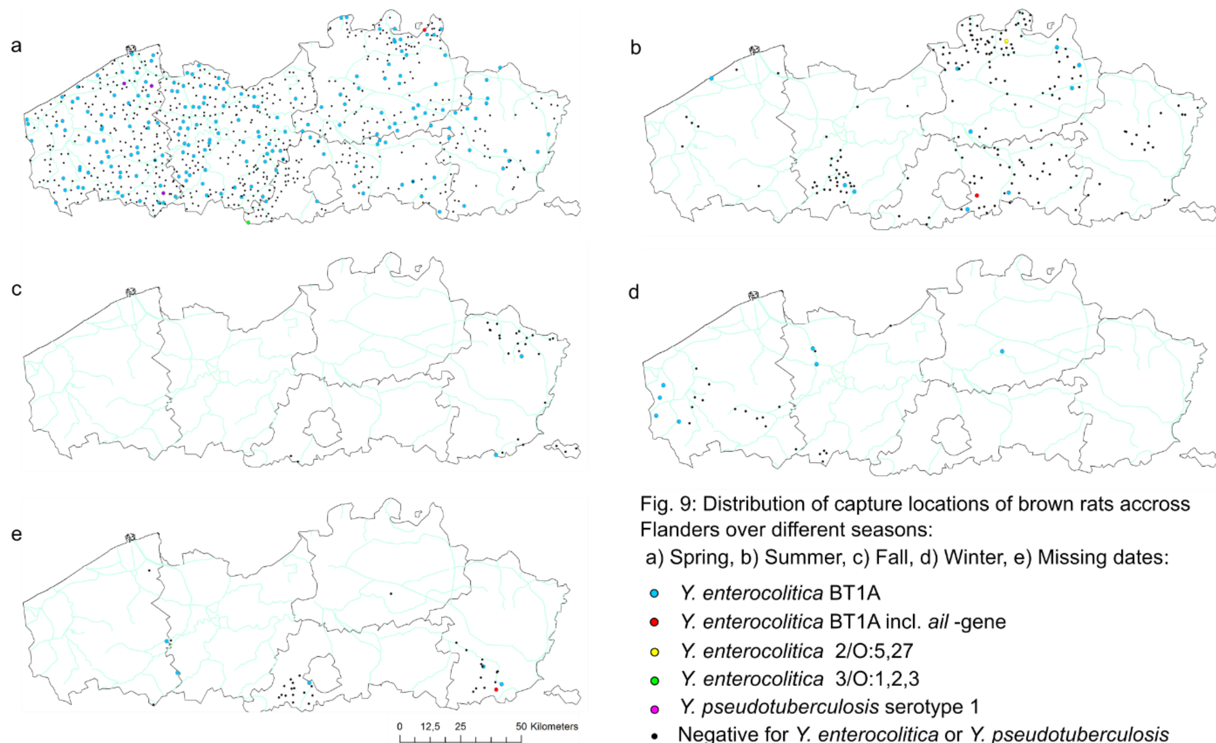

Fig. 9: Distribution of capture locations of brown rats accross Flanders over different seasons:
a) Spring, b) Summer, c) Fall, d) Winter, e) Missing dates:

MALDI-TOF MS and PCR on the combination of chromosomal- and plasmid-borne virulence genes have previously been used for the identification of (enteropathogenic) *Y. enterocolitica* and *Y. pseudotuberculosis* (Thoerner et al., 2003; Singh and Virdi, 2004; Ayyadurai et al., 2010; Kraushaar et al., 2011; Stephan et al., 2011; Stephan et al., 2013; Van Damme et al., 2015). Although MALDI-TOF MS has proven to accurately perform species specific identification of *Yersinia* spp. (Ayyadurai et al., 2010; Stephan et al., 2011), the sensitivity and specificity of the technique highly depends on the validation of the reference library used to identify the different species (Seng et al., 2009; Ayyadurai et al., 2010). Since only *Y. enterocolitica* and *Y. pseudotuberculosis* were accurately validated in the Bruker Daltonik MALDI Biotyper at the Department of Clinical Microbiology, resulting in high specificity and sensitivity (Seng et al., 2009; Stephan et al., 2011), only these species will be further discussed. The results of the MALDI-TOF MS-identification of the *Yersinia* species other than *Y. enterocolitica* and *Y. pseudotuberculosis* have been provided within the supporting information (S2 Table), although these results have to be interpreted with caution due to the lack of validation of the MALDI-TOF reference database for the other *Yersinia* spp. (Seng et al., 2009; Ayyadurai et al., 2010).

Due to the psychrotolerant nature of *Yersinia* spp., the observed prevalence could be expected to vary among seasons (Tashiro et al., 1991). Indeed, our study found a significantly higher prevalence of *Y. enterocolitica* in brown rats during winter (26.9%) and spring (24.3%)

months, compared to summer (6.3%) (P-values = 0.007 and <0.001 respectively) (Table 7). This observation is in line with previous studies in rodents and other animals (Mair, 1973; Servan et al., 1979; Mackintosh and Henderson, 1984; Liang et al., 2015), although a high prevalence of *Y. enterocolitica* during the summer has also been reported in rats (Kaneko et al., 1978).

	Estimate	Std. Error	z value	Pr(>|z|)
spring - fall == 0	1.4306	0.7571	1.890	0.20901
summer - fall == 0	-0.1705	0.8005	-0.213	0.99611
winter - fall == 0	1.6211	0.8883	1.825	0.23658
summer - spring == 0	-1.6011	0.3164	-5.060	< 0.001 ***
winter - spring == 0	0.1905	0.4561	0.418	0.97220
winter - summer == 0	1.7916	0.5519	3.246	0.00535 **

Table 7. Seasonal comparisons of *Y. enterocolitica* prevalence

Tukey's post-hoc tests for multiple contrasts were used to establish Bonferroni-corrected significant differences in *Y. enterocolitica* prevalence between seasons. Results show that compared to the summer period, *Y. enterocolitica* prevalence was higher in the spring and in the winter. Prevalence did not significantly differ between other season comparisons.

The prevalence of *Y. enterocolitica* (20.5% = 223/1088) in brown rats is similar to other studies in rodents (Oda et al., 2015). In the vast majority (93.3% = 208/223), the presence of the *ystB*-gene was demonstrated, which has been inferred to be restricted to BT1A (Ramamurthy et al., 1997; Thoerner et al., 2003; Singh and Virdi, 2004). Of these *ystB* positive isolates, three possessed an additional *ail*-virulence gene (100% identity with Accession number: FR847859.1). Controversy exists about the pathogenicity of *Y. enterocolitica* BT1A. Since most BT1A strains do not possess the typical virulence plasmid pYV, lack the chromosomal virulence genes such as the *ail*-gene and are often isolated from the environment (Tennant et al., 2003; Stephan et al., 2013), BT1A has been regarded as nonpathogenic. However, the increasing isolation of this biotype from clinical cases draws more attention to BT1A (Tennant et al., 2003; Stephan et al., 2013). Although rare, the presence of the *ail*-gene has previously been demonstrated in BT1A isolates (Kraushaar et al., 2011; Liang et al., 2015). The presence of the enterotoxin gene *ystB* in combination with the *ail*-virulence gene could be an indication that these BT1A strains possess virulent characteristics (Miller et al., 1989). However, potential loss of gene function, related to horizontal gene transfer cannot be ruled out (Kraushaar et al., 2011). The high genotypic diversity of BT1A makes the classification in clinical and nonclinical isolates more problematic since other, yet unknown, virulence factors

could be contributing to the observed virulence in some strains (Tennant et al., 2003; Stephan et al., 2013; Campioni and Falcão, 2014).

Although the majority of *Y. enterocolitica* belonged to the supposedly nonpathogenic BT1A, two human pathogenic *Y. enterocolitica* (bioserotype 2/O:5,27 and 3/O:1,2,3) (Bottone, 1999; EFSA and ECDC, 2015), possessing the *virF-*, *ail-* and *ystA*-gene virulence gene, were isolated from rats living in the proximity of livestock farms. These results are in line with other studies, indicating that *Y. enterocolitica* BT1A is widespread in rodents, but human pathogenic bioserotypes are rather rare (Kapperud 1975; Liang et al., 2015; Oda et al., 2015). The recovery of human pathogenic *Y. enterocolitica* from rodents could be related to the presence of farmhouses, as was hypothesized for bioserotype 4/O:3 and 3/O:3, for which the presence in rodents is presumed to be related to pig farms and pig slaughterhouses (Kaneko et al., 1978; Backhans et al., 2011; Liang et al., 2015; Van Damme et al., 2015). Despite the isolation of the two human pathogenic bioserotypes in the proximity of livestock farms, no definitive conclusion can be made from this observation, since the animals on the respective farms were not tested for the presence of pathogenic *Y. enterocolitica*. Furthermore, bioserotype 2/O:5,27 has been isolated from a variety of animals, such as cattle, pigs, hares and wild boars, and no primary reservoir has been identified yet (Frederiksson-Ahomaa et al., 2006; Weiner et al., 2014). Bioserotype 3/O:1,2,3, alternatively called the “chinchilla-type” (Wuthe and Aleksic, 1992), has also previously been isolated from pigs (Kwaga and Iversen, 1993). No peaks discriminating between the different biotypes could be identified in the MALDI-TOF spectra (data not shown).

Three (0.3%) *Y. pseudotuberculosis* serotype I possessing the *inv-* and *virF*-virulence gene, the most frequently isolated *Y. pseudotuberculosis* in Europe, were isolated. This serotype has been reported to cause disease in humans and other animals, such as birds and rats (Hubbert, 1972; Kaneko et al., 1979; EFSA, 2007; Kangas et al., 2008). The low percentage of *Y. pseudotuberculosis* observed in our study is in line with the absence or sporadic detection of *Y. pseudotuberculosis* in rodents in other studies (Battersby et al., 2002; Backhans et al., 2011; Liang et al., 2015; Oda et al., 2015).

Although a large number of brown rats was screened for the presence of enteropathogenic *Yersinia* spp. in Flanders, the additional investigation of other wild living animals, as potential carriers or reservoirs for enteropathogenic *Yersinia*, could substantially improve our knowledge on the epidemiology and ecology of these pathogens and the potential risk these animals pose on farm-animals and human health.

In conclusion, our results demonstrate that rats are frequent carriers for *Yersinia* spp. such as non-pathogenic and human pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis*.

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Supporting Information

S2 Table. MALDI-TOF results of *Yersinia* spp. other than *Y. enterocolitica* and *Y. pseudotuberculosis*

N°	x	y	Date	MALDI-TOF Result 1	Score 1	MALDI-TOF Result 2	Score 2	Consistency/ Mismatch
1	163600	229400	20/08/2013	*Y. frederiksenii*	2.087	*Y. enterocolitica*	1.949	B/SM
2	181263	192602	4/04/2013	*Y. enterocolitica*	2.215	*Y. kristensenii*	2.176	B/SM
3	171950	235100	31/07/2013	*Y. enterocolitica*	2.146	*Y. intermedia*	2.110	B/SM
4	217425	172725		*Y. intermedia*	2.219	*Y. enterocolitica*	2.187	B/SM
5	234575	176825	27/05/2013	*Y. enterocolitica*	2.158	*Y. kristensenii*	2.127	B/SM
6	228500	179840	6/06/2013	*Y. frederiksenii*	2.203	*Y. enterocolitica*	2.163	B/SM
7	160200	237000	23/07/2013	*Y. enterocolitica*	2.161	*Y. frederiksenii*	2.117	B/SM
8	199253	230750	15/05/2013	*Y. intermedia*	2.388	*Y. enterocolitica*	2.166	B/SM
9	161700	231600	26/08/2013	*Y. intermedia*	2.224	*Y. enterocolitica*	2.198	B/SM
10	188000	202000	10/04/2013	*Y. intermedia*	2.527	*Y. enterocolitica*	2.142	B/SM
11	189820	195180	22/05/2013	*Y. enterocolitica*	2.031	*Y. kristensenii*	1.934	B/SM
12	185000	229150	24/05/2013	*Y. rhodei*	2.345	*Y. rhodei*	2.262	A/M
13	150800	191100	22/04/2013	*Y. kristensenii*	2.474	*Y. enterocolitica*	2.368	B/SM
14	152100	235200	3/07/2013	*Y. intermedia*	2.497	*Y. enterocolitica*	2.210	B/SM
15	167300	228200	7/06/2013	*Y. enterocolitica*	2.007	*Y. kristensenii*	1.918	B/SM
16	154000	220700	29/07/2013	*Y. frederiksenii*	2.411	*Y. frederiksenii*	2.192	B/SM
17	184400	212900	5/07/2013	*Y. intermedia*	2.412	*Y. enterocolitica*	2.198	B/SM
18	177000	238000	14/08/2013	*Y. enterocolitica*	2.012	*Y. frederiksenii*	1.923	B/SM
19	159900	238100	27/06/2013	*Y. intermedia*	2.240	*Y. enterocolitica*	1.971	B/SM
20	151600	196000	29/04/2013	*Y. enterocolitica*	2.038	*Y. enterocolitica*	1.935	A/M
21	148200	221700	7/08/2013	*Y. enterocolitica*	1.935	*Y. kristensenii*	1.930	B/SM
22	176800	234000	5/07/2013	*Y. intermedia*	2.542	*Y. enterocolitica*	2.134	B/SM
23	199500	227500	8/07/2013	*Y. rhodei*	2.430	*Y. rhodei*	2.325	A/M
24	164400	230700	21/08/2013	*Y. frederiksenii*	2.273	*Y. enterocolitica*	2.202	B/SM
25	193600	215900	30/07/2013	*Y. enterocolitica*	2.167	*Y. frederiksenii*	2.126	B/SM
26	118150	163100	28/06/2013	*Y. enterocolitica*	2.229	*Y. frederiksenii*	2.192	B/SM
27	97080	159060	19/03/2013	*Y. intermedia*	2.202	*Y. enterocolitica*	2.149	B/SM
28	93760	166300	10/07/2013	*Y. enterocolitica*	2.146	*Y. enterocolitica*	1.942	A/M
29	110900	203600	25/07/2013	*Y. intermedia*	2.367	*Y. enterocolitica*	2.135	B/SM

30	165740	175300	1/07/2013	*Y. enterocolitica*	2.027	*Y. aleksiciae*	1.902	B/SM
31	135300	176600	27/06/2013	*Y. intermedia*	2.351	*Y. enterocolitica*	2.214	B/SM
32	218150	186350	15/05/2013	*Y. frederiksenii*	2.359	*Y. frederiksenii*	2.209	B/SM
33	128100	155600	9/07/2013	*Y. frederiksenii*	2.093	*Y. enterocolitica*	2.001	B/SM
34	167130	181190	7/06/2013	*Y. intermedia*	2.536	*Y. intermedia*	2.241	B/SM
35	155610	179120	18/06/2013	*Y. enterocolitica*	2.081	*Y. enterocolitica*	2.054	B/SM
36	97300	169720	1/08/2013	*Y. enterocolitica*	1.992	*Y. kristensenii*	1.821	B/SM
37	124250	159350	28/06/2013	*Y. intermedia*	2.313	*Y. intermedia*	2.076	B/SM
38	160660	179000	26/06/2013	*Y. intermedia*	2.199	*Y. enterocolitica*	2.146	B/SM
39	101420	164840	21/06/2013	*Y. intermedia*	2.396	*Y. enterocolitica*	2.213	B/SM
40	127800	154450	9/07/2013	*Y. frederiksenii*	2.227	*Y. frederiksenii*	2.055	B/SM
41	121525	156400	27/06/2013	*Y. enterocolitica*	1.969	*Y. intermedia*	1.886	A/M
42	95000	210700	9/04/2013	*Y. mollaretii*	2.207	*Y. enterocolitica*	2.167	B/SM
43	142160	161660		*Y. enterocolitica*	2.033	*Y. frederiksenii*	1.905	B/SM
44	62500	216900	23/04/2013	*Y. intermedia*	2.315	*Y. intermedia*	2.066	B/SM
45	65400	203500	8/05/2013	*Y. intermedia*	2.106	*Y. intermedia*	2.093	B/SM
46a	99700	218500	26/03/2013	*Y. kristensenii*	2.471	*Y. enterocolitica*	2.416	B/SM
46b	99700	218500	26/03/2013	*Y. intermedia*	2.163	*Y. enterocolitica*	2.054	B/SM
47a	81000	213000	26/04/2013	*Y. kristensenii*	2.213	*Y. enterocolitica*	2.204	B/SM
47b	81000	213000	26/04/2013	*Y. enterocolitica*	2.180	*Y. frederiksenii*	2.163	B/SM
48	136500	167260		*Y. intermedia*	2.445	*Y. intermedia*	2.241	B/SM
49	100900	203500	27/03/2013	*Y. kristensenii*	2.386	*Y. enterocolitica*	2.147	B/SM
50	97400	214900	16/05/2013	*Y. mollaretii*	2.114	*Y. enterocolitica*	2.079	B/SM
51	99500	198900	5/04/2013	*Y. enterocolitica*	2.051	*Y. kristensenii*	2.049	B/SM
52	73100	221600	24/04/2013	*Y. kristensenii*	2.179	*Y. enterocolitica*	2.149	B/SM
53	131400	159600		*Y. mollaretii*	2.132	*Y. enterocolitica*	2.091	B/SM
54	83200	209700	6/05/2013	*Y. intermedia*	2.216	*Y. enterocolitica*	2.083	B/SM
55	238800	165300	30/08/2013	*Y. frederiksenii*	2.057	*Y. enterocolitica*	2.020	B/SM
56	74700	220280	18/04/2013	*Y. frederiksenii*	2.526	*Y. frederiksenii*	2.285	B/SM
57	98200	195200	19/04/2013	*Y. intermedia*	2.356	*Y. enterocolitica*	2.156	B/SM
58	89200	203900	4/06/2013	*Y. intermedia*	2.414	*Y. enterocolitica*	2.164	B/SM
59	96200	207400	8/04/2013	*Y. intermedia*	2.409	*Y. enterocolitica*	2.182	B/SM
60	126123	173259	14/06/2013	*Y. enterocolitica*	2.088	*Y. kristensenii*	1.898	B/SM
61	72100	180000	4/06/2013	*Y. intermedia*	2.198	*Y. intermedia*	1.868	A/M
62	60300	170640	25/06/2013	*Y. intermedia*	2.449	*Y. enterocolitica*	2.140	B/SM
63	132122	166980	20/06/2013	*Y. intermedia*	2.226	*Y. frederiksenii*	1.825	A/M
64	112400	206900	26/06/2013	*Y. enterocolitica*	2.222	*Y. frederiksenii*	2.217	B/SM
65	78840	180060	21/06/2013	*Y. intermedia*	2.322	*Y. enterocolitica*	2.080	B/SM
66	129304	170965	14/06/2013	*Y. intermedia*	2.237	*Y. enterocolitica*	2.094	B/SM
67	137948	196073	28/05/2013	*Y. intermedia*	2.419	*Y. enterocolitica*	2.213	B/SM
68	129910	163115	20/06/2013	*Y. enterocolitica*	2.231	*Y. frederiksenii*	2.220	B/SM
69	194000	182000	9/08/2013	*Y. enterocolitica*	2.279	*Y. frederiksenii*	2.260	B/SM
70	173550	237250	29/04/2013	*Y. enterocolitica*	2.222	*Y. kristensenii*	2.145	B/SM
71	209560	160800	26/06/2013	*Y. intermedia*	2.080	*Y. kristensenii*	2.073	B/SM
72	191500	172080	13/06/2013	*Y. kristensenii*	2.024	*Y. enterocolitica*	1.979	B/SM

73	214369	197746	30/04/2013	*Y. intermedia*	2.286	*Y. enterocolitica*	2.213	B/SM
74	175150	235000	25/04/2013	*Y. mollaretii*	2.197	*Y. kristensenii*	2.019	B/SM
75	131065	188981	18/06/2013	*Y. intermedia*	2.463	*Y. enterocolitica*	2.140	B/SM
76	140586	197390	23/04/2013	*Y. kristensenii*	2.397	*Y. enterocolitica*	2.308	B/SM
77	64465	168920	25/06/2013	*Y. intermedia*	2.356	*Y. enterocolitica*	2.093	B/SM
78	78720	156660	25/06/2013	*Y. intermedia*	2.128	*Y. enterocolitica*	2.101	B/SM
79	147814	188073	28/05/2013	*Y. enterocolitica*	2.105	*Y. bercovieri*	1.984	B/SM
80	157300	237700	20/08/2013	*Y. intermedia*	2.432	*Y. enterocolitica*	2.181	B/SM
81a	196000	235650	30/04/2013	*Y. mollaretii*	2.187	*Y. kristensenii*	1.987	B/SM
81b	196000	235650	30/04/2013	*Y. intermedia*	2.186	*Y. intermedia*	1.962	B/SM
82	131600	205600	19/06/2013	*Y. intermedia*	2.319	*Y. enterocolitica*	2.216	B/SM
83	128300	212000	2/07/2013	*Y. kristensenii*	2.285	*Y. enterocolitica*	2.269	B/SM
84	133070	187311	21/06/2013	*Y. intermedia*	2.160	*Y. intermedia*	2.106	B/SM
85	61200	180500	5/03/2013	*Y. enterocolitica*	2.123	*Y. enterocolitica*	1.971	A/M
86	177000	229180	4/06/2013	*Y. intermedia*	2.203	*Y. enterocolitica*	2.042	B/SM
87	154000	237250	24/07/2013	*Y. intermedia*	2.313	*Y. enterocolitica*	2.142	B/SM
88	73800	211000	14/05/2013	*Y. mollaretii*	2.236	*Y. enterocolitica*	2.117	B/SM
89	198120	232650	2/05/2013	*Y. intermedia*	2.180	*Y. enterocolitica*	2.010	B/SM
90	61700	202600	17/05/2013	*Y. intermedia*	2.297	*Y. enterocolitica*	2.217	B/SM
91	195900	238135	22/04/2013	*Y. rhodei*	2.454	*Y. rhodei*	2.351	A/M
92	87900	197500	8/05/2013	*Y. frederiksenii*	2.183	*Y. enterocolitica*	2.147	B/SM
93	206770	187185	26/04/2013	*Y. enterocolitica*	2.354	*Y. kristensenii*	2.343	B/SM
94	182000	175250	13/08/2013	*Y. intermedia*	2.251	*Y. enterocolitica*	1.967	B/SM
95	177750	224650	12/06/2013	*Y. intermedia*	2.248	*Y. enterocolitica*	2.044	B/SM
96	232800	202000	28/10/2013	*Y. intermedia*	2.288	*Y. enterocolitica*	2.025	B/SM
97	230800	195700	22/07/2013	*Y. kristensenii*	2.306	*Y. enterocolitica*	2.242	B/SM
98	193460	178680	11/06/2013	*Y. frederiksenii*	2.594	*Y. frederiksenii*	2.401	BSM
99	200630	161340	21/08/2013	*Y. enterocolitica*	2.017	*Y. frederiksenii*	1.886	B/SM
100	70100	200000	5/06/2013	*Y. intermedia*	2.314	*Y. enterocolitica*	1.978	B/SM
101	193270	168000	24/05/2013	*Y. kristensenii*	2.445	*Y. enterocolitica*	2.344	B/SM
102	125184	170205	13/06/2013	*Y. intermedia*	2.327	*Y. intermedia*	1.999	B/SM
103	75800	174800	13/06/2013	*Y. enterocolitica*	2.076	*Y. frederiksenii*	2.076	B/SM
104	75100	201800	7/05/2013	*Y. rhodei*	2.304	*Y. rhodei*	2.273	A/M
105	109899	161090	9/04/2013	*Y. enterocolitica*	2.323	*Y. kristensenii*	2.269	B/SM
106	53900	196500	25/04/2013	*Y. intermedia*	2.275	*Y. enterocolitica*	2.244	B/SM
107	54200	187700	28/03/2013	*Y. intermedia*	2.272	*Y. enterocolitica*	2.003	B/SM
108	112489	168576	2/04/2013	*Y. frederiksenii*	2.202	*Y. enterocolitica*	2.185	B/SM
109	56000	194800	23/04/2013	*Y. intermedia*	2.230	*Y. intermedia*	1.998	B/SM
110	26100	180000	8/04/2013	*Y. kristensenii*	2.343	*Y. kristensenii*	2.264	B/SM
111	43600	181300	18/02/2013	*Y. aleksiciae*	2.099	*Y. intermedia*	1.993	B/SM
112	43600	202000	17/05/2013	*Y. frederiksenii*	1.935	*Y. rhodei*	1.712	A/M
113	116768	167346	29/03/2013	*Y. mollaretii*	2.184	*Y. kristensenii*	1.998	B/SM
114	50600	174800	8/04/2013	*Y. intermedia*	2.231	*Y. enterocolitica*	2.154	B/SM
115	34300	186400	27/03/2013	*Y. enterocolitica*	2.051	*Y. kristensenii*	2.019	B/SM
116	116719	170098	12/04/2013	*Y. enterocolitica*	1.838	*Y. kristensenii*	1.831	B/M

117	111587	168492	8/04/2013	*Y. intermedia*	1.974	*Y. enterocolitica*	1.952	B/SM
118	26600	188300	25/04/2013	*Y. intermedia*	2.310	*Y. frederiksenii*	1.951	B/SM
119	206125	185375	4/07/2013	*Y. intermedia*	2.250	*Y. intermedia*	2.127	B/SM
120	34480	184550	11/04/2013	*Y. mollaretii*	2.199	*Y. enterocolitica*	2.138	B/SM
121	24600	197300	2/04/2013	*Y. intermedia*	2.362	*Y. intermedia*	2.185	B/SM
122	116333	165453	28/03/2013	*Y. intermedia*	2.005	*Y. intermedia*	1.912	B/SM
123	47900	184100	10/02/2013	*Y. enterocolitica*	1.946	*Y. enterocolitica*	1.739	A/M
124	114260	172395	12/04/2013	*Y. intermedia*	2.181	*Y. enterocolitica*	2.049	B/SM
125	112207	161427	28/03/2013	*Y. intermedia*	2.220	*Y. enterocolitica*	2.052	B/SM
126	51000	178720	8/04/2013	*Y. intermedia*	2.073	*Y. intermedia*	2.019	B/SM
127	238600	208700	29/10/2013	*Y. enterocolitica*	1.967	*Y. enterocolitica*	1.866	A/M
128	173400	178880	26/08/2013	*Y. enterocolitica*	1.988	*Y. enterocolitica*	1.866	A/M
129	157220	172580	26/07/2013	*Y. intermedia*	2.088	*Y. enterocolitica*	2.003	B/SM
130	158900	175230	8/08/2013	*Y. intermedia*	2.362	*Y. enterocolitica*	2.213	B/SM
131	234500	162500	4/11/2013	*Y. enterocolitica*	2.153	*Y. enterocolitica*	2.018	B/SM
132	206350	182720		*Y. intermedia*	2.174	*Y. enterocolitica*	2.062	B/SM
133	235500	205000	28/10/2013	*Y. enterocolitica*	2.139	*Y. enterocolitica*	1.951	A/M
134	158900	196200	11/04/2013	*Y. intermedia*	2.155	*Y. enterocolitica*	2.148	B/SM
135	231700	209500	28/10/2013	*Y. intermedia*	2.040	*Y. intermedia*	2.034	A/M
136	177350	168300	20/08/2013	*Y. intermedia*	2.221	*Y. enterocolitica*	2.035	B/SM
137	243900	180300	24/10/2013	*Y. intermedia*	2.245	*Y. enterocolitica*	2.147	B/SM
138	97560	163440	20/03/2013	*Y. intermedia*	2.356	*Y. intermedia*	2.181	B/SM
139	113060	203000	30/04/2013	*Y. intermedia*	2.022	*Y. enterocolitica*	1.992	B/SM
140	141700	172850	26/06/2013	*Y. mollaretii*	2.148	*Y. enterocolitica*	1.949	B/SM
141	104500	166640	28/06/2013	*Y. intermedia*	2.175	*Y. intermedia*	2.114	B/SM
142	97100	160900	13/06/2013	*Y. intermedia*	2.247	*Y. intermedia*	2.235	B/SM
143	137300	217150	31/05/2013	*Y. intermedia*	1.977	*Y. enterocolitica*	1.934	B/SM
144	116800	202700	7/05/2013	*Y. intermedia*	2.116	*Y. enterocolitica*	1.933	B/SM
145	122370	210150	22/05/2013	*Y. intermedia*	2.283	*Y. enterocolitica*	2.126	B/SM
146a	127100	198300	29/05/2013	*Y. mollaretii*	2.144	*Y. aleksiciae*	2.073	B/SM
146b	127100	198300	29/05/2013	*Y. enterocolitica*	2.144	*Y. frederiksenii*	2.106	B/SM
147	124100	204500	16/04/2013	*Y. intermedia*	2.255	*Y. enterocolitica*	2.092	B/SM
148	101980	167620	3/07/2013	*Y. intermedia*	2.345	*Y. enterocolitica*	2.071	B/SM
149	173050	168400	8/08/2013	*Y. intermedia*	2.125	*Y. enterocolitica*	2.085	B/SM
150	111100	196130	24/04/2013	*Y. enterocolitica*	1.808	*Y. enterocolitica*	1.698	B/M
151	134167	169576	17/06/2013	*Y. enterocolitica*	2.046	*Y. kristensenii*	2.007	B/SM
152	117000	192200	18/04/2013	*Y. intermedia*	2.324	*Y. enterocolitica*	2.181	B/SM
153	102290	165680	21/06/2013	*Y. intermedia*	2.464	*Y. enterocolitica*	2.254	B/SM
154	140180	168800		*Y. enterocolitica*	1.987	*Y. intermedia*	1.951	B/SM
155	135700	217200	18/06/2013	*Y. intermedia*	2.236	*Y. enterocolitica*	2.192	B/SM
156	125800	194200	25/04/2013	*Y. intermedia*	2.223	*Y. enterocolitica*	2.114	B/SM
157	56380	163780	11/06/2013	*Y. enterocolitica*	2.153	*Y. frederiksenii*	2.141	B/SM
158	66550	162200	3/07/2013	*Y. intermedia*	2.129	*Y. enterocolitica*	2.004	B/SM
159	60250	177500	31/05/2013	*Y. mollaretii*	2.264	*Y. enterocolitica*	2.064	B/SM
160	68000	177800	21/06/2013	*Y. intermedia*	2.136	*Y. intermedia*	2.011	B/SM

161	97000	179100	30/05/2013	*Y. enterocolitica*	2.208	*Y. kristensenii*	2.164	B/SM
162	45400	195600	22/08/2013	*Y. intermedia*	2.280	*Y. enterocolitica*	2.174	B/SM
163	102000	184600	29/05/2013	*Y. rhodei*	2.450	*Y. rhodei*	2.181	B/SM
164	79950	187200	22/05/2013	*Y. enterocolitica*	2.133	*Y. mollaretii*	2.099	B/SM
165	36000	166500	28/06/2013	*Y. intermedia*	2.379	*Y. enterocolitica*	2.163	B/SM
166	50800	202600	28/06/2013	*Y. enterocolitica*	2.080	*Y. intermedia*	2.073	B/SM
167	98900	188500	24/05/2013	*Y. bercovieri*	2.315	*Y. enterocolitica*	2.130	B/SM
168	39485	206862	9/07/2013	*Y. intermedia*	2.451	*Y. intermedia*	2.173	B/SM
169	38080	169550	3/07/2013	*Y. intermedia*	2.488	*Y. intermedia*	2.271	B/SM
170	79600	190100	27/06/2013	*Y. intermedia*	2.374	*Y. intermedia*	2.264	B/SM
171	90500	180400	10/04/2013	*Y. enterocolitica*	2.142	*Y. bercovieri*	2.078	B/SM
172	68600	185800	13/06/2013	*Y. intermedia*	2.233	*Y. intermedia*	2.088	B/SM
173	95900	177750	30/05/2013	*Y. intermedia*	2.223	*Y. intermedia*	2.202	B/SM
174	58500	216140	20/06/2013	*Y. intermedia*	2.387	*Y. enterocolitica*	2.221	B/SM
175	59000	167000	5/07/2013	*Y. intermedia*	2.245	*Y. enterocolitica*	2.094	B/SM
176	55800	210500	24/06/2013	*Y. mollaretii*	2.357	*Y. enterocolitica*	2.093	B/SM
177	80700	194600	25/06/2013	*Y. intermedia*	2.146	*Y. enterocolitica*	2.141	B/SM
178	101500	186150	29/05/2013	*Y. intermedia*	2.201	*Y. intermedia*	2.169	B/SM
179	90340	169500	31/05/2013	*Y. intermedia*	2.289	*Y. enterocolitica*	2.219	B/SM
180	83280	171650	31/05/2013	*Y. intermedia*	2.258	*Y. enterocolitica*	2.151	B/SM
181	33350	171280	22/04/2013	*Y. intermedia*	2.214	*Y. intermedia*	2.121	B/SM
182	66400	187600	6/06/2013	*Y. intermedia*	2.196	*Y. enterocolitica*	2.187	B/SM
183	54500	172780	4/06/2013	*Y. intermedia*	2.386	*Y. enterocolitica*	2.195	B/SM
184	79600	191100	1/07/2013	*Y. intermedia*	2.354	*Y. enterocolitica*	2.158	B/SM
185	81150	158850	25/06/2013	*Y. intermedia*	2.295	*Y. enterocolitica*	2.239	B/SM
186	46020	162200	11/04/2013	*Y. intermedia*	2.310	*Y. enterocolitica*	2.209	B/SM
187	34420	175340	7/06/2013	*Y. frederiksenii*	2.453	*Y. frederiksenii*	2.427	B/SM
188	88000	175300	24/05/2013	*Y. kristensenii*	2.305	*Y. enterocolitica*	2.293	B/SM
189	36275	171650	24/05/2013	*Y. enterocolitica*	2.196	*Y. intermedia*	2.167	B/SM
190	95100	174700	31/05/2013	*Y. frederiksenii*	2.159	*Y. enterocolitica*	2.037	B/SM
191	99100	181600	30/05/2013	*Y. rhodei*	2.426	*Y. rhodei*	2.329	B/SM
192	81850	178800	22/05/2013	*Y. rhodei*	2.257	*Y. rhodei*	2.158	A/M
193	61000	168800	23/05/2013	*Y. mollaretii*	2.171	*Y. enterocolitica*	2.079	B/SM
194	61600	193500	21/06/2013	*Y. intermedia*	2.490	*Y. enterocolitica*	2.248	B/SM
195	82800	169300	10/04/2013	*Y. intermedia*	2.344	*Y. enterocolitica*	2.212	B/SM
196	41040	160000	23/04/2013	*Y. intermedia*	2.512	*Y. intermedia*	2.287	B/SM
197	58100	169750	5/06/2013	*Y. intermedia*	2.194	*Y. intermedia*	2.127	B/SM
198	74400	185600	21/06/2013	*Y. intermedia*	2.346	*Y. enterocolitica*	2.174	B/SM
199	78400	217400	25/07/2013	*Y. intermedia*	2.240	*Y. enterocolitica*	2.008	B/SM
200	34650	180590	17/05/2013	*Y. aleksiciae*	2.230	*Y. kristensenii*	1.964	B/SM
201	82700	191700	26/06/2013	*Y. enterocolitica*	2.201	*Y. kristensenii*	2.134	B/SM
202	104100	202700	21/06/2013	*Y. intermedia*	2.229	*Y. enterocolitica*	2.039	B/SM
203	66800	175200	14/06/2013	*Y. frederiksenii*	2.110	*Y. enterocolitica*	2.054	B/SM
204	105700	212000	15/06/2013	*Y. rhodei*	2.189	*Y. rhodei*	2.132	B/SM
205	83120	161520	5/06/2013	*Y. intermedia*	2.098	*Y. intermedia*	1.985	A/M

The top match and second best match (score 1 and 2 respectively), based on the mass spectra of a single spot for each isolate, are provided in the table. Results were assigned a consistency category based on the manufacturer's criteria.

- Species consistency (A) was attributed to a result when all matches scoring ≥1.9 show to be of the same species, and all matches scoring ≥1.7 are of the same genus.
- Genus consistency (B) is characterized by a top match score between 1.899 and 1.7, or by matches scoring ≥1.9 but that are not of the same species, taking into account that all matches scoring ≥1.7 have to be of the same genus.
- No consistency (C) in identification occurs when the top match score is <1.7, or matches scoring ≥1.7 are not of the same genus.

For each individual identification, the top 10 of best matches was also carefully assessed for mismatches:

- Mismatches (M) were defined as results on the list of the 10 best matches that differ with the top match result at genus level or species level.
- Significant mismatches (SM) indicate results within the top 10 best matches that differ with the top match result at genus level (score ≥ 1.7) or species level (score ≥ 1.9).

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GENERAL DISCUSSION

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1. Human disturbances

Being on the edge of a human induced sixth mass extinction (Ceballos et al., 2015), it is essential to identify important (anthropogenic) factors involved and how the current extinction rate can be decelerated, or preferentially stopped. Humans have played an important role in a variety of processes that either directly or indirectly alter population fitness and pathogen or disease occurrence (Wobeser, 2007). Some of these processes are:

1) disseminating pathogenic agents or their vectors voluntarily (e.g. myxoma virus in rabbits in Australia (Kerr, 2012)), or involuntary (e.g. avian malaria in Hawai (Atkinson and LaPointe, 2009), *Batrachochytrium salamandrivorans* in western Europe (Martel et al., 2014);
2) introducing alien species (e.g. cane toad (*Bufo marinus*) in Australia (Philips et al., 2006), ring-necked parakeets (*Psittacula krameri*) in Belgium (Strubbe and Matthysen, 2007));
3) aggregating animals with subsequent disease outbreaks (e.g. supplementary feeding systems (Brittingham and Temple, 1988), loss of wetlands for waterfowl (Smith and Higgins, 1990));
4) increasing the contact rate between humans, domestic animals with wildlife (e.g. Nipah virus in pigs and humans (Chua et al., 1999));
5) overharvesting (of e.g. horseshoe crabs (*Limulus polyphemus*) with subsequent declines in red knots (*Calidris canutus*) (Baker et al., 2004));
6) impacting (micro)climate (Kuttler, 2008);
7) intoxicating and contaminating the environment (e.g. endocrine disruptors (Schug et al., 2016)).
8) anthropogenic habitat destruction and fragmentation, two characteristics of urbanization, which represents one of the most intense and long-lasting anthropogenic modifications of natural systems, affecting entire ecosystems (Grimm et al., 2000; Evans et al., 2009), population health and host-pathogen interactions (Bradley and Altizer, 2007).

However, with respect to the latter point, there is a lack of research investigating the effect of urbanization on wildlife health, pathogen occurrence (which has mostly been focused on vector-borne diseases or those posing a high risk to human health), and host-pathogen interactions (e.g. Bichet et al., 2013; Gasparini et al., 2014; Giraudeau et al., 2014). Also the parameters potentially altering the infectious disease ecology are not well examined. Bearing this in mind, in this thesis we have attempted to assess how urbanization impacts enteropathogen occurrence in susceptible avian wildlife, whether or not differences in body

condition could be related to the level of urbanization or pathogen occurrence and assessed the role of other factors that might impact enteropathogen occurrence and avian health.

2. Urbanization: how to define the spatial scale?

How to measure the level of urbanization has been a matter of debate, since "urban" in one country does not necessarily mean "urban" in another country. Many different strategies have been used to classify different habitat patches, based on the identification and quantification of specific characteristics inherent to urbanized habitats (e.g. buildings, roads and vegetation), which can be identified using manual scoring of aerial images (Liker et al., 2008; Bókony et al., 2012), semi-automated scoring of satellite-images or GIS (e.g. using the European Union CORINE (Coordination of Information on the Environment) land cover database) (Seress et al., 2014). Seress et al. (2014) showed that the quantification of the urbanization level using these methods is highly comparable, although will depend on the quality and resolution of the images used. In our studies we have used the Large-scale Reference Database, which has a resolution that is far more fine grained compared to the 'CORINE land cover database' (resolution of 0.15m for LRD versus 100m for CORINE) (AGIV, 2013a and 2013b; Seress et al., 2014). Since the urban environment usually comprises an area covered by buildings and other built-up structures, the "built-up area" within a defined spatial scale has been widely used to assess the degree of urbanization solely or in combination with other variables (Blair, 1996; Marzluff et al., 2001; Bókony et al., 2012; Salleh Hudin et al., 2016). However, using the "most appropriate" spatial scale for the species of interest within which the calculation of the BU-density can be performed makes it even more complex (Wiens, 1989; Litteral and Shochat, 2017). A "body size dependent" spatial scale has previously been suggested, which assigns spatial scales to bird species according to their (allometrically corrected) bodyweight (e.g. smaller spatial scales for smaller birds, and larger scales for larger birds) (Wiens, 1989; Litteral and Shochat, 2017). Nevertheless, these calculations do not consider species specific traits and habitat use, as such the spatial scale of interest highly varies between different study species (Wiens, 1989). House sparrows, except for two subspecies (Summers-Smith, 1994), are extremely sedentary and have been shown to stay within 1-2 km of their breeding colony (Summers-Smith, 1963). This 'one kilometer difference' can be related to differences in environment and habitat use (e.g. house sparrows tend to have larger home ranges in rural areas compared to urban areas) (Wiens, 1989; Vangestel et al., 2010). Though one kilometer does not seem much, even small differences in radius may already cause considerable differences in the built-up area that can be measured within these circles (Table 8). This could lead to different

classifications of the urbanization levels (as shown in Table 8: radius of 50-400m: "urban", 800 and 3200m: "suburban", 1600m: "rural"), altering the results and conclusions of studies.

We must be careful comparing results of different studies and ideally the situation (landscape management and urban planning) of the country should be taken into account when comparing different studies, since even cities with similar built-up areas can differ quite a lot in their suitability for wildlife (Snep et al., 2016). To partly overcome this problem, the use of a multiscale approach has been suggested, using a core home range scale in combination with a landscape scale which provides information about the surrounding environment and gives a more complete image of the environment (Wiens, 1989; Melles et al., 2003; Litteral and Shochat, 2017). In our study we have relied on previous research, assessing the home range of house sparrows along an urban gradient, performed in Flanders (Vangestel et al., 2010; as explained in Chapter 1), which gives us the advantage that we have been able to estimate the "most appropriate spatial scale" for the house sparrows we are working on in Flanders, being a multi-scale approach using a local habitat (radius: 100m or 400m) in combination with a surrounding landscape (radius: 1600m). Although the 400m core home range was used in Chapter 2, for the models in Chapter 3 we have chosen to use the 100m core home range, since this spatial scale encompasses the most important foraging sites, and thus potential *Yersinia*-transmission sites, for the house sparrows. However, even when using this "pre-set-scale" (100m-1600m), differences between home ranges of populations potentially still affect the outcome and conclusions of our research. Ideally, the home ranges should be assessed for every house sparrow population, e.g. through radio-telemetry follow-up. As such specific and essential environmental parameters can be taken into account for each population.

Spatial Scale (in meters radius)	Built-Up-%	Color Code	Categorized Landscape scale
50	34,96	Red	Urban
100	22,17	Red	Urban
200	17,15	Red	Urban
400	12,62	Red	Urban
800	8,49	Yellow	Suburban
1600	4,69	Green	Rural
3200	5,41	Yellow	Suburban

Table 8. Variation of BU% according to the spatial scale used. Spatial scale is represented by the radius of the circle around the center of the main capture site: Hillegem in this example shows the highly variable Built-Up-% according to which scale is used when categorizing the different levels of urbanization (with the respective color codes)

Information regarding these environmental parameters is mostly limited to some abiotic environmental characteristics (e.g. the amount of built-up area, the percentage of impermeable surface, weather conditions), although other important factors such as pesticides and other contaminants, presence of supplementary feeding systems, and biotic features of the environment (e.g. presence of predators and invasive species, presence and ecology of disease-agents and reservoir hosts, cover habitat, competition for food) are often neglected. In our study (Chapter 3) we have tried to account for the presence of other granivorous birds (through the calculation of granivore-index), which gives an idea of the constitution of the avian community sharing the same environment as our house sparrows. Although due to logistic difficulties, no information regarding the density of these species was available which hampers the conclusions that can be made based on the correlations between pathogen presence and granivore-index.

In order to have an idea of the impact of the urbanization on pathogen occurrence and study species, a combination of representative (a)biotic characteristics of the environment should be taken into account.

3. Pathogen pressure and disease in relation to anthropogenic altered environments

Wild ranging animals, such as birds and rodents, are increasingly being recognized as carriers or even potential reservoirs for various diseases (Artois et al., 2001), with some of them, such as the bacterial enteropathogens we investigated (Chapter 2, 3 and 4), having a zoonotic potential. However, only few studies have been performed to actually assess the prevalence of *Yersinia* and *Salmonella* in apparently healthy wild living animals (e.g. Pennycott et al., 2002; Niskanen et al., 2003; Refsum et al., 2003; Kisková et al., 2011). In order to evaluate human health risks and food safety, most research is being performed in the surrounding environment of farms and slaughterhouses (Battersby et al., 2002; Kirk et al., 2002; Backhans et al., 2011; Andrès et al., 2013), or has been conducted as a consequence of a yersiniosis, or salmonellosis outbreak in animals or humans (Mackintosh and Henderson, 1984; Cízek et al., 1994; Refsum et al., 2003; Pennycott et al., 2006; Kangas et al., 2008; Lawson et al., 2010; Giovannini et al., 2013; Lawson et al., 2014). Nevertheless, one should be careful when appointing the primary source of infection, which could either be domesticated animals (such as livestock), wild animals (e.g. ranging on farms), humans, or the environment (e.g. spread of pathogens through floods). Since rodents and birds have been suggested to be a reservoir host of *Y. pseudotuberculosis, Y. enterocolitica* and *Salmonella* Typhimurium, these wild living animals

were rather easily (whether or not correctly) assigned as the most likely (primary) source of yersiniosis, or salmonellosis in domestic animals, livestock and humans (e.g. Mackintosh and Henderson 1984; Daniels et al., 2003; Kangas et al., 2008). Recent studies however are indicating that rodents and birds most likely acquire the infection on the farm from the primary source (e.g. infected livestock). Although in case of pathogens such as *Salmonella* and *Yersinia* wild animal populations are able to maintain the infection on a farm (within and in between batches of animals) for an extended period of time and can act as bridge species between farm and wild living animals, transmitting diseases from one area to the other (Wang et al., 2009; Backhans and Fellström, 2012; Liang et al., 2015). In a geographical context, the overall prevalence of pathogenic bacteria in wildlife decreases from areas close to e.g. pig premises to areas far from these agricultural fields (Henzler and Opitz, 1992; Andrés et al., 2013) following a concentric distribution around the primary animal reservoir (Wang et al., 2009; Liang et al., 2015). In order to assess the role of wild animals in the pathogen dynamics of enteropathogenic *Yersinia* species and *Salmonella* we focused on apparently healthy animals, not specifically related to agricultural areas. We did not isolate either *Salmonella* Typhimurium or enteropathogenic *Y. enterocolitica* from the apparently healthy house sparrows and only 2% of the birds were positive for *Y. pseudotuberculosis* (Chapter 2 and 3). The recovery of two *Salmonella* Typhimurium isolates (DT99 and DT195) from brain granulomas of house sparrows suggests an association of *Salmonella* Typhimurium infection with clinical disease which is most likely driven by the risk of exogenous exposure to pathogenic *Salmonella* Typhimurium strains (Chapter 2). Also in the brown rats only two human pathogenic *Y. enterocolitica* (bioserotype 2/O:5,27 and 3/O:1,2,3) and three *Y. pseudotuberculosis* serotype I were isolated, indicating a low prevalence of human pathogenic *Yersinia* isolates in brown rats in Flanders in 2013-2014 (Chapter 4). We did, however, find a high prevalence of other *Yersinia* species and of *Y. enterocolitica* BT1A both in the house sparrows (41% and 31% resp.) and in the brown rats (19% and 21% resp.) (Chapter 3 and 4). This supports the hypothesis that these animals are probably not the primary reservoir of these human pathogenic *Yersinia* species (house sparrows and rats) and *Salmonella* Typhimurium (house sparrows). However, they are potentially able to maintain *Yersinia* species within a population, at least temporarily, after coming into contact with the bacteria (Chapter 3 and 4; Pocock et al., 2001; Wang et al., 2009; Backhans and Fellström, 2012).

The potential rapid course of salmonellosis and yersininiosis in house sparrows in combination with the difficulty in finding diseased or dead wild living animals (Wobeser and

Wobeser, 1992) could have lead to an underestimation of the actual direct effect of *Salmonella* and *Yersinia* on the host populations. To enhance the chance of detecting a pathogenic agent in a population, measuring the incidence over a specific time-frame (preferentially covering all seasons over several years) in a population, in combination with the prevalence, would provide a more complete view of the presence of pathogens and their effect on the host population (Wobeser, 2006). Nevertheless, the susceptible population has to be known in advance and a high recapture rate would be essential, which is unlikely in the wary house sparrows.

4. Is there an interaction between host, environment and pathogen?

The effect of pathogens on host populations depends on the interaction between the pathogen (e.g. virulence, host restriction, capacity to survive in the environment), the host (e.g. susceptibility, host immune defense, population diversity and -density, stress) and the environment (e.g. aggregation of animals, food and water availability and -quality, suitability for vector replication, pathogen survival in environment, weather-conditions), also referred to as the epidemiological triangle (Wobeser, 2006; Vander Wal et al., 2014). Since anthropogenic alterations of habitats have a major impact on ecosystems, species assemblages and host communities (Evans et al., 2009; Ferenc et al., 2014), changes in host-pathogen interactions and disease outcomes are to be expected (Vander Wal et al., 2014; Pollack et al., 2017).

In sedentary birds the focus of most studies is placed on the assessment of pathogen prevalence along an urbanization gradient in birds, disregarding the effect on host health (e.g. Cork et al., 1995; Bradley and Altizer, 2007; Delgado-V and French, 2012; Hamer et al., 2012; Gasparini et al., 2014). Only few studies have attempted to assess the impact of pathogens on bird species along an urbanization gradient and have tried to determine variables most likely influencing these interactions (e.g. Chapter 3; Bichet et al., 2013; Giraudeau et al., 2014; Galbraith et al., 2017).

In our research we have evaluated if a correlation was present between “the environment” (the built-up density at two spatial scale, the environmental temperature, the dominant feeding strategy of the local bird assemblage, time of sampling), “the host” (sex, body condition) and “the pathogen” (*Y. pseudotuberculosis, Y. enterocolitica,* other *Yersinia* species) (Chapter 3).

4.1. The effect of environment on morphological changes

The urban habitat is, due to its inherent ecosystem alteration (e.g. increased noise, light and chemical pollution, reduced greenery, altered food quality and availability, different predation pressures), often perceived as a stressful environment for many wild living animals (Seress and Liker, 2015; Pollack et al., 2017). In this perspective many studies have tried to assess to what extent animals, experience this stressful environment, and in what manner they have been able to cope with these environmental changes (Peach et al., 2008; Bichet et al., 2013; Meillère et al., 2017; Ouyang et al., 2017).

Comparison of morphological changes, such as the body condition (SMI: Chapter 2 and 3), between birds living in urbanized and more natural or rural environments have been used in multiple studies (e.g. Liker et al., 2008; Bókony et al., 2012; Meillère et al., 2015b; Salleh Hudin et al., 2016), but the outcomes were not always straightforward. Different hypotheses for contrasting results have been suggested, where the use of different calculation methods for the assessment of the body condition index (Green, 2001) is of great importance and can in this perspective lead to different results. Liker et al. (2008) for example found that house sparrows inhabiting urban environments were smaller and in worse condition, based on a type-1 Ordinary Least Squares (OLS) linear regression, compared to house sparrows originating from rural habitats. On the other hand, Bókony et al. (2012), who studied the same house sparrows as mentioned in Liker et al. (2008) with the addition of 14 extra populations and using the SMI instead, did not find any relationship between the degree of urbanization and the body condition, although the house sparrows were confirmed to be smaller in the more urbanized regions. Most likely, and as was also suggested by Bókony et al. (2012), this contradiction is likely due to the different method used to calculate the body condition index, whereby the OLS-regression tends to inflate the residuals when the length parameter increases.

The urbanization (landscape) level had an influence on the hosts SMI. Higher body condition scores were measured in suburban areas compared to both, urban and rural environments. However, no difference in SMI was demonstrated between the house sparrows originating from the urban and rural habitats (Chapter 3). These results are in line with those of Bókony et al. (2012) and Meillère et al. (2015b, 2017). On the contrary, Salleh Hudin et al. (2016) did find that in southern France urban house sparrows were both leaner (as evidenced by a lower SMI) and smaller compared to the rural birds.

Various hypotheses have been suggested to explain variations in morphology in between different urbanization levels which can be related to intrinsic (i.e. genetic) factors or due to phenotypic plasticity (Alberti et al 2016), the ability of an animal with a specific genotype to change the phenotype in response to environmental changes (Fusco and Minelli, 2010). Low body conditions have since long been associated with stress and a lower overall health status, potentially mediated through environmental pollution and disease (Peig and Green, 2009; Bókony et al., 2012; reviewed in Pollack et al., 2017). As environmental factors, a specific urban microclimate (i.e. the heat island effect, lower wind velocity) lowers the energetic demands of smaller birds during winter and nighttime (Cuthill et al., 2000; Zuckerberg et al., 2011; Murthy et al., 2016). This could affect the body condition, by lowering the extra energy reserve needed in urban birds, or increase the daily weight gain when subjected to a less ideal climate in rural birds (Cuthill et al., 2000). In addition to the specific urban microclimate, food predictability, as a result of supplementary food sources in suburban and urban areas (Shochat, 2004; Robb et al., 2008; Salleh Hudin et al., 2016; Reynolds et al., 2017), and food quality, which in supplementary feeding systems is thought to be of lower quality ‘junk food’ compared to natural resources (Shochat, 2004; Heiss et al., 2009; Meillère et al., 2015b), could alter the body condition of the birds and potentially the overall fitness of the hosts. Salleh hudin et al. (2016), have tried to assess if the lower body condition of urban birds compared to their rural counterparts, was due to the predictability or the quality of the food by performing a field experiment providing “urban” or “rural” food to house sparrows originating from urban and rural environments using different ‘food type'-'bird origin’ combinations. Irrespective of the type of food, the rural birds dropped in body weight and SMI up to the level of the urban birds. They concluded that most likely, this observation was due to the predictability of the food supplementation, which, through an adaptive mass regulation, allows birds to lower their body weight (Salleh Hudin et al., 2016). Leaner birds, originating from urban areas, could in this perspective have an advantage when subjected to a higher predation risk through their ability to take-off faster and have better flight performance (Witter and Cuthill, 1993; Salleh Hudin et al., 2016). Nevertheless, although the density of predators (sparrowhawks, cats,…) is thought to be higher in urban areas (Shochat, 2004; Seress and Liker, 2015) and some studies have demonstrated behavioral changes in urban birds with respect to ‘flight initiation distance’ (Seress et al., 2011; Meillère et al., 2015a), the predation risk is not always believed to increase from rural to urban areas (Shochat, 2004; Seress and Liker, 2015).

As demonstrated above, different (interacting) factors will play a role in changing the animal's phenotype. The suburban areas, where the house sparrows with highest SMI were recovered from (Chapter 3), have been shown to support an increased species richness (Blair, 1996; Marzluff and Rodewald, 2008; McKinney, 2008; Seress and Liker, 2015), a higher bird density (Blair, 1996; Marzluff and Rodewald, 2008), and demonstrated an increased survival of certain urban adapter species (Evans et al., 2015), due to the combination of increased resource availability and the proximity and connectivity of various vegetation types (Marzluff and Rodewald, 2008; Vangestel et al., 2010; Evans et al., 2015). These intermediate areas of urbanization, compared to highly urbanized or rural areas, could be advantageous for urban, or suburban adapter species and should be taken into account when performing studies related to the impact of urbanization on wild living animals.

4.2. Is there a correlation between pathogens and morphology?

Besides the obvious effect a pathogen can have on individual animals and populations (clinical disease-death), subclinical effects can play an even greater role in population dynamics (Wobeser, 2006). When confronted with a disease agent, hosts need to make trade-offs which will depend on the condition (of the host, pathogen and environment at the time of exposure) and which can manifest in many different ways (e.g. altered SMI, decreased fitness) (Wobeser, 2006). This should definitely be considered when working with pathogens that have the potential for causing chronic disease, such as host adapted enteropathogens (Pasmans et al., 2003; Lawson et al., 2011; Eng et al., 2015), which can through long term stimulation of the immune system cause a deterioration of the body condition (Lochmiller and Deerenberg, 2000; Wobeser, 2006). In our study, the SMI of the house sparrows was not explained by presence of *Y. pseudotuberculosis, Y. enterocolitica* or other *Yersinia* species (Chapter 3). Since *Y. enterocolitica* BT1A and other *Yersinia* spp. have been suggested to be part of the normal avian microbiota (Niskanen et al., 2003; Kisková et al. 2011), a lack of effect on hosts SMI could be expected. Nevertheless, only limited research has focused on the pathogenicity of *Y. enterocolitica* BT1A and environmental *Yersinia* species in birds and subclinical effects could have gone undetected in wild ranging populations. Due to the known pathogenicity of *Y. pseudotuberculosis* an effect on the hosts SMI was expected. The low number of isolates could have masked this finding and although wild birds have been hypothesized to harbor *Y. pseudotuberculosis* at low levels subclinically, developing acute disease when exposed to stressful conditions (Niskanen et al., 2003), also here the subclinical effects, if present, should be assessed. On the other hand, animals which are known to be stressed, have been shown to

be more susceptible to and excrete higher numbers of pathogenic agents (Verbrugghe et al., 2012 and 2016). Since, as mentioned previously, urban environments are perceived as stressful, an effect on the hosts SMI could have been expected in highly urbanized areas with subsequent altered pathogen excretion. However, hosts SMI did not explain the presence of either *Yersinia* species (Chapter 3).

4.3. Pathogen pressure in relation to environment

Depending on which *Yersinia* species was modeled as response variable, other variables would have higher explanatory values (Chapter 3). Most likely this is related to the distinct metabolic flexibility of the *Yersinia* species tested (Reuter et al., 2014), influencing the life histories of the bacteria.

The presence of pathogenic *Y. pseudotuberculosis* was best explained by the granivore-index, for which a higher prevalence was detected when the local bird population was dominated by granivorous bird species. Also the landscape level of urbanization showed to have an influence on the presence of *Y. pseudotuberculosis,* which appeared to be more prevalent in suburban and to a lesser extent urban house sparrows, compared to rural individuals (Chapter 3). A higher faeco-oral transmission could be present in suburban and urban areas where bird densities are higher (Blair, 1996; Evans et al., 2009), are dominated by granivorous species (Chapter 3) using similar feeding strategies and of which some species such as members of the Fringillidae are known to be highly susceptible (Cork et al., 1999; Sandmeier and Coutteel, 2005), and where supplementary feeding systems are omnipresent (Brittingham and Temple, 1988; Pennycott et al., 2002; Robb et al., 2008).

The average environmental temperature, the granivore-index and to a lesser extent the urbanization-index at both the local and the landscape scale were the variables best explaining variation in *Y. enterocolitica* BT1A presence (Chapter 3). Lower environmental temperatures were positively correlated with the presence of these bacteria (Chapter 3), and are most likely related with the enhanced survival of *Y. enterocolitica* at lower temperatures (Tashiro et al., 1991). Similar observations related to environmental temperature were made for the other *Yersinia* species. As opposed to the effect of the degree of granivory on *Y. pseudotuberculosis,* the prevalence of *Y. enterocolitica* BT1A decreased when the local bird community consisted of more granivorous bird species (Chapter 3) which could be an indication that birds with other feeding patterns (e.g. omnivorous, insectivorous) could be more suitable hosts compared to granivorous birds (Novotný et al., 2007; Benskin et al., 2009). At the landscape scale, *Y.*

enterocolitica BT1A tends to be more prevalent in urban and rural areas (Chapter 3). Higher densities of urban exploiters in urban areas (Blair, 1996), and presence of other animals such as rodents, hares and livestock (Chapter 4; Frandölich et al., 2003; Vanantwerpen et al., 2014) possibly contributes to an increased occurrence of *Y. enterocolitica* BT1A in the examined house sparrows. Both *Y. enterocolitica* and other *Yersinia* species were less prevalent in home range scales which were more urbanized (Chapter 3). Since *Yersinia* has a higher survival in wet to damp soil (Tashiro et al., 1991), increased water runoff and increased evaporation in highly urbanized areas (Trusilova et al., 2008) will most likely decrease the survival of *Yersinia* in highly urbanized environment.

5. Main Conclusions

Our studies provide information regarding urban disease ecology, testing the effect of urbanization (measured along an urbanization gradient) on the presence of enteropathogens (*Salmonella* Typhimurium and *Yersinia* spp.) in an avian (house sparrows) host species, and assessing whether the host's body condition changes in relation to the presence of these enteropathogens or to the level of urbanization. In addition, we have assessed the role of a synantropic rodent species (the brown rat), often sharing food sources with granivorous house sparrows, as a potential reservoir species of pathogenic *Yersinia* spp..

No *Salmonella* could be isolated from the house sparrows that were sampled during the fieldwork and only one house sparrow was found to be positive for anti-*Salmonella* antibodies, indicating a low prevalence (<1.3%) of *Salmonella* Typhimurium in the wild living house sparrow populations. However, necropsy of 12 house sparrows obtained from bird rescue centers revealed salmonellosis (caused by *Salmonella* Typhimurium DT99 and DT195) in two birds. Disease related to salmonellosis was most likely linked to exogenous exposure to these *Salmonella* Typhimurium phage types. Since only a low prevalence was demonstrated in the host populations, no inference can be made regarding the effect of urbanization on *Salmonella* prevalence. On the contrary, by using isolation methods, the presence of enteropathogenic *Yersinia* spp. could be demonstrated in the house sparrow populations. Depending on the *Yersinia* spp. tested, *Yersinia* presence could be related to the degree of urbanization, average daily temperatures and the community of granivorous birds present at sparrow capture locations. No correlation between the host's body condition and presence of *Yersinia* spp. was detected. However, the body condition of house sparrows living in intermediate levels of urbanization (suburban habitats) was found to be higher compared to urban and rural house

sparrows. We conclude that two determinants of pathogen infection dynamics, body condition and enteropathogen (*Yersinia*) occurrence, vary along an urbanization gradient, potentially mediating the impact of urbanization on avian health. In addition, we found that brown rats are frequent carriers for various *Yersinia* spp., although they most likely present spill-over hosts for *Y. pseudotuberculosis* and (human pathogenic) *Y. enterocolitica,* the latter of which were more often isolated during winter and spring.

This low prevalence of enteropathogenic *Yersinia* and *Salmonella* Typhimurium in the house sparrows which were sampled in the context of the fieldwork might be regarded as not important on population level. Nevertheless, it must be stressed that since these pathogenic bacteria are present in the respective hosts, changes in (a)biotic environment, host (susceptibility, population structure) and pathogen virulence, could alter the disease dynamics and have devastating effects on populations. In addition, more subtle effects could be present, which were not detected using only the body condition as a parameter of health in the house sparrows.

6. Future perspectives

Since in most studies historical data are missing about the pathogen occurrence in host populations before a disease outbreak was detected and often information is lacking on the ecology of the host species in the context of a specific habitat (e.g. urban versus non-urban), only seldom explanatory variables can be appointed that could affect changes in host-pathogen dynamics. Also, subclinical (non-overt) disease is often neglected in studies, which hampers the assessment of the impact of a pathogen on a host population or an ecosystem.

Our exploratory studies provide information regarding pathogen dynamics in house sparrows in an urbanization context and regarding the role of brown rats as a carrier host for enteropathogenic *Yersinia.* However, since no maintenance reservoir was appointed, differential environmental pathogen survival in urban-suburban-rural areas, and the importance of other host species, should be assessed.

In general, to assess the effect of urbanization on wildlife populations (population dynamics), everything needs to be situated in a larger context and one should (in an ideal world) take the entire ecosystem into account. Multihost (including their immunity and microbiota)-multipathogen interactions within an ecosystem should be followed up on the long term and population structures, immunity, representative (a)biotic environmental characteristics

potentially influencing these interactions should be assessed, using a combination of incidence and prevalence data.

Translated to our studies, long term follow up of the house sparrow populations, mapping their home ranges, through radio-telemetry and by using citizen science data, and identifying hot spots for transmission (e.g. feeding sites) are one of the first important factors that should be determined. Subsequently, the environmental survival of the enteropathogens in these environmental hot spots should be assessed and compared between the different urbanization levels. The community (diversity and density) of domestic and wildlife species sharing the home ranges with the house sparrow populations should be assessed (including the inter- and intraspecies interactions) and checked for the presence of these (multi-host) enteropathogens. Furthermore at the host level, not only the house sparrow's health should be estimated using the combination of various parameters (e.g. body condition index, genetic diversity of the population, physiological parameters and blood chemistry and blood cell count), but also the house sparrow's fitness (reproduction success) should be taken into account in order to follow up population dynamics. The presence of other (non)infectious diseases, potentially interfering with host health and thus susceptibility to pathogens should be assessed. Last but not least, whenever mortality is detected in species inhabiting the same home range as the house sparrows, necropsy should be performed and pathogens present in the host species should be identified.

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Summary

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Urbanization represents one of the most intense and long-lasting anthropogenic modifications of natural environments. Many researchers, studying the effect of urbanization on wildlife and ecosystems, have been using bird communities as a study object, due to their ubiquity, ease of observation, capture and sampling. Many insights have been acquired regarding the effect of urbanization on bird-communities, most often leading to biotic homogenization of urban regions. Not much is known about the factors inducing these community changes and the effect of urbanization on individual and population level is often neglected. In addition, an underexplored aspect of urban ecology, required to better understand the ecological and evolutionary mechanisms driving population dynamics, is how urbanization impacts disease ecology, including its potential to alter wildlife-pathogen interactions and affect animal health. In this respect it is important to understand which animals could function as carrier-species or reservoirs of specific pathogens.

In this research, we have focused on the interaction between host (house sparrows (*Passer domesticus*)) and human enteropathogenic bacteria, known to be harmful to the host species, in an urbanization context (**Chapter 2**: *Salmonella* Typhimurium; and **Chapter 3**: *Yersinia pseudotuberculosis* and *Y. enterocolitica*) and have assessed the role of brown rats (*Rattus norvegicus*) as a potential reservoir host species (**Chapter 4**: *Y. pseudotuberculosis* and *Y. enterocolitica*) in different regions of Flanders, Belgium.

In **Chapter 2 and 3** we have used house sparrows, originating from 36 populations varying in their level of urbanization (measured at two spatial scales) in Flanders, as model species to disentangle effects of urbanization on host-pathogen interactions. House sparrows occur along the different urbanization levels (urban, suburban, rural) and are highly sedentary, making them an ideal study species to assess the effect of urbanization on an urban-exploiter species.

- No *Salmonella* Typhimurium could be isolated from the house sparrows, and only one bird was positive for anti-*Salmonella* antibodies. Necropsy performed on house sparrows, received from bird rescue centers (Merelbeke and Ostend), revealed the presence of brain granulomes in two out of 12 house sparrows. *Salmonella* Typhimurium DT99, a pigeon-adapted phage type, and DT195, were the causative agents of these lesions. These results suggest the apparent absence (prevalence: <1.3%) of *Salmonella* Typhimurium in apparently healthy house sparrows in the winter of 2013 in Flanders. Clinical disease associated with *Salmonella* Typhimurium infection is likely driven by the risk of exogenous exposure to pathogenic *Salmonella* Typhimurium strains (**Chapter 2**).

- *Yersinia pseudotuberculosis, Y. enterocolitica* and other *Yersinia* species could be isolated from respectively 2%, 31% and 41% of the apparently healthy house sparrows. We evaluated potential correlations between “the environment” (the built-up area at two spatial scales, the average environmental temperature, the granivore-index, the time of sampling), “the host” (sex, body condition) and “the pathogen” (*Y. pseudotuberculosis, Y. enterocolitica,* other *Yersinia* species) using generalized linear mixed models. The SMI of the house sparrows could not be explained by the presence of *Y. pseudotuberculosis, Y. enterocolitica* or other *Yersinia* species and vice versa. On the other hand, the urbanization, measured within the landscape level had an influence on the house sparrows SMI, with higher body condition scores measured in suburban areas compared to urban and rural environments. Depending on the *Yersinia* species tested other explaining variables were important. The presence of pathogenic *Y. pseudotuberculosis* was best explained by and positively related with the granivore-index. Also the landscape level of urbanization influenced the presence of *Y. pseudotuberculosis,* which appeared to be more prevalent in the faeces of suburban and to a lesser extent urban house sparrows, compared to rural individuals. The average environmental temperature (negative correlation), the granivore-index (negative correlation) and to a lesser extent the urbanization-index at both the local- (negative correlation) and the landscape scale were the variables best explaining variation in *Y. enterocolitica* presence. With respect to the landscape scale, *Y. enterocolitica* tends to be more prevalent in urban and rural areas, compared to suburban landscapes. Presence of other *Yersinia* species was best explained by the average daily temperature and by the percentage of urbanization of the home range level (both negatively correlated) (**Chapter 3**).

  In conclusion, we show that the level of urbanization, taking the suburban areas into account, affects body condition and pathogen occurrence, two determinants of pathogen infection dynamics, although no direct relationship between body condition and pathogen-occurrence was observed. Also the avian community structure (approximated by the granivore-index) should be accounted for (**Chapter 3**).

Since rats have been appointed as reservoirs of many zoonotic pathogens, we have assessed the role of brown rats as a potential reservoir host species of *Y. pseudotuberculosis* and *Y. enterocolitica* in Flanders, Belgium. Although *Yersinia* was isolated from 38.4% of 1088 rats, whereof 53.4% was designated *Y. enterocolitica*, only two rats harbored human pathogenic *Y. enterocolitica* (bioserotypes 2/O:5,27 and 3/O:1,2,3) and three were found to be positive for human pathogenic *Y. pseudotuberculosis*. In conclusion, our results demonstrate that rats are

frequent carriers of *Yersinia* spp. and although these animals are probably not the primary reservoir of these human pathogenic *Yersinia* species, they are potentially able to maintain (pathogenic) *Yersinia* species, at least temporarily, within a population after coming into contact with the bacteria (**Chapter 4**).

In this thesis, we have demonstrated that urbanization can impact pathogen-infection dynamics through 1) changing the body condition of the house sparrows and 2) affecting the presence of enteropathogenic bacteria. Nevertheless, no direct correlation between the enteropathogen presence and the host body condition was demonstrated. Despite the low prevalence of enteropathogenic bacteria, the exogenous exposure to certain enteropathogenic strains could cause mortality and should be considered as a risk factor. In addition, brown rats, frequently appointed as reservoirs for various infectious disease agents, were more likely acting as spill-over hosts than maintenance hosts.

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SAMENVATTING

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Van de effecten die de mens veroorzaakt op natuurlijke gebieden leidt de urbanisatie van het landschap tot één van de meest intense en langdurige veranderingen. Om de impact van urbanisatie op wilde dieren en ecosystemen na te gaan worden vogels frequent gebruikt als onderzoeksobject aangezien deze wijd verspreid aanwezig zijn en relatief gemakkelijk zijn om te observeren en te vangen. Hierdoor zijn er reeds veel inzichten verworven op gemeenschapsniveau, waarbij biotische homogenisatie van urbane vogelgemeenschappen een terugkerend fenomeen is. Onderzoek is echter nodig naar de factoren die leiden tot deze veranderingen alsook het effect van urbanisatie op populatie en individueel niveau. Een ander onderbelicht aspect van de urbane ecologie is de manier waarop urbanisatie een impact heeft op de ecologie van pathogenen, door onder andere te interfereren met gastheer-pathogeen interacties, waardoor de mate van urbanisatie een invloed kan hebben op de gezondheid van wilde dieren. Onderzoek naar pathogeen-ecologie in een urbane context is essentieel om een beter inzicht krijgen in ecologische en evolutionaire mechanismen die populatiedynamieken sturen in geürbaniseerde regio's, waarbij het opsporen van potentiële dragers en reservoirs van pathogenen een belangrijk luik vormt.

De focus van dit onderzoek werd gelegd op de interactie tussen huismussen (*Passer domesticus*) en enteropathogene bacteriën waarvan geweten is dat ze schadelijk kunnen zijn voor huismussen (**Hoofdstuk 2**: *Salmonella* Typhimurium; en **Hoofdstuk 3**: *Yersinia pseudotuberculosis* en *Y. enterocolitica*) langsheen een urbanisatie-gradiënt. Verder werd getracht de rol van bruine ratten (*Rattus norvegicus*) te bepalen als mogelijke reservoir-gastheer (**Hoofdstuk 4**: *Y. pseudotuberculosis* en *Y. enterocolitica*) in verschillende regio's in Vlaanderen, België.

Om de effecten van urbanisatie op gastheer-pathogeen interacties te bestuderen, werd er in **Hoofdstuk 2 en 3** geopteerd om met huismussen te werken van 36 populaties, verspreid over Vlaanderen, die verschillen in hun graad van urbanisatie (berekend op basis van twee spatiale schalen: een lokale en een landschapsschaal). Huismussen zijn in dit opzicht een ideale studiesoort, aangezien ze voorkomen langsheen een urbane gradiënt (urbaan, suburbaan, ruraal) en zeer sedentair zijn.

- *Salmonella* Typhimurium werd niet geïsoleerd uit de faeces van de huismussen en bij slechts één vogel konden anti-*Salmonella* antistoffen konden worden aangetoond. Wel kon na autopsie van 12 huismussen, verkregen via Vogelopvangcentra (Merelbeke en Oostende), *Salmonella* Typhimurium DT99 (een duif-geadapteerd faagtype) en DT195 worden geïsoleerd uit hersengranulomen van twee gestorven huismussen. Deze resultaten wijzen in

de richting van een schijnbare afwezigheid (prevalentie <1.3%) van *Salmonella* Typhimurium in gezond ogende huismussen tijdens de winter van 2013 in Vlaanderen. Klinische letsels, geassocieerd met een infectie door *Salmonella* Typhimurium, zijn hoogst waarschijnlijk te wijten aan een exogene blootstelling aan pathogene *Salmonella* Typhimurium stammen (**Hoofdstuk 2**).

- *Yersinia pseudotuberculosis, Y. enterocolitica* en andere *Yersinia* species konden uit de faeces van respectievelijk 2%, 31% en 41% van de gezond ogende huismussen geïsoleerd worden. Met behulp van "Gegeneraliseerde Lineaire Gemengde Modellen" werd nagegaan of er correlaties waren tussen de "omgeving" (bebouwingsgraad, de gemiddelde omgevingstemperatuur, de granivoren-index, de tijd waarop de mussen bemonsterd werden), de "gastheer" (geslacht, conditie uitgedrukt in scaled mass index (SMI)) en de aanwezigheid van "pathogenen" (*Y. pseudotuberculosis, Y. enterocolitica,* andere *Yersinia* species). De SMI van de huismussen kon niet worden verklaard door de aanwezigheid van *Y. pseudotuberculosis, Y. enterocoliticia* of andere *Yersinia* species en vice versa. De urbanisatie daarentegen, gemeten binnen de landschapsschaal, had wel een invloed op de SMI van de huismussen, waarbij mussen in suburbane gebieden een hogere SMI hadden vergeleken met mussen afkomstig uit urbane en rurale omgevingen. Afhankelijk van het *Yersinia* species dat getest werd, werden andere verklarende variabelen naar voor geschoven als zijnde belangrijk. De aanwezigheid van *Y. pseudotuberculosis* kon het best verklaard worden door, en was positief gecorreleerd met, de granivoren-index. Daarnaast bleek ook de urbanisatie (op landschapsschaal) een invloed te hebben op de *Y. pseudotuberculosis*-prevalentie, die meer aanwezig was in de faeces van suburbane en in mindere mate urbane huismussen, vergeleken met rurale dieren. De gemiddelde omgevingstemperatuur, de granivoren-index en de mate van urbanisatie op lokale schaal waren negatief gecorreleerd met de aanwezigheid van *Y. enterocolitica.* Ook de urbanisatie gemeten binnen de landschapsschaal had een invloed op de *Y. enterocolitica*-prevalentie, waarbij een hogere *Y. enterocolitica*-prevalentie werd waargenomen in urbane en rurale huismussen, vergeleken met suburbane mussen. De aanwezigheid van andere *Yersinia*-species werd het best verklaard door de gemiddelde omgevingstemperatuur en de lokale urbanisatie-schaal, waarbij voor beide een negatieve correlatie werd waargenomen (**Hoofstuk 3**).

  Er werd aangetoond dat de graad van urbanisatie een invloed heeft op de conditie van huismussen en de pathogeen-prevalentie, twee determinanten van pathogeen-infectie dynamieken. Er kon echter geen direct verband worden gelegd tussen de huismus-conditie

en de pathogeen-prevalentie. Verder dient ook de structuur van de vogelgemeenschap in de omgeving (welke hier werd benaderd door de granivoren-index) in rekening gebracht te worden (**Hoofstuk 3**).

Aangezien de bruine rat wordt aanzien als reservoir van een reeks zoönotische pathogenen, werd de rol van deze dieren als mogelijke reservoir-gastheer van *Y. pseudotuberculosis* en *Y. enterocolitica* in Vlaanderen nagegaan. Ondanks de isolatie van *Yersinia* uit 38.4% van 1088 ratten, waarvan er 53.4% als *Y. enterocolitica* werden geïdentificeerd, konden enkel twee enteropathogene *Y. enterocolitica* (bioserotype 2/O:5,27 en 3/O:1,2,3) en drie *Y. pseudotuberculosis* worden geïsoleerd. Onze resultaten tonen aan dat ratten dragers kunnen zijn van *Yersinia* spp. en hoewel de ratten waarschijnlijk niet het primaire reservoir zijn van de humane pathogene *Yersinia* species, kunnen ze (tijdelijk) (pathogene) *Yersinia* species dragen nadat de populatie in contact is gekomen met het pathogeen (**Hoofdstuk 4**).

In deze thesis werd aangetoond dat urbanisatie een invloed kan hebben op pathogeen-infectie dynamieken door het beïnvloeden van zowel 1) de gastheer conditie, als 2) de aanwezigheid van bepaalde enteropathogenen. Desondanks kon geen direct verband worden gelegd tussen de aanwezigheid van de enteropathogenen en gastheer-conditie. Daarenboven werd duidelijk dat, ondanks de lage prevalentie aan enteropathogenen, exogene blootstelling aan bepaalde enteropathogene stammen kan leiden tot sterfte en dit dus als een risico kan beschouwd worden. Verder bleken bruine ratten, die frequent worden aangeduid als reservoirs voor infectieuze ziekten, eerder te functioneren als een ‘spill-over’ gastheer dan als onderhoudsgastheer.

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CURRICULUM VITAE

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Lieze Oscar Rouffaer werd geboren op 25 november 1988 te Vilvoorde (België). Na het behalen van het diploma secundair onderwijs aan het Koninklijk Atheneum Etterbeek (KAE), richting Latijn-Wiskunde in 2006 vatte ze haar studies Diergeneeskunde aan, Universiteit Gent. In 2012 behaalde ze haar diploma 'Master in de Diergeneeskunde' (afstudeerrichting gezelschapsdieren) met grootste onderscheiding.

Tussen 2012 en 2013 heeft ze het internship "Zoological Medicine" afgelegd waarna ze het residentie "Wildlife Population Health" heeft aangevat.

In oktober 2013 begon ze aan haar doctoraatsopleiding bij de Vakgroep Pathologie, Bacteriologie en Pluimveeziekten (onderzoeksgroep: Wildlife Health Ghent) aan de Faculteit Diergeneeskunde, Universiteit Gent, in samenwerking met de Vakgroep Biologie (onderzoeksgroep: Terrestrische Ecologie), Faculteit Wetenschappen, Universiteit Gent. De studie werd gefinancierd door het Fonds Wetenschappelijk Onderzoek (FWO). Onder leiding van Prof. Dr. An Martel, Prof. Dr. Luc Lens en Prof. Dr. Frank Pasmans heeft ze onderzoek gedaan naar de effecten van urbanisatie op entero-pathogene bacteriën bij huismussen. Dit onderzoek maakt deel uit van het SPEEDY-project (SPatial and environmental determinants of Eco-Evolutionary DYnamics- anthropogenic environments as a model), gefinancierd door Belspo (Belgian Science Policy Office).

Ze nam deel aan verschillende nationale en internationale congressen en haar wetenschappelijk onderzoek leidde tot verschillende publicaties in internationale tijdschriften.

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Date: 26th of July 2015 – 30th of July 2015
Location: Twin Waters, Sunshine Coast, Queensland, Australia
Section: ***Living on the edge-Anthropogenic influences on wildlife health***
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"Enteropathogens in house sparrows along an urban gradient in Belgium" (Abstract n° 42)

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Date: 28th of October 2017
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Karen, Evy, Venessa, Lonneke (succes met je project!!), Iris, Wolf, altijd leuk om jullie tegen te komen in de bacteriologie-gangen! Een zwaaitje een babbelke… super ☺ Karen, merci om mijn gedachten net vóór de interne verdediging af te leiden ☺

Wouter, Mc Mark, Keely, Alex, Zhimin, Valarie, Moira, Pudsa, Queenie, Tijn and Jesse, the Wildlife Health Ghent group ☺. It was great to get to know you all ☺. Alex and Keely, you are almost there, keep on going. Alex, I'm curious to the final number of arthropods you have identified in your samples…! Wouter, although I preferred sitting in the Bacteriology building, it was a pleasure to have you as a neighbor in the VRB, hopefully in the future we can sometimes do some field-herping (?), or you can just show us the good spots ;-). Valarie, Ms. Ferrero Rocher!/Ms Cake!, I really hope everything will return to normal in Dominica and Good luck with the "Nederlandse-lessen" ☺ ! Moira, thanks to you I have learned a new "stylish" Cambridge-associated laugh ;-). Pudsa, you have almost finished, good luck with the last bits and pieces and good luck with your future in Thailand.

Serge en Koen, het olijke duo, jullie weten als geen ander hoe de sfeer erin te houden in en rond het labo en jullie staan altijd klaar voor…een onnozele grap ☺ Nathalie, Sarah, Arlette, Sofie en Julie, het was tof om jullie te leren kennen! Veel amusement nog in het labo en Arlette, zorg maar dat je snel terug beter wordt… Gunter M. en Jo, bedankt voor al de administratieve hulp en om elke ochtend 'goeiemorgen' te roepen of te zwaaien (tenzij Anderlecht verloren had…).

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Anneleen, wij gaan zeker nog afspreken, om te eten (super belangrijk ;-) en/of te puzzelen ☺. Ik denk niet dat ik iemand ken die zo begaan is met haar proefdieren als jij. Blijven volhouden, zeker niet opgeven. Ge kunt het! ☺

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Bruno, Elodie, Emily, Diane, Maarten, Branko, Niel, Garin, Debora, Maro, Rebecca, Martine, Yi, Emilie, Jenske… ik ga zeker meer tijd maken om af te spreken! Om te beginnen, Diane en Evert! Jullie trouw! Ik kijk er heel erg naar uit! ☺ Super spannend! ☺ Elodie, ik kom zeker eens naar het verre Nederland op bezoek, stapschoentjes aandoen aan kleine Clara en GAAN ☺ (heel benieuwd naar de gekke bekken ;-)), en Debora, ik passeer zeker ook in het verre Limburg voor de kleine Yarah ☺. Emily, we gaan dan eens eindelijk eens die koffie gaan drinken he ☺… gekoppeld aan een zoniënwoud-wandeling???

DT, DTa en DTh! Ja… daar zijn geen woorden voor… ☺ de uitbreiding van de familie is begonnen :-D dus DT(a) in Argentinië… we komen ZEKER op bezoek! En kunnen er weer een prachtige DT-reis aan breien!!! ☺ of nog wat reisjes in Europa met de hondjes ☺.

En dan de andere families… :-D Mama, papa, super erg bedankt om mij altijd te steunen en steeds opnieuw naar mijn mussen-verhalen te willen luisteren ☺ Geniet van jullie reis!! we maken er achteraf een leuk feestje van ☺ Brrroerr en zusie ☺ super dat jullie er altijd zijn voor de kleine zus, ik zie jullie (ja pink dat traantje maar weg ;-)) super graag!!! Peter Peter, Mieke, bedankt voor alles, EN voor het organiseren van de neven en nichtenfeestjes, spijtig dat het dees jaar niet lukte om er te geraken.

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