Influence of habitat fragmentation and microbial pressure on the reproductive success of great and blue tits

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

BGA	Brilliant Green Agar
DEI	Depth of Edge Infuence
DT	Definite Phage Type
ELISA	Enzyme-Linked Immunosorbent Assay
GLMM	Generalized Linear Mixed Model
HRP	Horseradish Peroxidase
Ig	Immunoglobulin
LMM	Linear Mixed Model
MLVA	Multi-Locus Variable number tandem repeat Analysis
OD	Optical Density
PBS	Phosphate Buffered Saline
RVS	Rappaport Vassiliadis medium supplemented with Soya
SI	Shape Index
SLOSS	Single Large or Several Small
SMA	Standardized Major Axis
SMI	Scaled Mass Index
spp.	species
subsp.	subspecies
TMB	Tetramethylbenzidine
XLD	Xylose Lysine Deoxycholate

INTRODUCTION

1. Disease triangle

The disease triangle consists of three major factors that are linked to disease dynamics: the host, the pathogen, and the environment. These three factors are connected to each other and their interactions determine the disease outcome (Wobeser, 2006b). The disease triangle model is commonly used to explain how variation in environmental factors, host susceptibility, and pathogen virulence lead to varying disease outcomes.

Understanding the disease triangle, the complex interplay between host-pathogen-environment is crucial for wildlife disease management and species conservation. For example, understanding how landscape changes can act as a selective pressure on host-pathogen dynamics; or how pathogens can induce adaptation in life-history traits, behaviour, resistance or tolerance and their consequent implications for host population dynamics.

This thesis aims to study how environmental changes i.e. habitat fragmentation effects the microbial pressure on eggs and juveniles of blue and great tits and the impacts of pathogens on their reproductive performances and how they cope with egg microbial pressure (Fig 1).

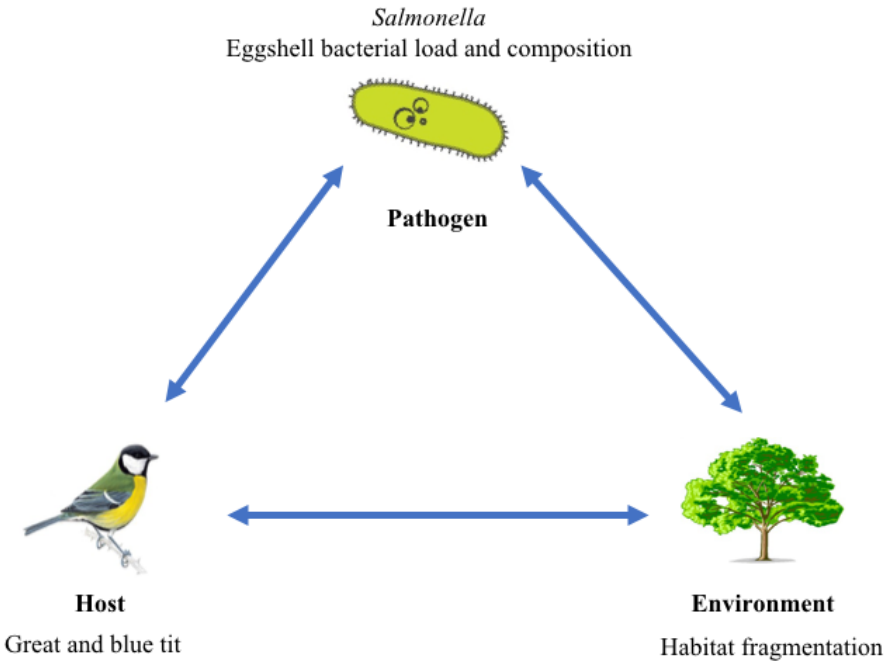

Figure 1: Representation of the disease triangle in this thesis. Host: great and blue tits; Pathogen: *Salmonella* and eggshell bacterial load and composition; Environment: Habitat fragmentation (fragment area).

2. Habitat fragmentation

As a result of the increasing human population which consists of 7.4 billion world citizens, and is growing by 1.18% (or 83 million people) annually (United Nations, 2015), landscape modifications (urbanization, agricultural land-use) with the resulting habitat destruction and fragmentation are a major concern (Fischer and Lindenmayer, 2007). When focusing on forest-ecosystems, a recent worldwide analysis revealed that 70% of forests are within one km of a forest edge (Haddad et al., 2015), which can have serious repercussions on the forest ecosystems (see below).

Flanders for example, with a population density of 475 inhabitants/km² (ENRD, 2015), is highly fragmented due to the extensive urban sprawl which started by the end of the 19th century (Tempels et al., 2012). Between 1775 and 2000, 9.7-12.2% of the total area in Flanders was occupied by forests. Only 16% of the forests that already existed in 1775 were still present in 2000 (the "ancient" forests). Furthermore, only 14% of the "recent" forests (planted/originated after 1775) were in physical contact with the ancient forests (De Keersmaeker et al., 2015). Although this contact facilitated the colonization of ancient forest specialist into new forests, even after a century, qualitative differences between the ancient and adjacent recent forests could be observed (Bossuyt et al., 1999). All these factors have contributed to increased 'forest fragmentation', with an increased number of forest fragments as a result (De Keersmaeker et al., 2015).

Wilcove et al. (1986) defines habitat fragmentation as "a process during which a large expanse of habitat is transformed into a number of smaller patches of smaller total area, isolated from each other by a matrix of habitats unlike the original". This definition pinpoints four aspects of landscape alteration: 1) the increasing number of habitat remnants, 2) the increased isolation of these remnants, 3) the reduction of the patch size, 4) the reduction in the total amount of habitat (Rolstad, 1991; Fahrig, 2003). The primary impact of fragmentation should be through the loss of continuity, not habitat loss *per se*. However, these concepts are closely connected, habitat loss is an important consequence of habitat fragmentation (Fig 2) (Rolstad, 1991; Andrén, 1997; Fahrig, 2017). Therefore, it is hard to separate the effects of habitat fragmentation from the effects of habitat loss. Eventhough, the impacts of hatbitat loss have been purposed to outweigh the impacts of habitat fragmentation *per se*, as such, care must be taken not to confound the consequences of either of these landscape modifications or their

combination (Haddad et al., 2015; Fahrig, 2017) which can have landscape management implications (Wilcox and Murphy, 1985; Saunders et al., 1991; Tjørve, 2010; Fahrig, 2017).

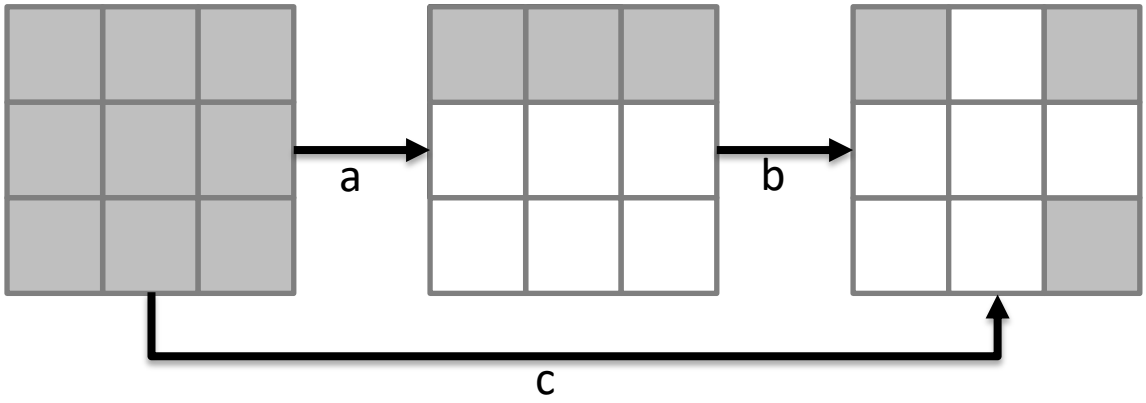

Figure 2: Habitat fragmentation versus habitat loss:

a. habitat loss;
b. habitat fragmentation (only possible if new forest would be planted);
c. habitat loss and fragmentation

Habitat fragmentation has become an important topic in conservation biology, but how this landscape alteration affects population dynamics and biodiversity is still under debate. In order to better understand the drivers changing the native fauna and flora, following characteristics should be considered: 1) the alterations in the microclimate and 2) isolation-status of the fragment, more specifically the time since isolation, the distance from other fragments and the connectivity between these fragments, 3) the fragment size and shape 4) and the surrounding landscape features (Saunders et al., 1991; Honnay et al., 1999; Tabarelli and Gascon, 2005; Haddad et al., 2015).

Forests, or other areas comprising of dense vegetation, act as a buffer to various climatic conditions outside these areas (Chen et al., 1993; Ewers and Banks-Leite, 2013). Clearing and fragmenting these forests will affect the microclimate in several ways which will be most prominent at the borders of the fragments, the 'edge-effects' (Saunders et al., 1991; Ewers and Banks-Leite, 2013; Haddad et al., 2015). The changes in microclimate are characterized by following variables:

Sunlight exposure, soil and air temperature: Due to an increased amount of solar radiation that is able to reach the ground surface in fragmented areas, the daytime temperature will be higher and the overnight temperature-loss will be faster and greater, resulting in a wider overall temperature-range in more fragmented areas (Chen et al., 1993; Davies-Colley et al., 2000;

Ewers and Banks-Leite, 2013). In the temperate zone, this effect is strongest at South-West facing forest-edges (Honnay et al., 2002).

Wind-velocity and direction: The overall exposure to wind increases with perimeter-to-area ratio and can have direct and indirect effects on the forest fragments. Direct damage consists of wind pruning and wind throwing of trees, which is especially important when the forest has recently been fragmented due to increased wind-forces on inadequately adapted trees (Saunders et al., 1991). Indirect effects comprise the enhanced desiccation of forest-edges (Davies-Colley et al., 2000) and increased dispersal of plant-seeds, (agricultural) pollutant throughfall into the forest fragment edges (Saunders et al., 1991; Honnay et al., 2002; Wuyts et al., 2008).

Precipitation and humidity: Due to altered evapotranspiration and precipitation, the humidity measured at the edge of fragments will be lower compared to measurements within the forests, with increased susceptibility to desiccation (Saunders et al., 1991; Chen et al., 1993), making the fragments more prone to wild-fires (Cochrane and Laurance, 2002).To what distance these edge-effects can be measured within the forest fragments depend on different factors, such as the perimeter to area-ratio, the edge shape and orientation, the season, forest-type and composition of the native vegetation and the maturity of the edge, whether or not the edge has been able to form a buffer against the surrounding environment (Laurance and Yensen, 1991; Chen et al., 1993; Young and Michell, 1994; Davies-Colley et al., 2000; Honnay et al. 2002; Wuyts et al., 2008; Ewers and Banks-Leite, 2013; Haddad et al., 2015). These edge effects can extent from a few meters up to a few hundred meters into the forest (Chen et al., 1993; Young and Michell, 1994; Davies-Colley et al., 2000; Honnay et al., 2002; Ewers and Banks-Leite, 2013; Haddad et al., 2015).

With respect to the effect of the type of forest, Wuyts et al. (2008) showed that compared to deciduous forests (oak (*Quercus* spp.), birch (*Betula* spp.), and their mixture), the throughfall of eutrophying and acidifying ions (NO_3^- and SO_4^{2-}) in coniferous forests (*Pinus* spp.) was larger for both the edge as for the interior of the forest, leading to increased soil acidification, eutrophication, nitrogen saturation, which can be related to lower biodiversity in coniferous forests (Gärtner and Reif, 2004).

To account for these edge-effects, Laurance and Yensen (1991) proposed a 'Core-Area model', later re-examined by Didham and Ewers (2012). The model extracts the core-area from the total area in a forest, based on the calculation by the fragment shape index (SI) and the depth of edge influence (DEI). Thus, the Core-Area model enables accurately assessing the

impacts of edge effects e.g. when assessing the effect of forest fragmentation on species biodiversity.

2.1 The effect of habitat fragmentation on bird populations and their health status

Fragmentation can have immediate and long term effects on populations, which could lead to population declines and eventually extinctions (Wilcox and Murphy, 1985; Rolstad, 1991; Saunders et al., 1991; Fischer and Lindenmayer, 2007). The factors contributing to these declines can to a greater or lesser extent be correlated to the climatic and habitat alterations/disturbance outlined above and will more specifically be related to the following points:

1) Changes in nest predation: Some studies describe a higher risk of nest predation (Nour et al., 1993; Hinsley et al., 1995a; Huhta et al., 2004; Borges and Marini, 2010) and brood parasitism (Borges and Marini, 2010) in fragmented areas. Nevertheless, this predation risk depends on the local predator community and the ones present in the surrounding landscape, and can be attributed to different predator species (Nour et al., 1993; Hinsley et al., 1995a; Huhta et al., 2004). However, some studies indicate the opposite, stating that nest predation decreases in fragmented areas. For example, a study in the United States showed higher nest predation in continuous forests compared to fragmented forests, since predator density in undisturbed forests was shown to be higher (Tewksbury et al., 1998).

2) Changes in climatic conditions such as increased turbulence in edge-fragments can lead to breeding failure through wind throwing of nestlings and increasing the difficulty of landing on the nests by fledglings (Reville et al., 1990; Saunders et al., 1991).

3) Reduced daily (between habitat-movements), or seasonal dispersal opportunities (Saunders et al., 1991; Fischer and Lindenmayer, 2007) and higher local recruitment in more isolated patches (Matthysen et al., 1995; Matthysen et al., 2001), which could lead to reduced pair success (Cooper and Walter, 2002) and loss of genetic diversity and eventually (although sometimes only seasonal (Van de Casteele et al., 2002)) inbreeding depression (Kempenaers et al., 1996; Dudash and Fenster, 2000; Gibbs et al., 2001).

4) Lower abundance (Zanette et al., 2000) and genetic diversity (Van Dongen et al., 1998) of the arthropod prey items for insectivorous bird species. Van Dongen et al. (1998) demonstrated a lower genetic diversity in winter moths (*Operophtera brumata*), likely resulting

in a lower adult weight in more isolated fragments, compared to those inhabiting continuous forests. This lower genetic diversity could lower the fitness of the population potentially interfering with their ability to synchronize the larval hatching with the bursting of oak (*Quercus robur*) buds (Van Dongen et al., 1994; Matthysen et al., 1995; Van Dongen et al., 1998). This mismatch could lower the abundance of arthropods which subsequently lowers the foraging efficiency of the insectivorous predator species, with a higher number of bird nests being abandoned due to scarce food availability (Matthysen et al., 1995; Zanette et al., 2000).

5) The increased prevalence of pollutants and decreased availability of important micro-elements could be related to fragmentation and/or forest type (Rolstad, 1991; Saunders et al., 1991; Goosem, 2007; Wuyts et al., 2008). Depending on the surrounding environment, animals/plants living in fragments have been found to be exposed to higher levels and a higher variety of pollutants (e.g. heavy metals, pesticides used in agriculture) (Goosem, 2007), which have been demonstrated to be concentrated in the forest-edges (Weathers et al., 2001). Besides the pollutants, deficiencies in micro-elements have been observed. Deficiencies in Calcium (Ca^{2+}) in areas with acidic soils, such as pine forests, or acidified areas due to acid rain (Wuyts et al., 2008) or due to the lower availability of snail shells (Graveland, 1996; Mänd et al., 2000a and b), can have major consequences on nestling and fledgling development and calcium deposition in the egg yolk and shell, with reduced chick growth and overall breeding success in tits (Graveland, 1996; Mänd et al., 2000a and b; Tilgar et al., 2005).

6) Stochastic events can have huge impacts on small populations in fragmented areas, potentially accelerating the extinction events (Hinsley et al., 1995b; Van Dongen et al., 1998).

7) Changes in infection pressure: Some studies describe an increasing risk of microbial infections by influencing species movement, dispersal, and resource availability in degrading habitats. Consequently, the stress levels of the animals increased and immunological functions starts to weaken, hence increasing the chances to get infected, resulting in advances for the disease and symbiont transmission (e.g. Eley et al., 1989; Ashford, 1996). A risk of infection can also be modified by changes in population densities and species richness, which are affected by habitat fragmentation. For example, a study in the Atlantic forest of Brazil revealed that bird diversity and richness was associated with larger forest fragments, and tick prevalence on birds was inversely correlated with bird diversity and richness. However, tick infections were not statistically different between forest patch sizes suggesting easier transmission of parasites occurring in small forest patches (Ogrzewalska et al., 2011). The other way around,

some studies indicate a higher disease prevalence in larger habitats. A study on rain forest birds in Australia describes a higher prevalence of haemosporidian infections in continuous forests compared to fragmented forests. Ecological traits including diet, foraging height, habitat specialization and distribution ranges were suggested to be associated with infections, which is in accordance with a study in Cameroon (Chasar et al., 2009; Laurance et al., 2013).

It thus has been shown that habitat degradation can lead to changes in infection dynamics (host-pathogen interactions), both in a positive and negative way. However, this aspect is still underexplored and contradictory. Nevertheless, the understanding of these host-pathogen interactions along a fragmentation gradient, with an increased access to anthropogenic resources in more fragmented areas, is of great importance to unravel the population dynamics in changing environments (Daszak et al., 2000; McCallum and Dobson, 2002; Keesing et al., 2006; Becker et al., 2015).

3. Egg and Offspring-infection

Microbial infection is considered to be one of the most important life threatening risks from fertilization until death. In oviparous animals, non-motile eggs are encounter with predation and parasites (Clutton-Brock, 1991), including microbes, with bacteria being the most diverse group (Mlot, 2004). The egg-content and offspring can become infected through different ways:

1) From parents:

1a) True vertical transmission occurs when the bacteria can infect the reproductive tract of the birds and subsequently the eggs during egg-formation (Keller et al., 1995; Wigley et al., 2005; Wobeser, 2006c; Hafez, 2013).

1b) Pseudo-vertical transmission can be categorized as a horizontal infection, although since the infection occurs within a short time-frame after oviposition (e.g. trans-shell infection) (Messens et al., 2005; Wobeser, 2006c; Hafez, 2013), the transmission can be mistakenly classified as a vertical transmission.

2) From environment or other animals:

Horizontal transmission also occurs after oviposition or hatching through contamination of the eggs or offspring from other animals (e.g. parents, nest visitors) or from the (nest) environment (Messens et al., 2005; Wobeser, 2006c; Hafez, 2013).

As such, both the reproductive tract (Keller et al., 1995; Wigley et al., 2005; Gantois et al., 2009), nest environment (e.g. nest lining material, faeces, feathers, skin, nest visitors, airborne-bacteria) which can be influenced by life-history traits and habitat (e.g. Cook et al., 2003 and 2005a; Mennerat et al., 2009a and 2009b; Goodenough and Stallwood, 2010; Martínez-García et al., 2016), and climatic conditions (e.g. Berrang et al., 1999; Cook et al., 2003; Beissinger et al., 2005; Wang et al., 2011; Walls et al., 2012) pose a risk of egg and nestling contamination/infection.

Shortly after laying, the eggshell is highly susceptible to pathogen penetration (Miyamoto et al., 1998; Gantois et al., 2009; Cox et al., 2012). The temperature difference between the egg and the environment induces a contraction of the egg-content (due to cooling), which creates a vacuum and negative pressure within the egg and enhances the entry of bacteria present upon the shell into the eggshell and membranes (Miyamoto et al., 1998; Berrang et al., 1999; Cook et al., 2003; Beissinger et al., 2005; Messens et al., 2005). Moist environments increase the potential of bacteria to enter the egg through the provision of a watery transport vehicle for the bacteria (Berrang et al., 1999; Cook et al., 2003; Beissinger et al., 2005; Messens et al., 2005; Cox et al., 2012). In this perspective, the eggshell bacterial load as well as the bacterial composition have been positively correlated with trans-shell infections and decreased hatching success (Berrang et al., 1999; Cook et al., 2003 and 2005a; Shawkey et al., 2009). Although not all studies have found a correlation between the bacterial load and the hatching success (e.g. Peralta-Sanchez et al., 2010; Ruiz-de-Castañeda et al., 2011).

Egg infection by opportunistic or pathogenic bacteria can greatly reduce the reproductive success of birds through e.g. embryonic death resulting in hatching failure (Pinowski et al., 1994; Stewart and Rambo, 2000; Cook et al., 2003 and 2005a and 2005b; Peralta-Sánchez et al., 2012 and 2014; d'Alba et al., 2016), or affecting the condition or morphology of the young (Mennerat et al., 2009a and 2009b; González-Braojos et al., 2012; Møller et al., 2013; Jacob et al., 2015). In order to increase the reproductive success, birds use different methods to decrease the bacterial load on the eggshells and increase the antibacterial capacity of the eggs to protect their offspring, which will be discussed below.

3.1. Resisting the egg and offspring infection: egg-related factors

To minimize embryonic contamination, the composition of the egg (eggshell and membranes, albumen and egg yolk) creates a natural physical and chemical barrier against bacterial infection (Sparks, 1994; Berrang et al., 1999; D'Alba and Shawkey, 2015; D'Alba et al., 2014, 2016 and 2017).

3.1.1. Eggshell and shell membranes

The eggshell and shell membranes consist of different layers (Fig 3) (from inner to outermost): Shell membranes (inner and outer shell membrane), the true egg shell (mammillary cones, palisade zone, vertical crystal layer) and the cuticle layer which spans and covers the eggshell pores (Hincke et al., 2012; D'Alba et al., 2014 and 2017). Besides the physical barrier against microbial invasion, some layers additionally have chemical or structural antimicrobial effects (Board and Fuller, 1974; Wellman-Labadie et al., 2008; D'Alba and Shawkey, 2015; D'Alba et al., 2014 and 2017).

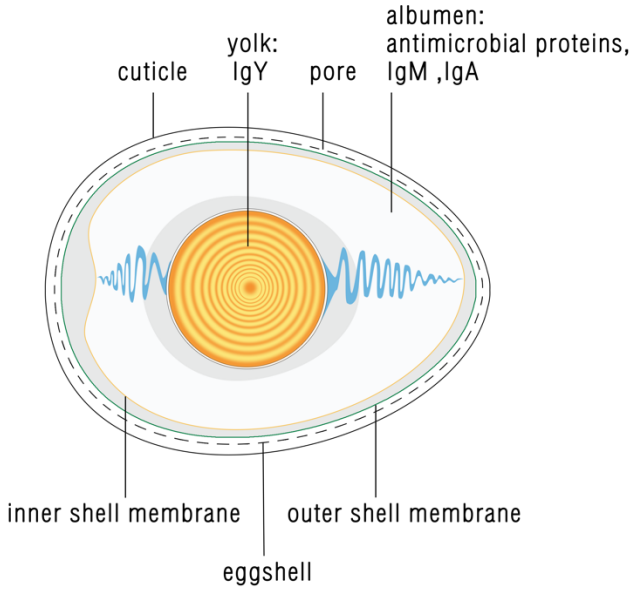

Figure 3: Egg compositions with immunological antibacterial properties indicated for the albumen and egg yolk (Adapted from Hinkce et al., 2012)

Many proteins have been identified in the eggshell and membranes, some of which are eggshell/membrane specific (e.g. ovocalyxins and ovocleidins (Mann et al., 2006; Gautron et al., 2007; Mann and Mann, 2011)), others are abundant in other parts of the egg as well, such

as the egg albumen (e.g. lysosyme, ovotransferrin) (Valenti et al., 1982; Hincke et al., 2000; Gautron et al., 2001; Ahlborn et al., 2006; Ahlborn and Sheldon, 2006; Mann, 2007; Wellman-Labadie et al., 2008; D'Alba and Shawkey, 2015). Although the function of most proteins is still unknown, some of these proteins have an antibacterial function, e.g. lysozyme, ovotransferrin, ovocalyxin (Gautron et al., 2007; Wellman-Labadie et al., 2008; D'Alba et al., 2016). Besides the presence of antibacterial-proteins in the cuticle and the physical closure of the egg-pores, some additional properties can be attributed to the egg cuticle. Depending on the nest-ecology of the bird species, nanospheres, present in the cuticle of some bird species, prevent the attachment of bacteria on the eggshell surface (D'Alba et al., 2014, 2016 and 2017; D'Alba and Shawkey, 2015). Some cuticles are hydrophobic and prevent flooding of the egg and/or protect the eggs against solar radiation (D'Alba et al., 2014, 2016 and 2017; D'Alba and Shawkey, 2015). Additionally, in some bird species such as great and blue tits, eggshell cuticle contains pigments (proto-porphyrin and/or biliverdin) (Higham and Gosler, 2006; Martínez-de la Puenta et al., 2007) which have been associated with reduced water loss and increased eggshell thickness (Higham and Gosler, 2006).

3.1.2. Albumen and egg yolk

The pH of the albumen plays an important role in the protection of the embryo. After oviposition, the albumen pH changes in a couple of days from pH 7.6 to pH 9, creating an alkaline environment which is not well supported by many bacteria (Board and Fuller, 1974; Reijrink et al., 2008). Furthermore, the female birds can allocate antibacterial proteins and immune factors, such as immunoglobulins (Ig) to the egg albumen and egg yolk in order to protect her offspring (D'Alba et al., 2010a).

3.1.2.1 Antimicrobial proteins

Antimicrobial proteins are present in the albumen and can be classified in the ones:

1) chelating vitamins or minerals, making these substances unavailable for microbial growth:

   - the glycoprotein 'avidin' binds to biotin, an essential growth factor (vitamin-B) for many bacteria, making it unavailable for these bacteria (Board and Fuller, 1974; Nau et al., 2007; D'Alba and Shawkey, 2015).

   - 'Ovotransferrin', produced in the oviduct, acts as a chelator of ferric ions, making these ions unavailable for bacterial growth. Furthermore, it can act as a bactericidal protein

through the direct binding to and damaging of bacterial membranes of gram-negative and gram-positive bacteria (Board and Fuller, 1974; Ibrahim et al., 1998 and 2000; Superti et al., 2007; D'Alba and Shawkey, 2015).

2) degrading microbial components:
   - ovotransferrin (see above).
   - Lysozyme (muramidase) is a hydrolase which has bactericidal capacities by hydrolyzing the linkage between N-acetylmuramic acid and N-acetylglucosamine, present in the peptidoglycan cell wall of, mostly gram-positive, bacteria (Board and Fuller, 1974; Lesnierowski and Kijowski, 2007; D'Alba and Shawkey, 2015; Javůrková et al., 2015).
3) inhibiting bacterial proteases, e.g. ovostatin, ovomucoid (D'Alba and Shawkey, 2015)

Depending on different factors (e.g. laying order, timing in the season, sex of the offspring), the bird species, and potentially the infection status of the female bird (although in wild living birds more research is needed), female birds can change the allocation of antimicrobial proteins to the eggs (Saino et al., 2002; Shawkey et al., 2008; D'Alba et al., 2010a; Bonisoli-Alquati et al., 2010; Bedrani et al., 2013; Horrocks et al., 2014).

3.1.2.1. Immunogolobulins

Besides the allocation of antimicrobial proteins, immunoglobulins (Ig) are transferred from the mother to the egg and offspring (Hamal et al., 2006; King et al., 2010). Females can transfer the IgY antibodies to the egg yolk, and IgM and IgA to the egg albumen, although the latter two can also be found in lower quantities in the egg yolk (Hamal et al., 2006; Staszewski et al., 2007; King et al., 2010). The detectability and half-life of immunoglobulins and the production of endogenous antibodies by the offspring varies between species (King et al., 2010). King et al. (2010) observed a clear difference between precocial chickens and altricial house sparrow (*Passer domesticus*) nestlings, with altricial youngs achieving immunological independence earlier than precocial offspring.

As was observed for the antimicrobial proteins, the investment of the mother bird to transfer immunoglobulins to the offspring depends on different factors. A positive correlation has been found between the environmental infection pressure of a specific pathogen and the Ig-titre in the plasma of the female bird (Gasparini et al., 2001 and 2002; Lindström et al., 2004). These birds subsequently confer the passive immunity and protection to their eggs and offspring, who can benefit from this passive maternal immunity when encountering the same infectious agent

as their mother (Buechler et al., 2002; Gasparini et al., 2001 and 2002; Grindstaff, 2008). Besides the pathogen pressure, also the perceived predation risk has been demonstrated to alter the transfer of immunoglobulins to the eggs, with more Ig's being allocated to the eggs when nesting in habitats with continuous predation pressure (Morosinotto et al., 2013). However, Hargitai et al. (2006) found a negative correlation between stress and immunoglobulin-transfer to the eggs. Furthermore, not all the eggs receive the same amount of immunoglobulins. The females body condition plays a role in the transfer of immunoglobulins to the eggs and offspring with more Ig's being transferred when the mother bird had a better body condition (Hargitai et al., 2006). Additionally, the laying order can also affect the Ig concentration in the egg, although contrasting results are available. Higher Ig concentrations have been observed when the egg was laid later in the clutch (Hargitai et al., 2006), but also when the egg was laid earlier in the clutch (Blount et al., 2002).

3.2. Resisting the egg and offspring infection: parental-related factors

3.2.1. Incubation

Incubation of the eggs has been shown to affect the bacterial community and abundance on avian eggshells, which can subsequently limit the possibility of trans-shell infections (Cook et al., 2003, 2005a and 2005b; Shawkey et al., 2009; D'Alba et al., 2010b; Peralta-Sánchez et al., 2012; Brandl et al., 2014; Giraudeau et al., 2014; Grizard et al., 2014; Lee et al., 2014). In this perspective, early incubation has been suggested as a mechanism of the parental birds to increase the egg-viability of the early laid eggs, despite the increased possibility of hatching asynchrony (Cook et al., 2003, 2005a and 2005b). Although contradictive reports have been published regarding the bacterial load on eggshells ('lower loads on incubated eggs': Cook et al. (2005a), Shawkey et al. (2009), D'Alba et al., 2010b; 'no significant difference in bacterial load': Wang et al. (2011), Brandl et al. (2014); 'higher bacterial loads': Peralta-Sánchez et al. (2012), Giraudeau et al. (2014), Grizard et al. (2014), Lee et al. (2014)), most studies that have looked into the bacterial assemblage did find a reduction of pathogenic bacteria (harmful gram-negative and/or hemolytic bacteria) compared to the commensal, non-harmful bacteria on incubated eggs (Cook et al., 2005a; Shawkey et al., 2009; Brandl et al., 2014; Grizard et al., 2014; Lee et al., 2014). How incubation alters the microbial load and diversity is still a matter

of debate, although the reduction of humidity on incubated eggs has been proposed to limit bacterial growth (D'Alba et al., 2010b).

3.2.2. Uropygial gland secretion

The uropygial gland secretion has been shown to harbor antimicrobial properties (Soler et al., 2008, 2012 and 2014; Ruiz-Rodríguez et al., 2012 and 2013; Møller et al., 2010). Especially during the breeding season the characteristics of this secretion has been shown to change in hoopoes (*Upupa epops*) (Soler et al., 2008). *Enterococcus* spp., *E. faecalis* in particular, are symbiotic bacteria within the uropygial gland, especially during the breeding season, producing antimicrobial substances such as bacteriocins, competitively excluding a broad spectrum of pathogenic bacteria (Soler et al., 2008; Ruiz-Rodríguez et al., 2012 and 2013). Through the combinatory effect of the increase in abundance and diversity of feather mites and chewing lice (Møller et al., 2010; Soler et al., 2012) and the production of antimicrobial substances, the uropygial gland secretion has:

- been associated with a growth inhibition of feather degrading bacteria, e.g. *Bacillus licheniformes* (Shawkey et al., 2003; Reneerkens et al., 2008; Soler et al., 2008; Ruiz-Rodríguez et al., 2009),
- been shown to lower the egg-infection and increase the hatching success when smeared onto the eggs through preening (Soler et al., 2008, 2012 and 2014; Møller et al., 2010).

3.2.3. Nest building behavior

Differences in nest building behaviour in avian species have been suggested to protect the eggs and offspring against pathogens such as:

- nest selection site: hatching success has been shown to differ between open versus cavity nests, with lower hatchability in open nests (Godard et al., 2007) and depends on the position of the nests within the environment (e.g. presence of plants with antibacterial properties) (Møller et al., 2013).

- nest sanitation (Singleton and Harper, 1998).
- selection of nesting materials (Clark and Mason, 1985 and 1988; Gwinner and Berner, 2005; Mennerat et al., 2009a and 2009b; Peralta-Sánchez et al., 2010, 2011, 2012 and 2014; Ruiz-Castellano et al., 2016).

Nevertheless, most research in this area has been performed on nest lining materials in different bird species. Some avian species which often reuse nests (such as cavity nesting birds) are potentially more exposed to bacteria and ectoparasites (Godard et al., 2007). These birds have learned to use environmental aromatic plants with volatile antimicrobial compounds (e.g. *Achillea millefolia*, *Mentha suaveolens*, *Heracleum sphondylium*, *Salix alba*, *Lavandula stoechas*, and *Helichrysum italicum*) to reduce the bacterial load on the eggs (Ruiz-Castellano et al., 2016) and nestlings, with a positive effect on the nestling growth and condition (Clark and Mason, 1988; Gwinner and Berger, 2005; Mennerat et al., 2009a and 2009b). Also feathers have, besides the thermoregulatory function, antimicrobial properties, reducing the bacterial load and affecting the bacterial composition in the nests and on the eggshells (Peralta-Sánchez et al., 2010, 2011, 2012, 2014; Ruiz-Castellano et al., 2016). Besides the number of feathers, also the colour of the feather seems to play a role in the bacterial composition and load on the eggs (Peralta-Sánchez et al., 2010 and 2014), with white feathers having a higher antimicrobial activity (Peralta-Sánchez et al., 2014) and nests lined with white feathers having a higher hatching success (Peralta-Sánchez et al., 2011).

In the following parts, I will focus on how opportunistic and avian pathogenic bacteria (e.g. *Salmonella enterica* subspecies *enterica* serotype Typhimurium) can infect the avian host and what defense mechanisms birds are using in the battle agains bacterial infections. Depending on host characteristics, the virulence of the bacterium and environmental factors (Wobeser, 2006b; Vander Wal et al., 2014), pathogen infection can be the cause of clinical or subclinical disease in the host. Far too often only obvious clinical disease is reported, although subclinical disease could decrease avian fitness and/or affect the reproductive success (e.g. through infection of the reproductive organs, increased hatching failure, retarded nestling and fledgling growth, reduced offspring survival) (Faddoul and Fellows, 1965; Cook et al., 2005a; Wobeser, 2006a; Peralta-Sánchez et al., 2012) and can play an important role in population dynamics (Wobeser, 2006b).

3.3. Pathogen: *Salmonella* Typhimurium in passerines

Salmonella is a genus of rod-shaped gram-negative bacteria, which belongs to the family of the *Enterobacteriaceae*. The genus *Salmonella* comprises three species, *Salmonella bongori, Salmonella subterranea* and *Salmonella enterica.* The latter can be subdivided into six subspecies: namely, *Salmonella enterica* subspecies *enterica* (I), subspecies *salamae* (II), subspecies *arizonae* (IIIa), subspecies *diarizonae* (IIIb), subspecies *houtenae* (IV), subspecies *indica* (VI) (Heyndrickx et al., 2005; Evangelopoulou et al., 2013). The genus *Salmonella* can be further classified into serotypes, according to flagella (H), somatic (O) and capsular (Vi) antigens, and phage types, according to their susceptibility to specific phages (Heyndrickx et al., 2005; Baggesen et al., 2010).

Salmonella enterica subspecies *enterica* serotype Typhimurium (*Salmonella* Typhimurium) has the potential to cause disease outbreaks in endothermic animals, e.g. wild living passerines and humans (Alley et al., 2002; Refsum et al., 2002; Hughes et al., 2008; Pennycott et al., 2010; Lawson et al., 2014). The transmission mainly occurs through the faecal-oral route and can easily be spread through pathogen-accumulation on bird feeders (Brittingham and Temple, 1988; Refsum et al., 2003; Krawiec et al., 2015). This accumulation is possible thanks to the well-developed survival and adaptation strategies of the bacterium (Spector and Kenyon, 2012), which can survive in the environment (e.g. in soil and faeces) outside the host for months (Davies and Breslin, 2003). Nevertheless, vertical transmission of *Salmonella enterica* to the eggs and offspring has been observed in chickens (Keller et al., 1995; Wigley et al., 2005).

Depending on the host-adaptivity and infection dose of *Salmonella,* on the characteristics of the host species and on environmental conditions, the pathogenesis and outcome of a *Salmonella* infection will be different. After oral ingestion, *Salmonella* Typhimurium can colonize the intestinal tract and penetrate into the cytoplasm of epithelial cells lining the intestinal tract. Since *Salmonella* Typhimurium is a facultative intracellular bacterium, it has the ability to survive within the host cells, even within macrophages for the more host-adapted strains (Pasmans et al., 2003; Eng et al., 2015). These host adapted strains are characterized by their ability to cause systemic infections through the systemic spread within macrophages and can as such reach various internal organs (Rabsch et al., 2002; Pasmans et al., 2003).

Infections with *Salmonella* Typhimurium in birds can have different outcomes going from an asymptomatic intestinal carrier stage over a chronic localized infection (with persistence of *Salmonella* in the granulomatous lesions within the different organs) that may or may not be clinically apparent to an acute fatal septicemia with or without enteritis (Alley et al., 2002;

Pennycott et al., 2002; Connolly et al., 2006; Hughes et al., 2008; Verbrugghe et al., 2012 and 2016). In Passeriformes, acute death is the most observed scenario and is associated with following pathological lesions: hepatosplenomegaly, presence of necrotic lesions in the oesophagus and/or crop, liver, spleen and brain (Alley et al., 2002; Refsum et al., 2003; Giovannini et al., 2013). Almost all *Salmonella* outbreaks in wild birds occur during stress periods (Alley et al., 2002; Refsum et al., 2002; Pennycott et al., 2006; Lawson et al., 2010) with some phage types (DT)40, DT56(v) and DT160 accounting for most of the outbreaks in passerines in Britain (Pennycott et al., 2006; Lawson et al., 2010 and 2014). Most studies on *Salmonella* Typhimurium in wild birds have focused on the outbreak scenarios (e.g. Alley et al., 2002; Refsum et al., 2003; Giovannini et al., 2013; Lawson et al., 2010), and only few on the prevalence of *Salmonella* in wild living birds (Pasmans et al., 2004; Hamer et al., 2012; Haesendonck et al., 2016; Rouffaer et al., 2016).

4. Study system

In this thesis, I investigated the relationship between habitat fragmentation, microbial pressure (total eggshell bacterial load and *Salmonella* presence) on the eggs, and the health and reproductive parameters of great and blue tits. In the following parts, I will focus on the host species (great and blue tits), the influence of fragmentation on microbial pressure and health parameters in these animals, and the study site that I used to investigate these parameters.

4.1. Host species: Great and blue tits

Great (*Parus major)* and blue tits (*Cyanistes caeruleus*) belong to the order of the *Passeriformes,* family *Paridae* (IUCN, 2016 and 2017). Together with other passerines (e.g. blackbirds (*Turdus merula),* robins (*Erithacus rubecula*)), birds of the order *Accipitriformes* (e.g. sparrowhawks (*Accipiter nisus*)), *Columbiformes* (e.g. wood pigeons (*Columba palumbus*), *Cuculiformes* (e.g. common cuckoo (*Cuculus canorus*)), *Strigiformes* (e.g. tawny owl (*Strix aluco*), they inhabit (mixed) deciduous forests, forest edges and can (occasionally for the blue tit) be found in coniferous forests throughout Europe (Fig. 4) (Cramp et al., 1993; IUCN, 2016 and 2017). Furthermore, they can be found in parks and gardens of (sub)urban areas, if sufficient breeding places are available. They are hole-breeders and usually nest in tree-cavities (Cramp et al., 1993; Newton, 1994; IUCN, 2016 and 2017). The female tit will build the nest with (plant) material found in the environment (e.g. moss, grasses, bark strips),

animal hair and feathers (IUCN, 2016 and 2017). They are mainly insectivorous, but also feed on spiders, seeds and fruit (Torok, 1985; IUCN, 2016 and 2017).

The clutch size of blue and great tit in Europe ranges from 7-13 and 6-11, respectively (Gosler et al. 2013). Their dispersal ranges from 0.66 to 4.4 km. Youngs that fledged early in the breeding season are more likely to recruit into the breeding population than young that fledged late (Verboven and Visser, 1998). Local recruitment is higher among male than female birds, and the difference is more pronounced in blue tits. In addition, local recruitment is higher in larger plots and in plots with a higher population density (Matthysen et al., 2001).

Great and blue tits are resident birds that can reach high densities (several pairs per hectare) in forest fragments and readily breed into nest boxes. As such, they are easy to follow-up and they constitute a good model for investigating the reproductive success and health status of forest birds along different forest fragments.

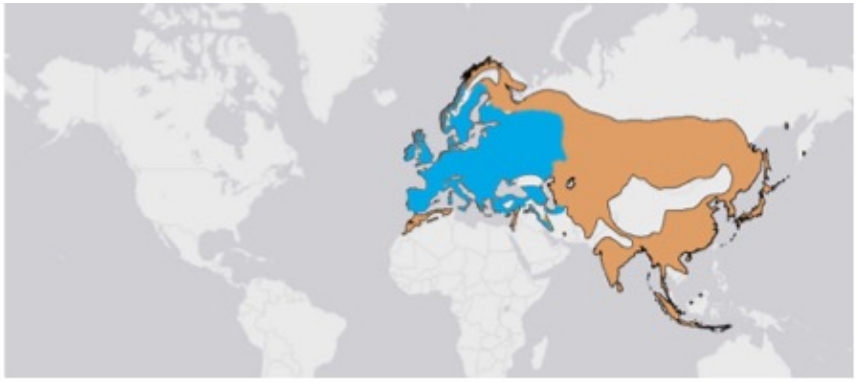

Fig 4. Distribution great and blue tit

Great tit (*Parus major*)

Great tit + blue tit (*Cyanistes caeruleus*)

BirdLife International. 2016. *Parus major*. The IUCN Red List of Threatened Species 2016: e.T22735990A87431138. Accessed on the 6th of December 2017

BirdLife International. 2017. *Cyanistes caeruleus*. The IUCN Red List of Threatened Species 2017: e.T103761667A118689415. Accessed on the 6th of December 2017

4.2. The effect of habitat fragmentation on Great and blue tits

In general, studies focusing on the effects of habitat fragmentation on tit populations (health status, reproductive parameters, host-pathogen interactions) are very limited, highlighting the need for thorough research about this topic. I here describe the few studies concerning these themes.

4.2.1. Effect of habitat fragmentation on health and reproductive paramaters of great and blue tits

Habitat fragmentation has been linked to differences in breeding onset of great and blue tits (Matthysen et al., 1995; Hinsley et al., 1999). These differences could depend on the fragment size, or on the isolation status of the fragment and have been shown to advance (Matthysen et al., 1995), or delay (Hinsley et al., 1999) the breeding onset, compared to tits breeding in continuous forests. This difference in timing can be related to their attempt to synchronize their breeding with the caterpillar availability (Lambrechts et al., 1997). If mismatch does occur, nestlings can be affected, resulting in decreased nestling body mass and condition and eventually decreased breeding success (Lambrechts et al., 1997; Hinsley et al., 1999). In addition, tits that have fledged later in the season will potentially be exposed to higher competition with tits that have fledged earlier, decreasing their survival chances and eventually their chance of successful breeding (Verhulst et al., 1995; Hinsley et al., 1999).

4.2.2. Effect of habitat fragmentation on bacterial pressure of great and blue tits

Several studies have surveyed the prevalence of bacterial infections in great and blue tits, including *Salmonella* spp. (e.g. Vikøren et al., 2010; Beckman et al., 2014; Krawick et al., 2015). However, most of these studies focused on death birds or apparently healthy adult birds, but not the egg or juvenile infection pressure. A study on microbiota of great and blue tit nests revealed that *Staphylococcus hyicus* and *Enterobacter cloacae* are the most abuntdant pathogenic bacteria in great and blue tit nests (Goodenough and Stalwood, 2010). These bacteria have been associated with infections in poultry and black-bellied whistling ducks (*Dendrocygna autumnalis*) (Aguirre et al., 1992; Silvanose et al., 2001).

Although literature in great and blue tits is limited, there are some indications that habitat fragmentation can have an influence on the bacterial pressure of great and blue tits.

- *Brood parasitism:* It has been shown that there is a higher risk of nest predation (Nour et al., 1993; Hinsley et al., 1995a; Huhta et al., 2004; Borges and Marini, 2010) and brood parasitism (Borges and Marini, 2010) in fragmented areas. Besides, brood parasitism has been associated with increased bacterial contamination of host eggs (Soler et al., 2011). Furthermore, Barrientos et al. (2015) studied the occurrence of facultative interspecific brood parasitism in

great and blue tit by monitoring 38 forest plots over 3 years. They found a prevalence of 3.0%, which reached a prevalence of 7.2% in small woodlands. As such, fragmentation and changes in brood parasitism can lead to altered host-pathogen interactions in great and blue tits.

-*Feeder visiting*: In a recent paper, it has been shown that the north American great tit (Black-capped Chickadees) in highly fragmented forests visits feeders more often (Latimer et al., 2018). Besides, it is already documented that pathogen acquisition and transmission are associated with feeder visiting (Adelman et al. 2015). As such, fragmentation and changes in feeder visiting, can lead to altered host-pathogen interactions in great and blue tits.

4.3. Study Site

Studies of blue and great tits were performed in 53 study plots of the TREEWEB research platform (Fig 5; Table 1). All study plots (30 x 30 m) were established in 2014 to study effects of forest fragmentation and tree species diversity on food web dynamics (De Groote et al., 2017). The study plots have a similar land-use history (continuously forested since at least 1850) and developmental stage (mature stands; >60 years). All plots are located on a similar relatively dry, sandy loam soil located outside river valleys with the soil parent material varying from light sandloam to sandloam in the south of Ghent (coordinates: 50°57'19"N, 3°43'31"E), northern Belgium. The 15 km × 30 km study window has a total forest cover of c. 3000 ha (forest index 6.8 %), covering both larger forest patches as well as small forest patches. Forest fragments varied in size (range: 1.3 to 90.4 ha) and tree layer (3 focal species; Pedunculate oak (*Quercus robur*), Red oak (*Q. rubra*) and Beech (*Fagus sylvatica*) in monocultures, 2 species mixtures or 3 species mixtures). To avoid edge effects of adjacent, different stands, we aimed for a buffer zone of minimum 10 m wide around the plots.

In my studies, I focused on the effect of fragment area as a marker for fragmentation, but not on habitat quality effects via tree composition, plant diversity or insect abundance.

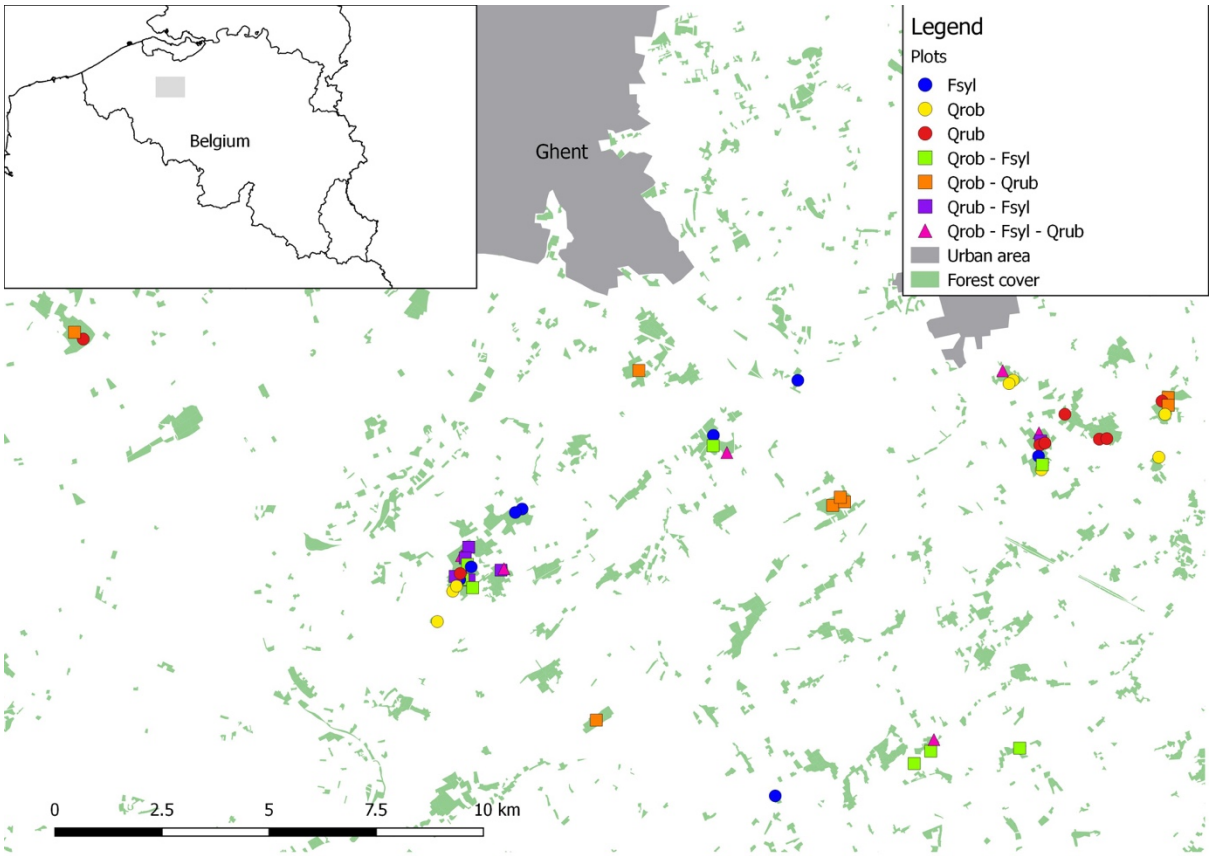

Figure 5: Map showing the location of all the study plots. The study of great and blue tit nests was performed in 53 study plots, established in 19 forest fragments. Different colours and shapes represent different tree species composition.

Table 1: Summary of sampled great (PM) and blue (PC) tit eggs in the different study plots

Forest	Plot	Latitude	Longitude	Surface area (ha)	tree diversity (spp.)	Nestbox	spp.
Lemberge	1	50.990342	3.7732	16.68	2	1.2	PM
						1.3	PC
						1.4	PM
Nerenbos (Merelbeke)	2	50.961391	3.73474	41.74	2	2.1	PC
						2.2	PC
						2.3	PM
						2.4	PM
	3	50.960503	3.73237	41.74	2	3.2	PM
						3.4	PC
Heilig Geestgoed (Merelbeke)	4	50.948551	3.728218	27.49	2	4.1	PM
						4.3	PM
	5	50.948747	3.728979	27.49	2	5.1	PM
						5.2	PM
Makegembos (Merelbeke)	7	50.951324	3.715364	83.77	2	7.1	PM

Forest	Plot	Latitude	Longitude	Surface area (ha)	tree diversity (spp.)	Nestbox	spp.
						7.2	PC
						7.3	PM
	8	50.950905	3.716254	83.77	1	8.2	PC
	9	50.949559	3.716739	83.77	1	9.1	PC
						9.3	PM
						9.4	PM
	10	50.94883	3.718443	83.77	1	10.3	PM
Harentbeekbos (Merelbeke)	12	50.9464	3.717465	83.77	1	12.1	PC
						12.3	PM
						12.4	PM
	13	50.946545	3.715697	83.77	1	13.2	PC
						13.3	PC*
	16	50.947438	3.714835	83.77	1	16.1	PM
						16.2	PC
						16.4	PM
	17	50.944474	3.718904	83.77	2	17.1	PC
						17.2	PM
						17.3	PC
	18	50.943761	3.712352	83.77	1	18.1	PM
						18.2	PM
						18.3	PM
						18.4	PM
	19	50.9449	3.713682	83.77	1	19.3	PM
						19.4	PC
Wannegatstraat (Gavere)	20	50.937592	3.707042	3.03	1	20.1	PM
						20.2	PC
						20.3	PC
Bueren (Melle)	21	50.9886	3.82614	6.19	2	21.1	PC
						21.2	PM
Aalmoezenijbos (Oosterzele)	22	50.976081	3.798739	23.57	2	22.1	PM
						22.2	PM
						22.4	PC
	23	50.974748	3.797965	23.57	2	23.1	PM
						23.3	PM
	24	50.973663	3.802786	23.57	1	24.1	PM
						24.2	PM
						24.4	PM
Spiegeldries bos (Oosterzele)	25	50.916874	3.760309	11.37	2	25.1	PM
						25.2	PM
						25.3	PM

Forest	Plot	Latitude	Longitude	Surface area (ha)	tree diversity (spp.)	Nestbox	spp.
Zottegem	26	50.901508	3.819877	3.53	3	26.2	PM
						26.3	PM
						26.4	PM
St-Lievens-Houtem	27	50.908521	3.865333	1.31	2	27.4	PM
	28	50.911148	3.871161	1.59	2	28.2	PC
						28.3	PC
						28.4	PM
	29	50.913516	3.872813	5.63	1	29.2	PC
						29.4	PM
	30	50.91155	3.901021	12.04	1	30.1	PC
						30.3	PM
						30.4	PC
	31	50.973112	3.946005	9.21	1	31.3	PM
Nonnenbos (Serskamp)	32	50.985475	3.949129	32.69	2	32.2	PM
Serskamp	36	50.976824	3.926348	58.9	2	36.2	PC
						36.3	PM
						36.4	PC
	37	50.976977	3.9288	58.9	2	37.3	PM
						37.4	PM
Oud smetlede	38	50.978195	3.906263	47.77	1	38.1	PM
						38.3	PM
						38.4	PM
	39	50.976306	3.907334	47.77	1	39.4	PM
	40	50.975601	3.906863	47.77	2	40.1	PM
	41	50.976082	3.908319	47.77	3	41.3	PM
	43	50.970567	3.907196	47.77	1	43.1	PC
						43.2	PM
						43.3	PM
	44	50.971339	3.907868	47.77	1	44.1	PM
						44.3	PM
	45	50.982	3.914797	58.9	2	45.2	PM*
Hospicebossen (Nazareth)	46	50.99087	3.894436	18.73	1	46.3	PC
	47	50.98917	3.897568	18.73	2	47.1	PM
						47.2	PM
	48	50.988468	3.89644	18.73	1	48.1	PC
						48.2	PM
						48.4	PM
Oosterzele	49	50.962551	3.838403	30.65	1	49.1	PM
						49.3	PM
						49.4	PC
	50	50.96349	3.842156	30.65	3	50.1	PC

Forest	Plot	Latitude	Longitude	Surface area (ha)	tree diversity (spp.)	Nestbox	spp.
						50.2	PM
						50.3	PC
						50.4	PM
	51	50.964019	3.840559	30.65	1	51.1	PM
						51.2	PC
						51.4	PC
Ooidonk (Deinze)	52	50.996011	3.588524	46.16	1	52.2	PM
	53	50.997431	3.585583	46.16	3	53.1	PC
						53.4	PM

An asterisk (*) indicates a nest where brood parasitism was observed.

4.4 Sample collection

During autumn 2014, 212 standard nest boxes for blue and great tits (dimensions 23 x 9 x 12 cm, entrance 32 mm) were installed at a height of 1.5 m, at each corner of a plot, of which 3 broke during the experiment. During the breeding season of 2015 (April-June 2015), all nest boxes were checked at least twice a week to determine the total number of eggs produced (clutch size) and the total number of nestlings and fledglings.

To avoid intra-clutch variation, the fifth egg per clutch of great and blue tits was collected using sterile gloves, stored in a sterile bottle and transported to the laboratory where the eggs were cracked under a laminar flow cabinet. The egg yolk and egg white were collected. The inside of the eggshells was washed with sterile phosphate buffered saline (PBS) to remove the adhering egg albumen in order to avoid antimicrobial activity of the albumen.

At 14-15 days of age, all fledglings were ringed and measured (tarsus (in mm) and weight (in g)). Body condition of juveniles was calculated using the scaled-mass index (SMI). Additionally, 20 µl of blood was collected from the basilic vein of 4 fledglings per nest. As blue tits are smaller than great tits, only great tit fledlging blood samples were collected. All samples were kept in Eppendorf tubes at -20 °C until analysis.

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SCIENTIFIC AIMS

The environment, host and pathogen are linked to each other by the so called disease triangle. Changes in one of these factors can alter all the interactions of the disease triangle. Environmental changes such as habitat fragmentation can have profound effects on host-pathogen interactions (e.g. changes in pathogen encounter rate and egg contamination, leading to changes in breeding performances and health status of the birds). Although the effects are not as obvious as the immediate effects on populations, the overall outcome can be disastrous for populations. Despite of the potential link, there is a lack of studies investigating the role of forest fragmentation on microbial pressure and reproductive success.

The **overall aim** of this thesis was to investigate the microbial infection pressure and reproductive success in blue (*Cyanistes caeruleus*) and great (*Parus major*) tits in 19 mature (> 60 years) deciduous forest fragments of Flanders (Belgium) and to what extent they are influenced by habitat fragmentation.

The **specific aims** of the different chapters are defined as follows:

CHAPTER I: *Salmonella enterica* subspecies *enterica* serovar Typhimurium is the most common cause of salmonellosis in passerines. Birds can get infected through vertical and horizontal transmission which can result in different disease outcomes.

In this chapter, I investigated **the potential of endemic *Salmonella* infections to reduce the reproductive success of great and blue tits in the different forest fragments** and determined:

1) the prevalence of *Salmonella* Typhimurium on eggshells, in the albumen and the egg yolk, and checked whether the strains could be passerine-adapted

2) the presence of anti-*Salmonella* antibodies in the fledglings

3) and whether the presence of *Salmonella* Typhimurium on the birds' eggs affect the reproductive parameters and the body condition of the fledglings.

CHAPTER II: During the pre-and post-hatching stages, microbial infections can lead to hatching failure and death in birds. Females however can influence the phenotype and fitness of their offspring through the transfer of immunoglobulins and antibacterial proteins to their eggs in order to protect the offspring against infections. Females have been suggested to be able to modify the levels of egg immune factors according to the infection risk.

In this chapter, I have determined **the impact of microbial pressure on great (*Parus major*) and blue (*Cyanistes caeruleus*) tit hatching success**.

First, I determined and compared (between free-ranging great and blue tits):

1) the bacterial infection pressure (load and microbiota diversity) on eggs,

2) the immune investment into the eggs (IgY, avidin, lysozyme and ovotransferrin),

3) the effect of this infection pressure and immune investment on the hatching success of either species.

Furthermore, I investigated whether the environmental factor "surface area of the forest fragments" is correlated to eggshell microbial pressure.

CHAPTER I

Salmonella Typhimurium DT193 and DT99 are present in Great and Blue Tits in Flanders, Belgium

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Abstract

Endemic infections with the common avian pathogen *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) may incur a significant cost on the host population. In this study, we determined the potential of endemic *Salmonella* infections to reduce the reproductive success of blue (*Cyanistes caeruleus*) and great (*Parus major*) tits by correlating eggshell infection with reproductive parameters. The fifth egg of each clutch was collected from nest boxes in 19 deciduous forest fragments. Out of the 101 sampled eggs, 7 *Salmonella* Typhimurium isolates were recovered. The low bacterial prevalence was reflected by a similarly low serological prevalence in the fledglings. In this study with a relatively small sample size, presence of *Salmonella* did not affect reproductive parameters (egg volume, clutch size, number of nestlings and number of fledglings), nor the health status of the fledglings. However, in order to clarify the impact on health and reproduction a larger number of samples have to be analyzed. Phage typing showed that the isolates belonged to the definitive phage types (DT) 193 and 99, and multi-locus variable number tandem repeat analysis (MLVA) demonstrated a high similarity among the tit isolates, but distinction to human isolates. These findings suggest the presence of passerine-adapted *Salmonella* strains in free-ranging tit populations with host pathogen co-existence.

Keywords: *Salmonella* Typhimurium, egg, passerine, reproductive success

Introduction

Infectious diseases pose an increasing threat to wildlife. Worldwide, *Salmonella* is one of the most important bacterial pathogens [1], affecting reptiles, birds and mammals [2-9]. *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) has a wide host range including humans, livestock, waterfowl, rodents and birds such as passerines [10-15].

In passerine birds, *Salmonella* Typhimurium is the most common cause of salmonellosis [3, 15, 16]. Birds can be infected through direct or indirect contact with other birds or animals, or through contact with contaminated environments [11, 15, 17-19]. Once birds are infected with this bacterium, it can be passed to their eggs during egg formation (vertical transmission) or during and after oviposition through eggshell contamination from the colonized gut or contaminated faeces (horizontal transmission) [20].

Within this serovar, the phage types DT40, DT41, DT56, and DT160 are potentially adapted to passerines and can result in endemic or context-driven epizootic infections [3, 16]. Until recently, the majority of research focused on clinical outbreaks of *Salmonella* in passerines, with clinical signs ranging from brief episodes of severe disease to acute death [11, 15, 19]. However, the poorly known and less obvious infections with host adapted strains have been suggested to have a profound impact on the birds' reproductive success [18, 21-26]. The latter effect on host health is counterintuitive since maintenance of host-adapted pathogens in the host population would benefit from having only a minimal cost on the infected host [27, 28].

In our study, we first determined whether passerine-adapted *Salmonella* strains circulate in populations of blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*), two closely related territorial hole-nesting passerines that are widely distributed throughout Europe, using molecular typing of bird-derived *Salmonella* isolates. We then correlated *Salmonella* presence on the birds' eggs and *Salmonella* seroprevalence in fledglings with health and reproduction parameters.

Materials and methods

Monitoring of nest boxes of blue and great tits

In the present study, 101 eggs were sampled in 53 (30 x 30 m) study plots located in 19 mature (> 60 years) deciduous forest fragments in the south of Ghent (co: 50°57'19"N, 3°43'31"E), northern Belgium (Fig 1). These study plots (30 x 30 m) have been established to

study the effects of tree species diversity and forest fragmentation on food web dynamics [29]. In the autumn of 2014, standard nest boxes for blue and great tits (dimensions 23 x 9 x 12 cm, entrance 32 mm) were installed at a height of 1.5 m, at each corner of a plot. In total, we installed 212 nest boxes of which 3 broke during the experiment. During the breeding season (April–June 2015), all nest boxes were checked twice a week to determine first-egg laying dates, then every other day to determine the laying order, the total number of eggs produced (clutch size) and the total number of nestlings and fledglings. To avoid intra-clutch variation, the fifth egg per clutch of great and blue tits was collected using sterile gloves, stored in a sterile bottle and transported to the laboratory where the eggs were cracked under a laminar flow cabinet. The egg yolk and egg white were collected. The inside of the eggshells was washed with sterile phosphate buffered saline (PBS) to remove the adhering egg albumen in order to avoid antimicrobial activity of the albumen. Bacteriological analysis of the eggshell, egg yolk and egg white was conducted as described below.

At 14-15 days of age, all fledglings were ringed and measured (tarsus (in mm) and weight (in g)). Body condition of juveniles was calculated using the scaled-mass index (SMI) [30]. Additionally, 20 µl of blood was collected from the basilic vein of 4 fledglings per nest for *Salmonella* antibody titre analysis. As blue tits are smaller than great tits, we only collected blood from great tits. All samples were kept in Eppendorf tubes at -20 °C until analysis.

Bacteriological analysis

Eggshells, including shell membranes, were transferred to an Eppendorf tube and crushed gently. Eggshell, egg yolk, and egg white samples were processed according to the ISO 6579-1:2017 method for the isolation of different *Salmonella* serovars, including *Salmonella* Typhimurium. Briefly, the samples were pre-enriched overnight in buffered peptone water at 37 ± 1°C, then enriched overnight in tetrathionate brilliant green broth (Merck, Belgium) and Rappaport Vassiliadis medium supplemented with soya (RVS) (Oxoid, UK) at 37 ± 1°C and 41.5 ± 1°C, respectively. Subsequently, the samples were plated on Brilliant Green Agar (BGA) (Oxoid, UK) and Xylose Lysine Deoxycholate (XLD) (Oxoid, UK) plates. Pink colonies on BGA or light transparent reddish with black center colonies on XLD were confirmed to be *Salmonella* based on their biochemical characteristics (glucose fermentation, H_2S production, lysine decarboxylation positive and urea negative) [31]. All the isolates were serotyped as *Salmonella* Typhimurium using slide agglutination, targeting the antigens O4, O5 and O12. Phage typing was performed at the *Salmonella* and *Escherichia coli* reference lab of

the Animal & Plant Health Agency (APHA), Weybridge, England. Multi-locus variable number tandem repeat analysis (MLVA) using the European 5-loci scheme [32] as further performed at the Scientific Institute of Public Health, Belgium [33].

Salmonella antibody titre analysis

To measure IgY-anti-*Salmonella* antibodies, we applied an indirect enzyme-linked immunosorbent assay (ELISA) [26]. In summary, ELISA plates (F96 maxisorp Nunc-immuno plates, Nunc, Denmark) were coated overnight at 4°C with 140 µL of a suspension containing formalin-inactivated *Salmonella* Typhimurium DAB69 (pigeon strain) bacteria diluted in coating buffer to an optical density (OD) of 660 nm, measured with a spectrophotometer (Ultraspec III®). Each whole blood sample was thoroughly centrifuged and then diluted 1/1000 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) and added to the wells (100 µL) for 1 hour at 37°C. The plates were then washed three times using washing buffer (0.6 g NaH2PO4·2H2O, 5.6 g NaH2PO4. 12H2O, 0.5 ml Tween 20, 12.5 g NaCl, 1000ml distilled water). Conjugate consisting of 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) was added and incubated at 37 °C for 1 h. The plate was developed using 100 µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma Aldrich Chemie Gmbh, Steinheim Germany) for 15 min and stopped by the addition of 100 µl stop solution (Sigma Aldrich Chemie Gmbh, Steinheim Germany). The optical density was measured using a Multiskan MS Reader (Labsystems Oy, Helsinki, Finland) with the Ascent Software, version 2.6. All measurements were performed in duplicate.

Statistical analysis

All statistical tests were performed with R statistical environment [34]. First, differences in *Salmonella* Typhimurium prevalence between great and blue tits were tested using a generalized linear mixed model (GLMM), R library lme4, [35]. Forest fragment identity was modeled as a random effect, species (i.e. blue versus great tit), forest fragment area size (ha), and first-egg laying date of each clutch (Julian day) were included as fixed-effect covariates while specifying a binomial error distribution. Second, to test whether *Salmonella* Typhimurium impacts upon reproductive parameters (egg volume, clutch size, the number of nestlings, the number of fledglings and SMI of fledged young), these parameters were specified

as dependent variables in linear mixed model (LMM) with as fixed effects presence or absence of *Salmonella* Typhimurium, fragment area, laying date. Forest fragment was again modelled as a random effect. When testing for impacts upon the number of nestlings and fledglings, clutch size was included as an additional covariate. When assessing impacts on individual fledgling SMI, we included the number of fledglings as a covariate and accounted for the non-independence of nestlings by including a nested random effect (nest box nested with forest fragment). Separate models were run for great and blue tits, and model residuals were normally distributed for all analyses (all Shapiro-Wilk $W > 0.91$). All continuous variables were standardized before analysis. Variable selection followed a frequentist approach whereby full models (i.e., models containing all explanatory variables considered) were reduced in a stepwise manner, by excluding the variable with the highest P-value until only $P < 0.05$ predictors remained. Reported statistics are derived from a minimal model (i.e. model with only the significant terms included, if any) where *Salmonella* Typhimurium presence or absence was fitted into.

Ethical considerations

All trapping and sampling protocols were approved by the Ethical Committee VIB Ghent site (EC2015-023).

Results

Low Salmonella Typhimurium prevalence in the nests of blue and great tits

Blue and great tits only occupied nest boxes in 51 out of the 53 study plots. Great tits occupied 112 (53.59%) nest boxes and laid eggs in 66 nest boxes (31.58%). Blue tits occupied 45 nest boxes (21.53%) and eggs were found in 37 nest boxes (17.70%). The other 52 nest boxes (24.88%) remained unoccupied. In total, 65 and 36 eggs of great and blue tits, respectively, were screened for the presence of *Salmonella*. Egg contents of the screened eggs were negative for *Salmonella*. The eggshell of seven eggs (6.93% with 0-11.9 95%CI), of which four originated from blue tits, and three from great tits, were positive for *Salmonella* Typhimurium. Although relative *Salmonella* prevalence was about 2.4 times higher for blue (11.11%) compared to great tits (4.62%), these differences failed to achieve statistical significance (z-value = -0.44, P = 0.66) (Table 1 and S1 Table). The *Salmonella* Typhimurium prevalence generally is low for both tit species, however the bacterium exhibits a wide distribution between the different forests and plots (Fig 1). Six of the seven positive eggshells

were found in different forests and they were all found in a different plot. Furthermore, anti-*Salmonella* antibodies were detected in four fledglings in four additional nests, in three different forest fragments and all in different plots, also suggesting a low prevalence with a wide distribution (Fig 1, Table 2, S2 Table).

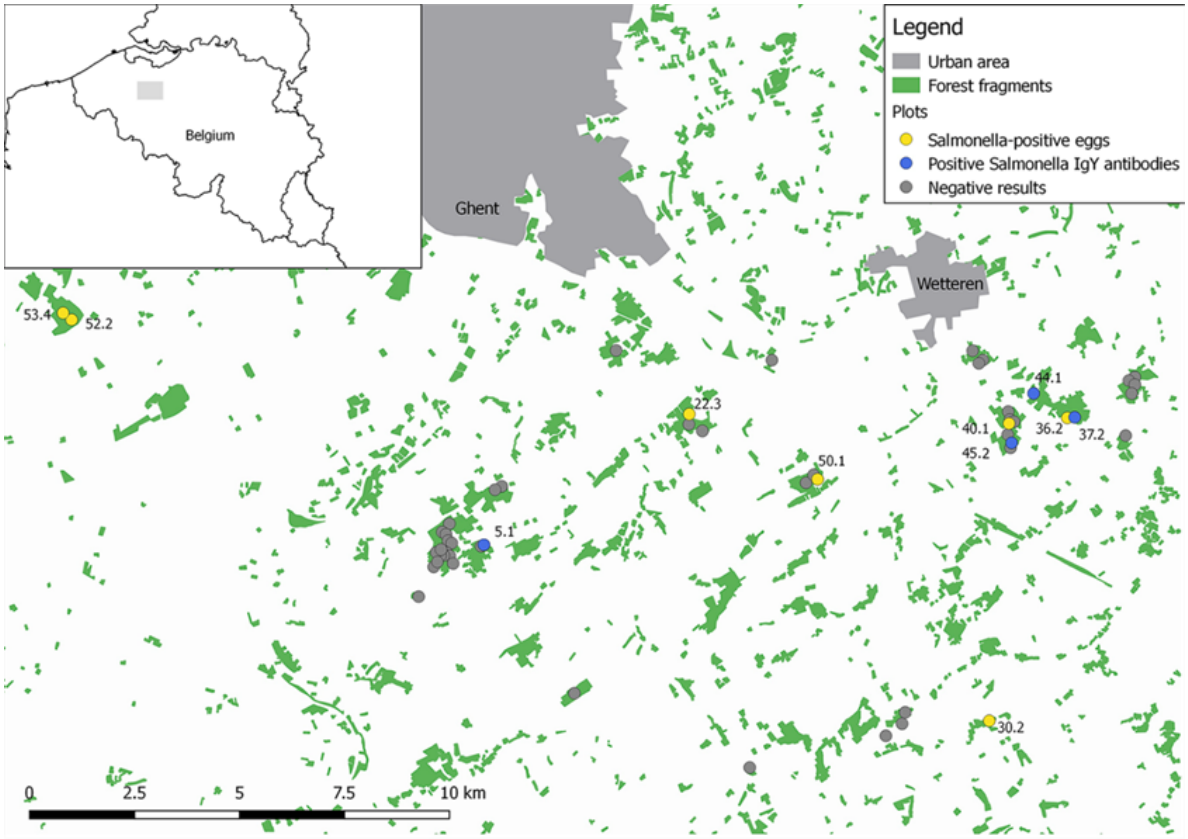

Fig 1: Map of the study plots showing the distribution of *Salmonella* Typhimurium. Shown are the study plots used to investigate *Salmonella* Typhimurium presence in blue and great tit nest boxes. Negative plots are indicated by grey dots, plots where *Salmonella* was found on the eggshell are represented by yellow dots and plots with nestlings carrying *Salmonella* IgY antibodies are depicted by blue dots.

Table 1: Reproductive parameters and SMI of blue and great tits originating from a nest containing a *Salmonella* positive eggshell. Shown is the egg volume of the *Salmonella* positive eggs of blue (PC) and great (PM) tits, found in 53 analyzed plots. Per positive nest box, the clutch size, number of nestlings, number of fledglings are given, as well as the mean scaled-mass index (SMI) ± stdev of the nestlings. Due to practical issues, some samples were

not collected (NC). If the number of fledglings was equal to 0, brood reduction and SMI could not be calculated (not applicable or NA).

Forest fragment	Plot	Nest Box number	Bird species	Egg volume (mm^3)	Clutch size	n° nestlings	n° fledglings	Mean SMI ± stdev
Aelmoeseneiebos (Melle)	22	22.3	PC	7602.914	11	9	8	NC
Borsbeke (Herzele)	30	30.2	PC	5445.613	12	10	7	10.43 ± 0.66
Serskamp	36	36.2	PC	6283.4	9	8	6	11.05 ± 0.97
Oud smetlede	40	40.1	PM	10254.51	8	0	0	NA
Moortelbos (Oosterzele)	50	50.1	PC	NC	7	6	0	NA
Ooidonk (Deinze)	52	52.2	PM	10254.51	10	8	NC	NC
Ooidonk (Deinze)	53	53.4	PM	12742.74	7	NC	4	16.84 ± 0.88

Table 2: IgY antibody assessment in blood of great tits. Indicated are fledglings having anti-*Salmonella* antibodies (IgY) in their blood at day 14-15.

Forest fragment	Plot	Nest box number	Bird species	*Salmonella* ELISA
Heilig Geestgoed (Merelbeke)	5	5.1	PM	positive
Serskamp	37	37.2	PM	Positive
Oud smetlede	44	44.1	PM	positive
Oud smetlede	45	45.2	PM	positive

Salmonella Typhimurium has no effect on the reproductive fitness and SMI of blue and great tits

We first analyzed whether *Salmonella* Typhimurium affects the reproductive parameters (egg volume, clutch size, number of nestlings and fledglings) of blue and great tits. The results are summarized in Table 3, and no significant association between the presence of *Salmonella* Typhimurium on the eggshell and any reproductive parameter could be detected (Table 4). Secondly, we analyzed whether the presence of *Salmonella* Typhimurium has a negative impact on the SMI of blue and great tits. The SMI of 113 blue tit fledglings, of which eight fledglings hatched in a nest containing an egg with a *Salmonella*-positive eggshell, was calculated. In total, 186 great tit fledglings were analyzed of which four were found in a nest where we detected *Salmonella* on the eggshell. The mean SMI of blue and great tits, hatched in nest boxes where no positive eggs were found, was 11.04 ± 1.09 and 17.51 ± 1.93,

respectively. The SMI in the nests containing an egg with a *Salmonella* positive eggshell, was reduced to 10.74 ± 0.84 and 16.84 ± 0.88, respectively (Table 3). However, statistical analysis showed no significant association between the presence of *Salmonella* Typhimurium on the eggshell and the SMI (Table 4).

Table 3: Health and reproductive parameters of blue and great tits. Shown are the mean health and reproductive parameters ± stdev, including SMI, egg volume, clutch size, number of nestlings and number of fledglings, of blue and great tits in nests containing an egg with a *Salmonella* negative or positive eggshell.

	Nests with a *Salmonella* negative eggshell		Nests with a *Salmonella* positive eggshell	
	Blue tits (n=32)	Great tits (n=62)	Blue tits (n=4)	Great tits (n=3)
SMI ± stdev	11.04 ± 1.09	17.51 ± 1.93	10.74 ± 0.84	16.84 ± 0.88
Egg volume (mm^3) ± stdev	8226.29 ± 2223.56	10993.25 ± 2314.26	6443.98 ± 1087.58	11083.92 ± 1436.58
Clutch size ± stdev	11.03 ± 1.69	8.55 ± 1.85	9.75 ± 2.22	8.33 ± 1.53
Number of nestlings ± stdev	8.87 ± 2.31	6.42 ± 2.31	8.25 ± 1.71	4.00 ± 5.66
Number of fledglings ± stdev	7.58 ± 3.09	5.13 ± 2.77	5.25 ± 3.59	2.00 ± 2.83

Table 4: Statistical analysis. We investigated the association between the presence of *Salmonella* Typhimurium on the eggshell of blue and great tits and the egg volume, clutch size, number of nestlings, number of fledglings and SMI. The results are represented as the estimate ± standard deviation (stdev), degrees of freedom (d.f.), t-value and P-value.

Bird species	Association	Estimate ± stdev	d.f.	t-value	P-value
	Salmonella Typhimurium ~ egg volume	0.0019 ± 0.61	63	0.003	0.99
	Salmonella Typhimurium ~ clutch size	0.13 ± 0.58	65	-0.23	0.82
	Salmonella Typhimurium ~ number of nestlings	-0.97 ± 0.70	66	-1.40	0.170
	Salmonella Typhimurium ~ number of fledglings	-0.51 ± 0.64	63	-0.80	0.42
Great tits	*Salmonella* Typhimurium ~ SMI	0.28 ± 1.02	42	0.28	0.78
	Salmonella Typhimurium ~ egg volume	-1.17 ± 1.03	22	-1.13	0.27
	Salmonella Typhimurium ~ clutch size	-1.35 ± 0.70	26.8	-1.93	0.064
	Salmonella Typhimurium ~ number of nestlings	-0.03 ± 0.49	16.5	-0.061	0.95
	Salmonella Typhimurium ~ number of fledglings	-0.51 ± 0.78	18.0	-0.66	0.52
Blue tits	*Salmonella* Typhimurium ~ SMI	-0.85 ± 0.98	21	-0.87	0.40

Salmonella Typhimurium isolates belong to bird adapted phage types DT99 and DT193

Phage typing of the seven *Salmonella* Typhimurium strains, isolated from positive eggshells (Fig 1), showed that three of them belonged to phage type DT99, whereas four belonged to phage type DT193. These isolates where further typed with MLVA, targeting five loci. Regardless of phage type, five isolates (30.2, 36.2, 50.1, 52.2, 53.4) showed identical MLVA profiles (2-16-5-13-112). For strains 22.3 and 40.1, an extra repeat was found for loci STTR5, indicating that these isolates are closely related to the other strains (Table 5). Comparing their patterns to 3239 human *Salmonella* Typhimurium isolates from 2010-2016 (database of the WIV), regardless of phage type, revealed a large distinction between the tit and human isolates (Fig 2).

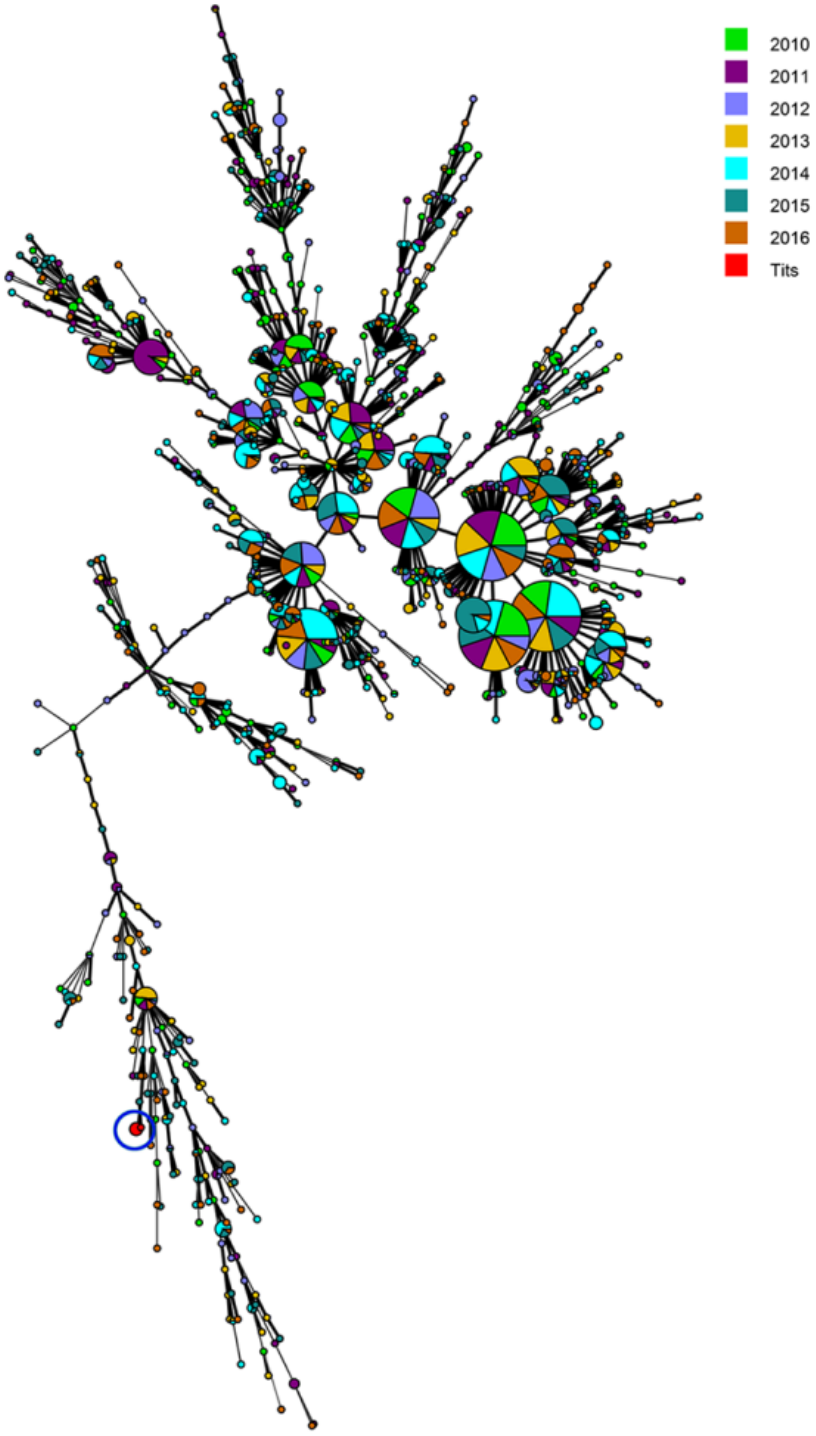

Fig 2: Minimum spanning tree based on MLVA data. Shown is a minimum spanning tree calculated for the MLVA profiles of the seven *Salmonella* Typhimurium isolates of blue and great tits, compared with 3239 *Salmonella* Typhimurium human isolates, regardless of phage type, in Belgium over the period of 2010-2016

Table 5: Phage and MLVA typing of *Salmonella* Typhimurium. Shown are the phage types and MLVA profiles of the seven *Salmonella* Typhimurium isolates targeting 5 loci.

Forest	Plot	Nest box number	bird species	Phage type	STTR9	STTR5	STTR6	STTR10	STTR3
Aelmoeseneiebos (Melle)	22	22.3	PC	DT99	2	17	5	13	0112
St-Lievens-Houtem	30	30.2	PC	DT99	2	16	5	13	0112
Serskamp	36	36.2	PC	DT99	2	16	5	13	0112
Oud smetlede	40	40.1	PM	DT193	2	17	5	13	0112
Moortelbos (Oosterzele)	50	50.1	PC	DT193	2	16	5	13	0112
Ooidonk (Deinze)	52	52.2	PM	DT193	2	16	5	13	0112
Ooidonk (Deinze)	53	53.4	PM	DT193	2	16	5	13	0112

Discussion

This study found a broad distribution at a low prevalence (± 7%) of two *Salmonella* Typhimurium phage types (DT99 and DT193) in populations of apparently healthy great and blue tits in Flanders, Belgium. The low prevalence was also confirmed by the seroprevalence of the fledglings. Surprisingly, none of the fledglings originating from a nest where we detected *Salmonella* on the eggshell was seropositive and conversely, all *Salmonella* seropositive fledglings were not from a nest containing a positive eggshell. This could have different reasons. Firstly, it is possible that the isolated strains have a limited chance of trans-shell infection, explaining the fact that egg yolk and white were negative for *Salmonella*. Secondly, nestlings became infected after hatching. This could occur through contact with contaminated nest material, which we did not screen for the presence of *Salmonella*. Thirdly, as we only screened one egg per nest, it possible that we missed other positive eggs in the nest, or that the other eggs in the nest were negative. Therefore, it is possible that our results are an underestimation of the *Salmonella* prevalence. Fourth, it is possible that the detected antibodies are maternal antibodies from an earlier infection. In chickens, around 3-4 days after hatching the juveniles begin to synthesize their own antibodies, however maternal antibodies can persist in the chick's circulation for 14 days after hatching [36]. Therefore, it is possible that the maternal immunity biased the serological results.

DT99 is usually considered a pigeon-adapted variant of *Salmonella* Typhimurium, which circulates endemically in feral pigeons in Belgium [37] but has also been associated with

mortality in passerines [26, 38]. Infections can cause systemic disease associated with high mortality rates in pigeons [14]. Clinical manifestations include gastroenteritis, arthritis, oophoritis or orchitis and systemic granulomatous inflammation [39]. Feral pigeons may serve as a source of infection for passerines especially in high aggregation areas such as bird feeding stations. DT193 is commonly associated with human infections [33, 40-44]. Additionally, this phage type has also been associated with disease outbreaks in birds on a few occasions [3]. Wild birds are considered as carriers of *Salmonella*, causing salmonellosis in both humans and domestics animals [10, 13]. The MLVA typing of both tit phage types in comparison with human isolates of *Salmonella* Typhimurium showed a high level of genetic similarity between the different tit isolates, but a large distinction between human and tit isolates. It is therefore possible that the isolates belonging to DT193 in this study represent avian-adapted *Salmonella* Typhimurium strains in free-ranging tits. If so, these *Salmonella* Typhimurium tit isolates possibly have a low impact on humans. However, more epidemiological data are needed to confirm the host range and to support our hypothesis. Furthermore, our data cannot be generalized as nest location and contact with human-made environment are important factors that can influence the epidemiology of specific *Salmonella* strains [3, 4]. Possibly, providing nest location in the surrounding of human settlements could lead to the isolation of *Salmonella* isolates with an MLVA profile closely linked to human isolates and with zoonotic and epizootic potential. Taking this in account, it is very likely that the distinction that we observe between the human and tit isolates is related to the sampling location. Therefore, it would be interesting to investigate the difference in *Salmonella* presence and their impact in both rural and urban forests.

Although pathogen persistence in specific host populations is an essential mechanism of host-adapted pathogens [45], costs and benefits for the host population during a state of pathogen endemism have been poorly studied. Host adaptation has been associated with systemic disease and increased severity of infection [46, 47]. On the contrary, hosts can benefit from host–pathogen coevolution, as it can lead to a lower pathogenicity and mortality [27, 28]. We did not observe any health or reproduction-related impacts from the presence of *Salmonella* Typhimurium on the eggshells. This finding is in line with the hypothesis that a limited impact of pathogen burden on host health allows host-pathogen co-existence and pathogen population maintenance in its primary niche, the host. However, the limited number of *Salmonella* positive nests in our study raises the need for extra experimental or field studies with a much bigger positive sample size.

In summary, our results indicate that *Salmonella* Typhimurium is present in free-ranging tit populations, without representing a major risk for reproductive success and health status. It is possible that, by limiting the impact of the pathogen burden on host health, *Salmonella* is able to persist and establish a wide distribution pattern. Although there is limited evidence that these strains currently have epizootic and/or zoonotic potential, we cannot state that free-ranging tits cannot transmit *Salmonella* Typhimurium to humans and other non-human animals. Since the host-pathogen interaction is driven by host characteristics, pathogen virulence and environmental drivers, including static and dynamic pathogen reservoirs, changes in any of these compounds of the disease triangle [48] may shift the state of co-existence towards the epizootics that have been described before [16, 49]. Future studies on the drivers of infection and disease dynamics are thus vital to understand the impact of *Salmonella* infections in wild birds.

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Supporting information

S1 Table: Overview of the *Salmonella* status, reproductive parameters and SMI in nests of blue and great tits. Shown are the nests with eggs of blue (PC) and great (PM) tits in 53 different plots. Every 5th egg was weighted (volume) and bacteriologically analyzed for the presence of *Salmonella* (negative or positive). Per nest box, the clutch size, number of nestlings and number of fledglings are given, as well as the mean scaled-mass index (SMI) ± stdev of the nestlings. Due to practical issues, some samples were not collected (NC). If the number of fledglings was equal to 0, brood reduction and SMI could not be calculated (not applicable or NA). An asterisk (*) indicates a nest box that was occupied twice by both PC and PM.

Forest fragment	Forest fragment surface area (ha)	Plot	Nest box	Spp	Eggshell *Salmonella* status	Egg white *Salmonella* status	Egg yolk *Salmonella* status	Egg volume (mm^3)	Clutch size	N° nestling	N° fledgling	Mean SMI ± stdev
Vurtzak (Merelbeke)	16.68	1	1.2	PM	negative	negative	NA	10254.51	8	7	7	15.78 ± 0.15
			1.3	PC	negative	NA	negative	NC	11	0	0	NA
			1.4	PM	negative	NA	NA	12034.81	9	8	8	NC
Nerenbos (Merelbeke)	41.74	2	2.1	PC	negative	negative	negative	7602.91	11	10	9	11.60 ± 0.70
			2.2	PC	negative	NA	negative	NC	8	7	7	11.79 ± 3.33
			2.3	PM	negative	negative	negative	12742.74	8	5	5	18.03 ± 0.62
			2.4	PM	negative	negative	negative	9651.30	8	4	4	16.76 ± 1.13
		3	3.2	PM	negative	NA	NA	7602.91	5	4	4	18.55 ± 1.20
			3.4	PC	negative	negative	negative	15599.59	10	7	4	10.45 ± 1.15
Heilig Geestgoed (Merelbeke)	27.49	4	4.1	PM	negative	negative	negative	12034.81	8	5	5	20.29 ± 2.70
			4.3	PM	negative	negative	negative	11326.88	10	7	0	NA
		5	5.1	PM	negative	negative	negative	8109.77	7	2	2	17.83 ± 1.29
			5.2	PM	negative	negative	negative	10254.51	6	4	4	16.36 ± 0.52
Makegembos (Merelbeke)	83.77	7	7.1	PM	negative	negative	negative	12742.74	9	7	3	13.33 ± 1.00
			7.2	PC	negative	negative	negative	6283.40	10	9	9	11.83 ± 0.40
			7.3	PM	negative	negative	negative	11460.92	9	8	8	17.15 ± 0.89
		8	8.2	PC	negative	negative	negative	5864.51	10	9	9	9.49 ± 0.70
		9	9.1	PC	negative	negative	NA	8109.77	10	9	9	NC
			9.3	PM	negative	negative	negative	16965.18	8	7	7	16 54 ± 0.39
			9.4	PM	negative	negative	negative	10857.72	7	5	5	16.97 ± 0.93
		10	10.3	PM	negative	negative	negative	10857.72	10	6	6	NC
Harentbeek bos (Merelbeke)	83.77	12	12.1	PC	negative	negative	negative	7602.91	8	6	5	11.98 ± 0.56
			12.3	PM	negative	negative	negative	12742.74	9	7	7	17.62 ± 1.17
			12..4	PM	negative	negative	NA	12034.81	9	8	8	15.97 ± 0.39

Forest fragment	Forest fragment surface area (ha)	Plot	Nest box	Spp	Eggshell *Salmonella* status	Egg white *Salmonella* status	Egg yolk *Salmonella* status	Egg volume (mm^3)	Clutch size	N° nestling	N° fledgling	Mean SMI ± stdev
		13	13.2	PC	negative	negative	negative	8109.77	11	10	10	11.71 ± 0.51
			13.3	PC*	negative	negative	negative	7602.91				12.71 ± 2.36
				PM*	NC	NC	NC	NC	11	9	8	14.88 ± 0.39
		16	16.1	PM	negative	negative	negative	9630.36	6	5	4	16.59 ± 0.31
			16.2	PC	negative	negative	negative	7602.91	9	7	7	10.74 ± 0.94
			16.4	PM	negative	negative	negative	10857.72	8	7	5	16.57 ± 0.76
		17	17.1	PC	negative	negative	negative	6283.40	14	13	13	9.83 ± 0.36
			17.2	PM	negative	negative	negative	10857.72	8	6	6	16.90 ± 0.82
			17.3	PC	negative	negative	negative	14778.56	12	10	9	10.80 ± 0.51
		18	18.1	PM	negative	negative	negative	10254.51	11	10	10	18.75 ± 1.80
			18.2	PM	negative	negative	negative	9123.50	8	6	6	16.89 ± 1.00
			18.3	PM	negative	negative	negative	11326.88	8	5	5	NC
			18.4	PM	negative	negative	negative	10254.51	10	7	7	17.11 ± 1.09
		19	19.3	PM	negative	negative	negative	6283.40	9	8	8	16.99 ± 0.66
			19.4	PC	negative	negative	negative	7841.68	14	9	9	11.30 ± 0.68
Wannegatstrt (Gavere)	3.03	20	20.1	PM	negative	negative	negative	12742.74	13	11	9	NC
			20.2	PC	negative	negative	negative	7602.91	11	9	7	NC
			20.3	PC	negative	negative	NA	6283.40	13	11	0	NA
Bueren (Melle)	6.19	21	21.1	PC	negative	NA	negative	8109.77	12	9	9	10.41 ± 0.56
			21.2	PM	negative	negative	negative	10254.51	7	5	5	17.40 ± 0.89
Aelmoesenei bos (Melle)	23.57	22	22.1	PM	negative	negative	negative	13450.66	8	6	6	NC
			22.2	PM	negative	NA	NA	14778.56	6	4	0	NA
			22.3	**PC**	**positive**	**negative**	**negative**	7602.91	**11**	**9**	**8**	**NC**
			22.4	PC	negative	NA	negative	8109.77	11	8	7	9.52 ± 1.87
		23	23.1	PM	negative	NA	NA	6283.40	8	7	6	23.57 ± 1.57
			23.3	PM	negative	negative	negative	6283.40	8	5	5	22.25 ± 1.56
		24	24.1	PM	negative	negative	negative	10254.51	6	5	5	NC
			24.2	PM	negative	negative	negative	6702.29	9	8	0	NA
			24.4	PM	negative	negative	negative	12742.74	10	9	7	NC
Spiegeldries bos (Oosterzele)	11.37	25	25.1	PM	negative	NA	negative	12742.74	10	9	8	18.72 ± 1.00
			25.2	PM	negative	negative	negative	9048.10	10	9	7	17.78 ± 0.76
			25.3	PM	negative	negative	NA	10254.51	9	0	0	NA
St-Lievens-Houtem	1.31	27	27.4	PM	negative	negative	negative	12034.81	9	4	0	NA
	1.59	28	28.2	PC	negative	negative	negative	8109.77	12	9	7	NC
			28.3	PC	negative	negative	negative	6283.40	NC	NC	NC	NC
			28.4	PM	negative	negative	negative	10254.51	8	6	5	18.02 ± 0.70
	5.63	29	29.2	PC	negative	negative	negative	NC	10	9	9	11.80 ± 1.47
			29.4	PM	negative	negative	negative	10254.51	10	9	9	17.29 ± 0.65

Forest fragment	Forest fragment surface area (ha)	Plot	Nest box	Spp	Eggshell *Salmonella* status	Egg white *Salmonella* status	Egg yolk *Salmonella* status	Egg volume (mm^3)	Clutch size	N° nestling	N° fledgling	Mean SMI ± stdev
Borsbeke (Herzele)	12.04	30	30.1	PC	negative	negative	negative	8109.77	13	8	5	11.69 ± 0.56
			30.2	**PC**	**positive**	**negative**	**negative**	5445.61	**12**	**10**	**7**	**10.43 ± 0.66**
			30.3	PM	negative	NA	negative	15599.59	11	7	5	NC
			30.4	PC	negative	NA	NA	NC	12	11	11	10. 98 ± 0.47
	9.21	31	31.3	PM	negative	negative	negative	8109.77	8	7	7	18.23 ± 1.83
Nonnenbos (Serskamp)	32.69	32	32.2	PM	negative	negative	negative	6283.40	11	9	0	NA
Serskamp	**58.90**	**36**	**36.2**	**PC**	**positive**	**negative**	**negative**	6283.40	**9**	**8**	**6**	**11.05 ± 0.97**
			36.4	PC	negative	negative	negative	8109.77	7	6	5	11.53 ± 0.13
		37	37.2	PM	negative	negative	negative	13450.66	9	7	2	14.96 ± 0.33
			37.3	PM	negative	negative	negative	10254.51	7	6	3	17.10 ± 1.78
			37.4	PM	negative	negative	negative	10857.72	8	5	3	17.52 ± 1.02
Oud smetledebos (Smetlede)	47.77	38	38.1	PM	negative	negative	negative	11326.88	11	10	10	16.50 ± 0.58
			38.3	PM	negative	negative	negative	12742.74	6	4	4	18.33 ± 1.08
			38.4	PM	negative	negative	negative	10254.51	8	7	7	17.95 ± 2.20
		39	39.4	PM	negative	NA	negative	13957.53	7	6	0	NA
		40	**40.1**	**PM**	**positive**	**negative**	**NA**	10254.51	**8**	**0**	**0**	**NA**
		41	41.3	PM	negative	NA	negative	NC	8	7	6	17. 72 ± 0.79
		43	43.1	PC	negative	negative	negative	8109.77	12	10	10	11.47 ± 0.61
			43.2	PM	negative	negative	negative	12742.74	8	7	7	17.89 ± 0.85
		44	44.1	PM	negative	negative	negative	12742.74	8	7	6	17.36 ± 0.67
	58.90	45	45.2	PM*	negative	negative	negative	7602.91				14.66 ± 0.82
				PC*	NC	NC	NC	NC	17	14	10	8.77 ± 1.22
Hospiesbos (Wetteren)	18.73	46	46.3	PC	negative	negative	negative	8109.77	11	10	9	12.34 ± 0.27
		47	47.1	PM	negative	NA	negative	NC	7	5	5	17.36 ± 0.69
			47.2	PM	negative	negative	negative	10254.51	8	5	5	17.30 ± 2.42
		48	48.1	PC	negative	negative	negative	NC	10	9	9	10.48 ± 1.18
			48.2	PM	negative	NA	NA	12742.74	8	6	6	18.12 ± 0.15
			48.4	PM	negative	NA	NA	12742.74	8	7	0	NA
Moortelbos (Oosterzele)	30.65	49	49.1	PM	negative	NA	NA	7602.91	6	5	5	18.63 ± 1.21
			49.3	PM	negative	NA	negative	12742.74	8	6	6	16.49 ± 0.87
			49.4	PC	negative	negative	negative	10618.95	12	9	9	11.51 ± 0.41
		50	**50.1**	**PC**	**positive**	**NA**	**negative**	NC	**7**	**6**	**0**	**NA**
			50.2	PM	negative	negative	negative	12742.74	10	9	8	16.52 ± 0.60
			50.3	PC	negative	negative	negative	6702.29	13	12	0	NA
			50.4	PM	negative	negative	negative	12742.74	10	0	0	NA
		51	51.1	PM	negative	negative	negative	10254.51	9	7	7	16.60 ± 0.77
			51.2	PC	negative	NA	NA	7841.68	12	10	10	NC
			51.4	PC	negative	NA	negative	8109.77	9	9	9	11.02 ± 0.74
Ooidonk (Deinze)	**46.16**	**52**	**52.2**	**PM**	**positive**	**negative**	**negative**	10254.51	**10**	**8**	**NC**	**NC**

Forest fragment	Forest fragment surface area (ha)	Plot	Nest box	Spp	Eggshell *Salmonella* status	Egg white *Salmonella* status	Egg yolk *Salmonella* status	Egg volume (mm^3)	Clutch size	N° nestling	N° fledgling	Mean SMI ± stdev
		53	53.1	PC	negative	NA	NA	8616.64	13	11	11	11.12 ± 1.07
			53.4	**PM**	**positive**	**negative**	**negative**	12742.74	**7**	**NC**	**4**	**16.84 ± 1.01**

S2 Table: IgY antibody assessment in blood of great tits. Using a *Salmonella* specific ELISA, as described in the materials and methods section, the presence of IgY antibodies in the blood of blue tits (PM) was analyzed as negative or positive. Due to practical issues, some samples were not collected (NC). If the number of fledglings was equal to 0, no blood could be taken (not applicable or NA).

Forest	Plot	Nestbox	Spp.	Juvenile	*Salmonella* ELISA
Vurtzak (Merelbeke)		1.2	PM	58V92692	negative
				58V92693	negative
				58V92694	negative
				58V92698	NC
		1.4	PM	58V92930	negative
				58V92932	negative
				58V92933	negative
Nerenbos (Merelbeke)	2	2.3	PM	58V92852	negative
				58V92853	negative
				58V92854	negative
				58V92855	NC
				58V92966	negative
		2.4	PM	58V92857	negative
				58V92858	negative
				58V92859	NC
	3	3.2	PM	58V92861	negative
				58V92862	negative
				58V92863	negative
				58V92864	NC
Heilig Geestgoed (Merelbeke)	4	4.1	PM	58V92812	negative
				58V92813	NC
				58V92814	negative
				58V92815	negative
				58V92816	NC
		4.3	PM	NA	NA
	5	5.1	PM	58V92796	negative
				58V92797	**positive**
		5.2	PM	58V92601	NC
				58V92602	negative
				58V92798	negative
				58V92799	negative
Makegembos (Merelbeke)	7	7.1	PM	58V92545	negative
				58V92546	NC
				58V92547	NC
		7.3	PM	58V92537	negative

Forest	Plot	Nestbox	Spp.	Juvenile	*Salmonella* ELISA
				58V92538	negative
				58V92539	negative
				58V92541	negative
	9	9.3	PM	58V92605	negative
				58V92607	negative
				58V92609	negative
				58V92611	negative
		9.4	PM	58V92549	negative
				58V92550	negative
				58V92551	negative
				58V92552	NC
	10	10.3	PM	NC	NC
Harentbeekbos (Merelbeke)	12	12.3	PM	58V92866	negative
				58V92867	negative
				58V92868	negative
				58V92869	negative
		12..4	PM	57V84782	negative
				57V84783	negative
				57V84784	negative
				57V84785	negative
	13	13.3	PM	58V92581	NC
				58V92583	negative
				58V92584	NC
				58V92586	negative
	16	16.1	PM	58V92615	negative
				58V92616	negative
				58V92617	negative
				58V92618	negative
		16.4	PM	58V92620	negative
				58V92621	negative
				58V92622	NC
				58V92624	negative
	17	17.2	PM	58V92628	negative
				58V92629	negative
				58V92630	negative
				58V92633	NC
	18	18.1	PM	58V92873	negative
				58V92874	negative
				58V92875	negative
				58V92876	negative
				58V92877	negative
				58V92878	negative
				58V92879	negative

Forest	Plot	Nestbox	Spp.	Juvenile	*Salmonella* ELISA
				58V92880	negative
		18.2	PM	58V92642	negative
				58V92643	negative
				58V92644	NC
				58V92645	negative
		18.3	PM	NC	NC
		18.4	PM	57V84790	NC
				57V84791	negative
				57V84792	negative
				57V84793	negative
	19	19.3	PM	58V92634	negative
				58V92635	negative
				58V92638	NC
				58V92639	negative
Wannegatstraat (Gavere)	20	20.1	PM	NC	NC
Bueren (Melle)	21	21.2	PM	58V92671	negative
				58V92672	negative
				58V92674	negative
				58V92675	negative
Aelmoeseneiebos (Melle)	22	22.1	PM	NC	NC
		22.2	PM	NA	NA
	23	23.1	PM	58V92656	negative
				58V92657	NC
				58V92660	negative
				58V92662	negative
		23.3	PM	58V92663	negative
				58V92664	negative
				58V92667	negative
				58V92668	NC
				58V92669	negative
	24	24.1	PM	NC	NC
		24.2	PM	NA	NA
		24.4	PM	NC	NC
Spiegeldriesbos (Oosterzele)	25	25.1	PM	58V84797	negative
				58V84798	negative
				58V84799	NC
				57V84800	negative
		25.2	PM	58V92805	NC
				58V92806	NC
				58V92807	NC
				58V92808	NC
		25.3	PM	NA	NA
St-Lievens-Houtem	27	27.4	PM	NA	NA

Forest	Plot	Nestbox	Spp.	Juvenile	*Salmonella* ELISA
	28	28.4	PM	58V92990	negative
				58V92991	NC
				58V92992	negative
				58V92993	negative
	29	29.4	PM	58V92701	negative
				58V92704	negative
				58V92997	negative
				58V92998	negative
Borsbeke (Herzele)	30	30.3	PM	58V92766	negative
				58V92768	negative
	31	31.3	PM	58V92742	negative
				58V92744	negative
				58V92745	negative
Nonnenbos (Serskamp)	32	32.2	PM	NA	NA
Serskamp	37	37.2	PM	58V92504	NC
				58V92505	negative
				58V92506	NC
				58V92507	**positive**
		37.3	PM	58V92501	negative
				58V92502	NC
				58V92503	negative
				58V92900	negative
		37.4	PM	58V94773	negative
				58V94774	negative
Oud smetledebos (Smetlede)	38	38.1	PM	57V84756	NC
				57V84757	NC
				57V84758	NC
				57V84759	NC
		38.3	PM	58V92758	negative
				58V92759	negative
				58V92760	NC
				58V92761	negative
		38.4	PM	57V84766	NC
				57V84767	NC
				57V84768	NC
	39	39.4	PM	58V92883	NC
				58V92884	negative
	40	40.1	PM	NA	NA
	41	41.3	PM	58V92787	negative
				58V92788	negative
				58V92789	negative
				58V92790	negative
	43	43.2	PM	58V92763	negative

Forest	Plot	Nestbox	Spp.	Juvenile	*Salmonella* ELISA
				58V92764	NC
				58V92765	NC
				58V92766	NC
				58V92776	negative
				58V92898	NC
				58V92899	negative
	44	44.1	PM	58V92777	negative
				58V92778	negative
				58V92779	negative
				58V92781	**positive**
	45	45.2	PM	58V92571	NC
				58V92572	negative
				58V92575	**positive**
				58V92577	negative
Hospiesbos (Wetteren)	47	47.1	PM	58V92710	negative
				58V92711	negative
				58V92712	negative
				58V92713	negative
		47.2	PM	58V92733	negative
				58V92734	negative
				58V92736	negative
				58V92737	NC
	48	48.2	PM	58V92683	negative
				58V92685	negative
				58V92687	negative
				58V92688	negative
		48.4	PM	NA	NA
Moortelbos (Oosterzele)	49	49.1	PM	58V92820	NC
				58V92821	negative
				58V92822	negative
				58V92823	negative
		49.3	PM	58V92564	negative
				58V92565	NC
				58V92566	negative
				58V92568	NC
	50	50.2	PM	58V92532	negative
				58V92533	NC
				58V92535	negative
		50.4	PM	NA	NA
	51	51.1	PM	58V92827	negative
				58V92828	NC
				58V92829	negative
				58V92830	negative

Forest	**Plot**	**Nestbox**	**Spp.**	**Juvenile**	***Salmonella* ELISA**
Ooidonk (Deinze)	52	52.2	PM	NC	NC
	53	53.4	PM	58V92983	NC
				58V92984	negative
				58V92985	negative
				58V92986	Negative

CHAPTER II

Mitigating the impact of microbial pressure on great *(Parus major*) and blue (*Cyanistes caeruleus*) tit hatching success through maternal immune investment

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Abstract

The hatching success of a bird's egg is one of the key determinants of avian reproductive success, which may be compromised by microbial infections causing embryonic death. During incubation, outer eggshell bacterial communities pose a constant threat of pathogen translocation and embryo infection. One of the parental strategies to mitigate this threat is the incorporation of maternal immune factors into the egg albumen and yolk. It has been suggested that habitat changes like forest fragmentation can affect environmental factors and life-history traits that are linked to egg contamination. This study aims at investigating relationships between microbial pressure, immune investment and hatching success in two abundant forest bird species and analyzing to what extent these are driven by extrinsic (environmental) factors. We here compared (1) the bacterial load and composition on eggshells, (2) the level of immune defenses in eggs, and (3) the reproductive success between great (*Parus major*) and blue (*Cyanistes caeruleus*) tits in Belgium and examined if forest fragmentation affects these parameters. Analysis of 70 great tit and 34 blue tit eggshells revealed a similar microbiota composition (*Enterobacteriaceae*, *Lactobacillus* spp., *Firmicutes* and *Bacteroidetes*), but higher bacterial loads in great tits. Forest fragmentation was not identified as an important explanatory variable. Although a significant negative correlation between hatching success and bacterial load on the eggshells in great tits corroborates microbial pressure to be a driver of embryonic mortality, the overall hatching success was only marginally lower than in blue tits. This may be explained by the significantly higher levels of lysozyme and IgY in the eggs of great tits, protecting the embryo from increased infection pressure. Our results show that immune investment in eggs is suggested to be a species-specific adaptive trait that serves to protect hatchlings from pathogen pressure, which is not directly linked to habitat fragmentation.

Keywords: forest fragmentation, IgY, lysozyme, passerine, pathogen pressure, reproductive success

Introduction

Embryonic development of birds is a process that is threatened by microbial invasion [1-4]. Vertical transmission of pathogens during egg formation [5-6] and horizontal transmission after oviposition [7-8] may threaten the individual fitness and viability of the embryo and result in hatching failure [3, 9-12]. Shortly after laying, the eggshell becomes susceptible to pathogen penetration [13-14]. As such, environmental factors such as nest materials, bacteria on the female's skin, feathers and feces, nest visitors, and airborne bacteria are important risks of egg contamination [15-17].

To minimize embryonic contamination, the composition of the egg creates a natural physical barrier against bacterial penetration [18-19], and together with antimicrobial substances within the egg yolk and albumen [20-21], they constitute a first line of defense. In birds, females can influence the phenotype and fitness of their offspring by modifying the egg composition through the transfer of immunoglobulins (e.g. IgY) and antibacterial proteins to their eggs [22-23]. These maternal immune factors protect the embryo against bacteria which have succeeded in penetrating the eggshell, and the hatchling after the resorption of the remaining egg yolk and albumen [24]. Amongst antimicrobial proteins in the egg albumen, lysozyme, ovotransferrin, and avidin are the three most abundant ones [25].

Several studies showed that the number of bacteria present on the eggshell is positively associated with the risk of trans-shell infection [1, 3, 26-27]. Not only the bacterial load is a forerunner of hatching failure, but also the composition of the bacterial community and certainly the presence of pathogenic bacterial strains could play a role [28]. In the gastrointestinal tract of avian species, the phyla *Firmicutes* (including *Lactobacillus*) and *Bacteroidetes* and the family of *Enterobacteriaceae* are amongst the most abundant bacterial groups [29-30]. Most of the enteric bacteria have established a commensal status [31-33] however, some members are also known as pathogens, especially *Enterobacteriaceae* such as *Escherichia coli*, *Salmonella* spp. and *Yersinia* spp., but also *Clostridium perfringens* belonging to the *Firmicutes* phylum [34-36].

It has been suggested that habitat change, such as fragmentation of large, homogenous habitat blocks into small, isolated patches, can affect both extrinsic (environmental traits) and intrinsic (life-history traits) factors linked to egg contamination. For instance, brood parasitism [37], climatic conditions [1], dispersal opportunities, and feeder visiting are all presumed to alter host-pathogen dynamics, breeding performance and risk of trans-shell infection [38-39]. Although some research has been performed on the effect of human encroachment on natural

environments of wild bird populations and its effect on host-pathogen interactions, how extrinsic (environmental) drivers shape relationships between infection pressure, immune investment and breeding performance in forest birds, remains poorly known.

To fill this knowledge gap, this study aims at investigating these relationships in great and blue tits, two relatively closely-related [40] forest species with strongly overlapping ecological niches that are widely distributed and abundant throughout Europe. We first examined bacterial infection pressure (load and community composition) on eggs of free-ranging blue and great tits in 19 mature deciduous forest fragments in East-Flanders (Belgium) and analyzed to what extent bacterial loads varied with fragment area. Next, we analyzed variation in maternal immune investment (IgY, avidin, lysozyme and ovotransferrin) into eggs and the extent to which this was correlated with hatching success.

Materials and Methods

Study design and study site

We performed a study of blue and great tits in 53 study plots located in 19 mature (> 60 years) deciduous forest fragments in the south of Ghent (coordinates: 50°57'19"N, 3°43'31"E), northern Belgium (Fig 1, S1 Table). All study plots (30 x 30 m) were established in 2014 to study effects of tree species diversity and forest fragmentation on food web dynamics [as explained in 41]. Forest fragments in which these plots were located, strongly varied in size (range: 1.3 to 90.4 ha). Surface area sizes for each forest fragment were calculated from detailed GIS layers.

During autumn 2014, 212 standard nest boxes for blue and great tits (dimensions 23 x 9 x 12 cm, entrance 32 mm) were installed at a height of 1.5 m, at each corner of a plot, of which 3 broke during the experiment [see 42 for more information]. During the breeding season of 2015, all nest boxes were checked at least twice a week to determine the total number of eggs produced (clutch size) and the total number of hatchlings (S1 Fig). To avoid intra-clutch variation, the fifth egg of each great and blue tit clutch was collected using sterile gloves, stored in a sterile bottle and transported to the laboratory where the eggs were cracked under a laminar flow cabinet. Egg yolk and egg white were collected and stored separately at -20°C. In order to avoid antimicrobial activity of the albumen, the inside of the eggshells was washed with sterile phosphate buffered saline (PBS) to remove the adhering egg albumen. The eggshell, including shell membranes, was transferred to an Eppendorf tube and stored at -20°C.

The percentage of hatching failure was calculated as ((1-(number of hatchlings/(clutch size – 1)))*100).

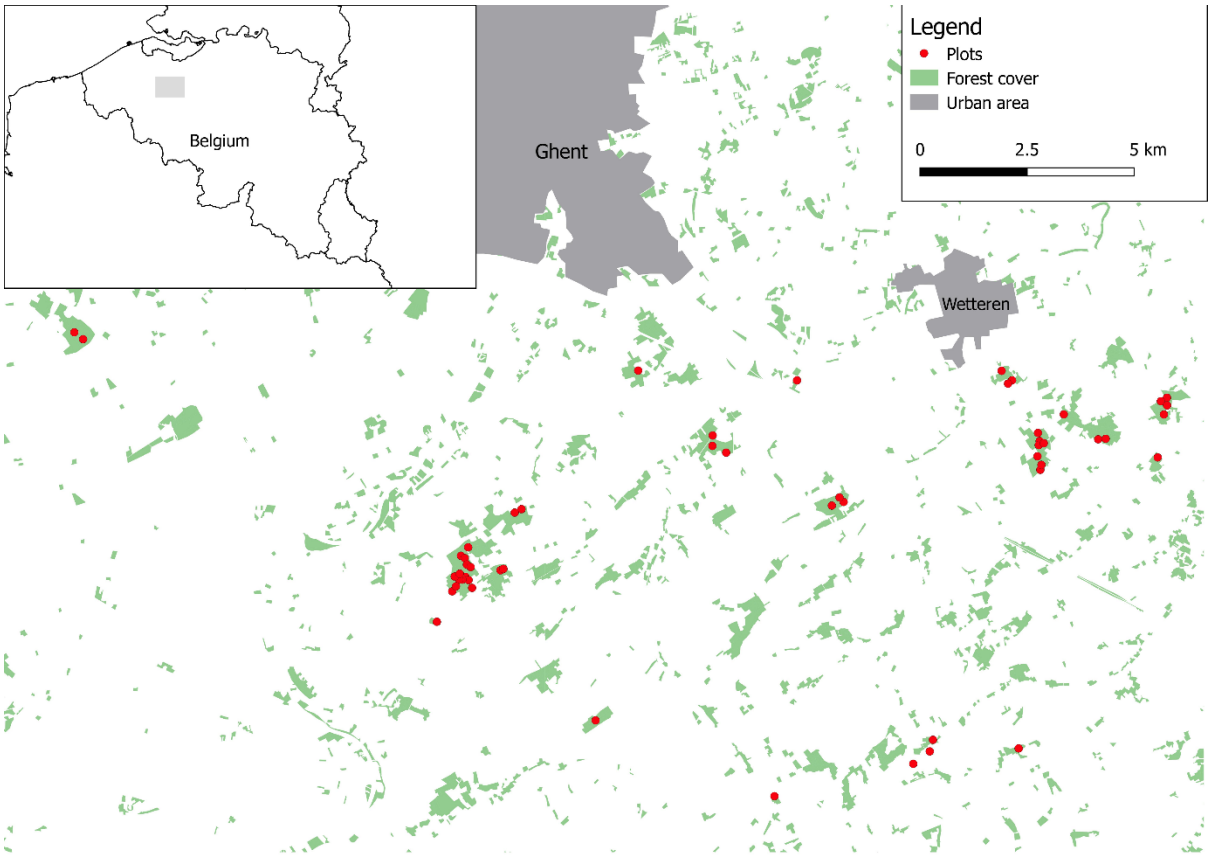

Figure 1: Map showing the location of all the study plots. The study of great and blue tit nests was performed in 53 study plots, established in 19 forest fragments.

Antimicrobial assays: lysozyme, avidin and ovotransferrin

We assessed lysozyme concentrations following Ruuskanen et al. (2011) [43]. Briefly, albumen was diluted in phosphate buffer (67 mM, pH 6.2, dilution 1:500). A *Micrococcus lysodeikticus* (Sigma-Aldrich, Darmstadt, Germany) suspension was prepared in phosphate buffer (0.5 mg/ml). A hundred µl of the diluted albumen and 100 µl of the *Micrococcus* suspension were added to a 96 well plate (MaxiSorp Nunc-Immuno™ plate, Thermo Fisher Scientific, Massachusetts, USA) and the absorbance was measured every 2 minutes, during 30 min at room temperature and at 450 nm using a Multiskan MS Reader (Labsystem Diagnostics Oy, Vantaa, Finland) with the Ascent Software, version 2.6. Each sample was analyzed in duplicate and before each measurement, the plate was mixed for 10 s. The results, given as

Unit/mg protein, were calculated from the changes in absorbance per minute and compared to the standards (lysozyme from chicken egg white, Sigma-Aldrich).

To measure avidin, we used a modified version of the colorimetric method of Gan & Marquardt (1999) [44]. Therefore, each albumen sample was diluted 1:4 in carbonate–bicarbonate buffer (Sigma-Aldrich) and 100 µl of each 10-fold serial dilutions was added to a 96 well plate (MaxiSorp Nunc-Immuno^{TM} plate), until a dilution factor of 11 was achieved. Serial dilutions of avidin (5 – 0.002 µg/ml, Sigma-Aldrich) were used as a standard. The plates were incubated at 4 °C overnight and then rinsed 3 times with phosphate-buffered saline (PBS)/0.05% Tween-20 (Sigma-Aldrich). Superblock buffer (Pierce, Rockfords, USA) was added for 30 s at room temperature to prevent nonspecific binding. This was repeated twice. Subsequently, we added 100 µl of a 1:4000 dilution of biotin/horseradish peroxidase (Sigma-Aldrich) in Superblock/0.05% Tween-20 to each well. The plates were incubated for 25 min at room temperature, followed by a wash step with PBS/0.05% Tween-20. After washing the plate 5 times, 100 µl of blue peroxidase (POD) substrate (Roche, Reinach, Switzerland) were added to each well before incubating the plates at room temperature for 30 min. Finally, the absorbance was measured at 450 nm using a Multiskan MS Reader. The concentration of avidin (µg/ml) in each sample was calculated by comparison of absorbance values to those in the standard curve.

The concentration of ovotransferrin was determined using the total iron binding capacity assay of Yamanishi et al. (2002) [45]. Therefore, 125 µl of a 1:500 dilution of an iron-standard solution (1000 mg/ml; Sigma-Aldrich) in a buffer (pH 8.4) containing 300 mmol/l Tris (Thermo Fisher Scientific), 150 mmol/l sodium hydrogen carbonate (EMD Millipore, Darmstadt, Germany), and 4.2 g/l Triton X-100 (Sigma-Aldrich) was added to 24 µl of each albumen sample in wells of a 96-well plate (Nunc MaxiSorp). After 5 min of incubation at 37 °C, a second reagent (pH 4.0) containing 10 mmol/l ferrozine (Baker, Maine, USA) and 32.6 mmol/l L-ascorbic acid (Thermo Fisher Scientific) in 50 mmol/l Tris buffer were added to each well and incubated at 37 °C for 5 min. Subsequently 100 µl of a third reagent containing 600 mmol/l citric acid (Baker) and 25.6 mmol/l thiourea (Baker) was added. The absorbance was measured every 20 s at 570 and 660 nm for 6.2 min using Multiskan MS Reader. To calculate ovotransferrin concentration, we determined the difference in absorbance at 570/660 nm at the beginning and end of the 6.2-min period. The absolute ovotransferrin concentration (mg/ml) was calculated by comparing these values with those in a standard curve.

Antibody titre analysis (IgY)

The antibody (IgY) level was determined using an indirect enzyme-linked immunosorbent assay (ELISA), modified from Morosinoto et al. (2013) [46]. Briefly, ELISA plates (MaxiSorp Nunc-Immuno^{TM} plates) were coated overnight at 4°C with 50 µl anti-chicken IgG (produced in rabbit) diluted 1/2000 in carbonate coating buffer. Egg yolk was diluted 1/3 with distilled water and supernatant was collected after centrifugation at 13 000 rpm for 15 min (4°C). Subsequently, the supernatant diluted 1/2000 in 1% bovine serum albumin in phosphate buffered saline (BSA-PBS) was added to the wells (50 µl) and incubated for 3 hours at room temperature. An alkaline phosphatase conjugated rabbit anti-chicken IgY antibody (1/2000) (Sigma-Aldrich) was added overnight at 4°C as a secondary antibody. The plate was developed using p-nitrophenyl phosphate for 30 min. The optical density was measured at 405 nm using a Multiskan MS Reader.

Bacteriological analysis: Enumeration of bacterial load by qPCR

DNA was extracted from the eggshell using a PowerLyzer® PowerSoil® DNA Isolation Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's guidelines. The abundance of total bacteria, *Firmicutes* and *Bacteroidetes* phyla, *Enterobacteriaceae* family and *Lactobacillus* spp. were quantified using the primers and PCR protocols described in Table 1. Amplification and detection were performed using the CFX384 Bio-Rad Real-time PCR detection system (Bio-Rad, Nazareth, Belgium). Each reaction was done in duplicate in a 12-µl total reaction mixture using 2 x SensiMix SYBR No-ROX mix (Bioline, Luckenwalde, Germany) and 2 µl of DNA.

Table 1: Primers and qPCR protocols used to quantify the total bacteria, *Firmicutes*, *Enterobacteriaceae*, *Bacteroidetes* and *Lactobacillus* spp.

Bacterial groups	Reference	Primers (5' -3')	Primer concentration	PCR program
Firmicutes	[68]	F: GGA GYA TGT GGT TTA ATT CGA AGC A	0.5 µM	10' 95°C; (30" 95°C, 30" 60°C) x 40; 15" 95°C
		R: AGC TGA CGA CAA CCA TGC AC	0.5 µM	
Enterobacteriaceae	[69]	F: CAT TGA CGT TAC CCG CAG AAG AAG C	0.5 µM	10' 95°C; (30" 95°C, 1' 63°C) x40; 15" 95°C
		R: CTC TAC GAG ACT CAA GCT TGC	0.5 µM	

Bacterial groups	Reference	Primers (5' -3')	Primer concentration	PCR program
Bacteroidetes	[70]	F: CRA ACA GGA TTA GAT ACC CT	0.75 µM	10' 95°C; (15" 95°C, 15" 61.5°C, 20" 72°C) x40; 15" 95°C
		R; GGT AAG GTT CCT CGC GTA T	0.75 µM	
Lactobacillus spp.	[71]	F: GGA ATC TTC CAC AAT GGA CG	0.5 µM	20" 95°C; (3"95°C, 30" 57°C) x40; 15" 95°C
		R: CGC TTT ACG CCC AAT AAA TCC GG	0.5 µM	
Total bacteria	[72]	F: CGG YCC AGA CTC CTA C GG G	0.5 µM	10' 95°C; (1' 94°C, 1' 53°C, 2' 60°C) x 40;15" 95°C
		R: TTA CCG CGG CTG CTG GCA C	0.5 µM	

Statistical analysis

First, in order to test whether total eggshell bacteria, *Firmicutes*, *Bacteroidetes, Enterobacteriaceae* and *Lactobacillus* numbers were influenced by fragment area, egg and nest characteristics (i.e. egg volume, clutch size and laying date) or species identity (i.e. great versus blue tit), linear mixed models ('glmer' function of R library 'lme4' [47] were run using bacteria counts as dependent variable. Forest fragment identity was included as a random effect to account for possible non-independence of nests within the same forest fragment, and models were run with a Poisson error distribution as bacterial loads were expressed as count data. An observation-level random effect was added in order to account for overdispersion present in the data [48]. Second, in order to test whether bacterial eggshell communities significantly differed between great tits and blue tits, we applied an analysis of dissimilarity (ADONIS, as implemented in the R library 'vegan' [49]. Third, to compare concentrations of egg protein concentrations (i.e. concentrations of egg lysozyme, IgY, avidin and ovotransferrin) between both species, Gaussian linear mixed models were applied, using egg protein concentrations as dependent variable and species as explanatory variable while including forest fragment identity as a random effect. All model residuals were normally distributed (Shapiro-Wilk $W > 0.90$). Lastly, to explore the relationship between egg immune factors and reproductive success, hatching failure was modelled as a binominal process, comparing success against failure. All statistical tests were performed with R [50].

Ethical considerations

All trapping and sampling protocols were approved by the Ethical Committee VIB Ghent site (EC2015-023).

Results

Bacterial abundance is higher on eggshells of great tits, but with a similar microbiota composition as in blue tits

The bacterial loads of 70 eggs of great tits collected from nest boxes in 42 plots, and of 34 eggs of blue tits collected in nests from 25 plots (summarized in S1 Table), were determined by qPCR. Bacterial loads were higher ($p < 0.001$) on the eggshells of great tits compared to those on the shells of blue tits (Fig 2 and S2 Table). The mean (± SE) eggshell total bacterial count (gene copies / eggshell) of great and blue tit eggs was $8.57 \times 10^5 \pm 1.83 \times 10^5$ and $3.67 \times 10^5 \pm 5.49 \times 10^4$, respectively. This corresponds to a Log_{10} value of 5.70 ± 0.05 for great tits and 5.42 ± 0.07 for blue tits (Table 2). Fragment area, egg volume, laying date and clutch size could not explain the pattern of bacterial load on eggs of great and blue tits (all $p > 0.05$; S3 Table). Although more bacteria were present on the eggshells of great tits, no significant differences were observed in the relative abundance of the composition of eggshell microbiota (Fig 3). Similar proportions (%) of *Enterobacteriaceae*, *Lactobacillus* spp., *Firmicutes*, and *Bacteroidetes* were observed relative to the total amount of bacteria present on the eggshell of both bird species (Table 2 and S2 Table).

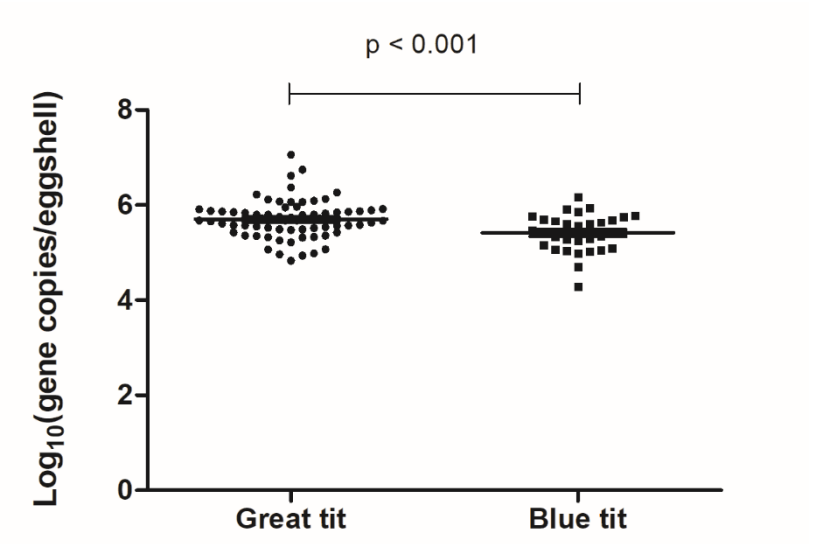

Figure 2: Number of total bacteria present on the eggshell of great and blue tits. The eggshells of great (n = 70; 42 different plots) and blue tits (n = 34; 25 plots) were analyzed using qPCR for bacterial presence. The results are expressed as the log_{10} of the copy number of the gene per eggshell. The whiskers represent the mean ± standard error of the mean. Statistical significance is shown by the p value.

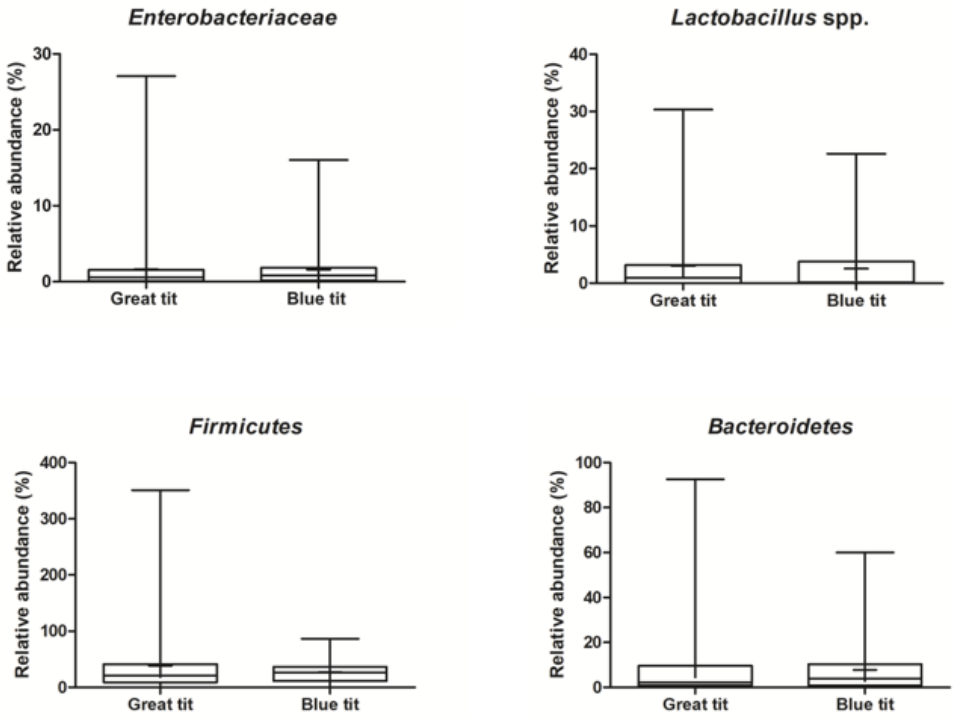

Figure 3: The relative abundance of bacterial groups present on the eggshell of great and blue tits. Box plots showing the relative abundance of *Enterobacteriaceae*, *Lactobacillus* spp., *Firmicutes* and *Bacteroidetes* compared to the total bacterial numbers present on the eggshell of great and blue tits, as assessed with qPCR. The whiskers represent the median, the minimum and maximum values, and the first and third quartiles. The plus indicates the mean value.

Higher immune factor concentrations in eggs of great tits

Egg lysozyme and IgY levels were significantly ($p < 0.001$) higher in egg albumen and egg yolk of great tits compared to blue tits (Fig 4 and S2 Table). Mean (± SE) concentrations of lysozyme and IgY were 68675.56 ± 5878.35 and 0.48 ± 0.037 in blue tits and 107952.81 ± 3991.08 and 0.74 ± 0.036 in great tits. These differences are species specific as "species" was shown to be a driver for IgY and lysozyme concentrations ($p < 0.001$; S4-S5 Table). Great tits eggs also tended to show higher concentrations of avidin and ovotransferrin, but without reaching statistical significance ($p > 0.05$; Table 2 and S2 Table).

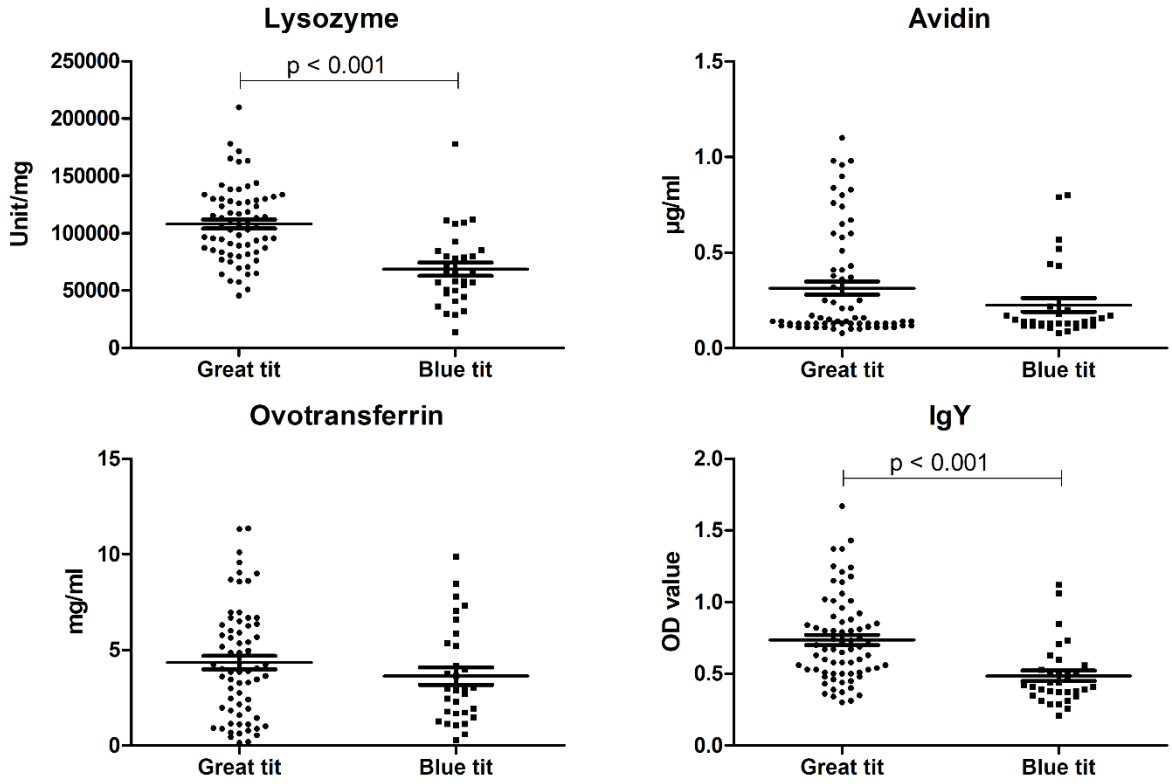

Figure 4: Concentration of antimicrobial proteins and IgY antibodies. Lysozyme, avidin, ovotransferrin and IgY was determined in the eggs of great and blue tits. The results are expressed as unit/mg lysozyme, µg/ml avidin, mg/ml ovotransferrin or as OD value for IgY. The whiskers represent the mean ± standard error of the mean. Statistical significance is shown by the p value.

Table 2: Difference in bacterial load, microbiota composition, IgY and presence of antibacterial proteins between blue and great tits. Shown in the mean ± SEM of the total eggshell bacterial load, the proportion (in %) of *Enterobacteriaceae*, *Lactobacillus* spp., *Firmicutes,* and *Bacteroidetes* and egg immune factors of blue and great tit eggs.

Measurement	Blue tit	Great tit
Eggshell total bacterial count (gene copies/eggshell)	$3.67 \times 10^5 \pm 5.49 \times 10^4$	$8.57 \times 10^5 \pm 1.83 \times 10^5$
Log10 eggshell total bacterial count (gene copies/eggshell)	5.42 ± 0.07	5.70 ± 0.05
Proportion of *Enterobacteriaceae* (%)	1.6 ± 0.49	1.6 ± 0.43
Proportion of *Lactobacillus* spp. (%)	2.5 ± 0.79	3.0 ± 0.68
Proportion of *Firmicutes* (%)	27.3 ± 3.90	38.5 ± 7.38

Measurement	Blue tit	Great tit
Proportion of *Bacteroidetes* (%)	7.8 ± 1.99	9.5 ± 2.17
IgY (OD)	0.48 ± 0.037	0.74 ± 0.036
Lysozyme (unit/mg)	68675.56 ± 5878.35	107952.81 ± 3991.08
Avidin (µg/ml)	0.23 ± 0.035	0.32 ± 0.034
Ovotransferrin (mg/ml)	3.63 ± 0.46	4.34 ± 0.35

Hatching success in great tits is only slightly impacted by the increased microbial pressure

Hatching success in clutches of great tits declined with increasing bacterial load of the fifth egg (p = 0.024; S6 Table). In blue tits, none of the studied variables were found to correlate with hatching success. At species level, hatching failure was significantly higher in great tits (16.66 ± 2.79) than in blue tits (10.02 ± 2.92 %) (p = 0.025; Fig 5 and S2 Table).

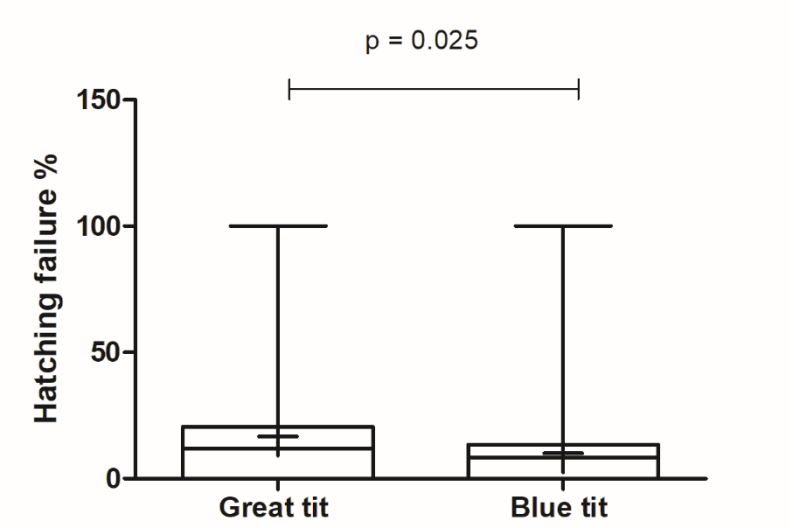

Figure 5: Hatching failure in nests of great and blue tits. Shown is the percentage of hatching failure. The whiskers represent the median, the minimum and maximum values, and the first and third quartiles. The plus indicates the mean value. Statistical significance is shown by the p value.

Discussion

We provide evidence for a higher infection pressure in great tit eggs than in those of the sympatric, ecologically similar blue tit, while the bacterial load of neither species was associated with variation in fragment area, egg volume, laying date or clutch size.

Avian species are known to apply different behavioral, chemical and physical strategies to control embryo infections, such as the use of intrinsic properties of plants to protect their nestlings against contamination with parasites and pathogens [19, 51-52]. Among passerines, blue tits have been reported to use aromatic plants as nest materials, possibly exploiting the antimicrobial properties of essential oils [51-52]. In our study, we detected leaves of the aromatic plant *Stachys sylvatica,* and pine needles in a number of blue tit nests, and essential oils of both plant species are believed to have antimicrobial activities [53-55]. As no such leaves or needles were detected in great tit nests, differential use of nest material may partly explain the observed differences in microbial pressure between both species. Alternatively, or in addition, differential bacterial accumulation may result from differences in nest sanitization, and results of our study would point towards a higher nest hygiene in blue tits. However, this hypothesis contradicts the results of Goodenough & Stallwood (2010) showing higher bacterial loads in blue tit nests than in those of great tits, hence more empirical studies are needed to test this hypothesis [56].

In contrast to bacterial load, relative egg microbiota composition (*Enterobacteriaceae*, *Lactobacillus* spp., *Firmicutes* and *Bacteroidetes*) did not differ between great and blue tits, with the *Firmicutes* phylum being the most abundant in both species. Such pattern is in line with gastrointestinal microbiota sampled in adults from various bird species [30, 57-58], supporting the hypothesis that bacteria are transmitted from the female cloaca to the eggs [17, 28]. Although most members of these bacterial groups are commensals, several bacterial species are also known as primary or opportunistic pathogens. Especially bacteria of the *Enterobacteriaceae* family such as *E. coli*, *Salmonella*, *Yersinia*, *Klebsiella*, *Citrobacter* and *Enterobacter* have been reported to cause disease and mortality in nestling passerines [59-61]. Additionally, *Streptococcaceae* of the phylum *Firmicutes* has been reported to cause embryonic death and infections in nestlings [9, 62].

Transmission of antimicrobials and IgY antibodies to the egg constitutes an important chemical defense mechanism in birds [63]. Lysozyme catalyzes the lysis of cell walls of gram-positive bacteria [64] and plays an important role pre-hatching, whereas IgY antibodies particularly protect nestling post-hatching [65]. While still fairly speculative, some authors suggested that mothers may distribute antimicrobial proteins differentially within and among clutches [20, 24-25], based on food availability [66] and depending on male attractiveness [25, 67]. These studies also provide evidence that birds may have evolved to differentially transmitting antimicrobials to increase the probability of offspring survival. In our study, great tits incorporated more lysozyme and IgY into the eggs than blue tits, suggesting that great tits

females may manipulate their antimicrobial allocation to compensate for the higher pathogen load. This, in turn, may explain why the reproductive success of great tits was only moderately lower than that of blue tits, despite their larger infection pressure and the observed correlation between infection pressure and hatching failure. To the best of our knowledge, this is the first study providing evidence for different maternal immune adaptations between two closely related bird species in order to increase the probability of offspring survival at different pathogen exposure probabilities.

Summarized, our results show that although great and blue tits are relatively closely related and ecologically similar, eggs of great tits are exposed to higher microbial pressures, for which they have adapted their immunological transfer to the eggs in order to limit the negative effect on reproductive parameters.

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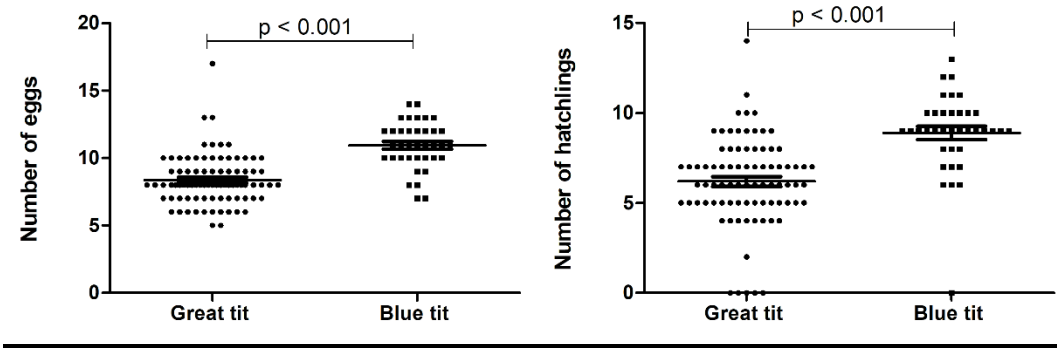
Supplementary Information:



S1 Figure: Overview of the clutch size and the number of hatchlings in the nests of great and blue tits.

S1 Table: Summary of sampled great (PM) and blue (PC) tit eggs in the different study plots

Forest	Plot	Latitude	Longitude	Surface area (ha)	tree diversity (spp.)	Nestbox	spp.
Lemberge	1	50.990342	3.7732	16.68	2	1.2	PM
						1.3	PC
						1.4	PM
Nerenbos (Merelbeke)	2	50.961391	3.73474	41.74	2	2.1	PC
						2.2	PC
						2.3	PM
						2.4	PM
	3	50.960503	3.73237	41.74	2	3.2	PM
						3.4	PC
Heilig Geestgoed (Merelbeke)	4	50.948551	3.728218	27.49	2	4.1	PM
						4.3	PM
	5	50.948747	3.728979	27.49	2	5.1	PM
						5.2	PM
Makegembos (Merelbeke)	7	50.951324	3.715364	83.77	2	7.1	PM
						7.2	PC
						7.3	PM
	8	50.950905	3.716254	83.77	1	8.2	PC
	9	50.949559	3.716739	83.77	1	9.1	PC
						9.3	PM
						9.4	PM
	10	50.94883	3.718443	83.77	1	10.3	PM
Harentbeekbos (Merelbeke)	12	50.9464	3.717465	83.77	1	12.1	PC

Forest	Plot	Latitude	Longitude	Surface area (ha)	tree diversity (spp.)	Nestbox	spp.
						12.3	PM
						12.4	PM
	13	50.946545	3.715697	83.77	1	13.2	PC
						13.3	PC*
	16	50.947438	3.714835	83.77	1	16.1	PM
						16.2	PC
						16.4	PM
	17	50.944474	3.718904	83.77	2	17.1	PC
						17.2	PM
						17.3	PC
	18	50.943761	3.712352	83.77	1	18.1	PM
						18.2	PM
						18.3	PM
						18.4	PM
	19	50.9449	3.713682	83.77	1	19.3	PM
						19.4	PC
Wannegatstraat (Gavere)	20	50.937592	3.707042	3.03	1	20.1	PM
						20.2	PC
						20.3	PC
Bueren (Melle)	21	50.9886	3.82614	6.19	2	21.1	PC
						21.2	PM
Aalmoezenijbos (Oosterzele)	22	50.976081	3.798739	23.57	2	22.1	PM
						22.2	PM
						22.4	PC
	23	50.974748	3.797965	23.57	2	23.1	PM
						23.3	PM
	24	50.973663	3.802786	23.57	1	24.1	PM
						24.2	PM
						24.4	PM
Spiegeldries bos (Oosterzele)	25	50.916874	3.760309	11.37	2	25.1	PM
						25.2	PM
						25.3	PM
Zottegem	26	50.901508	3.819877	3.53	3	26.2	PM
						26.3	PM
						26.4	PM
St-Lievens-Houtem	27	50.908521	3.865333	1.31	2	27.4	PM
	28	50.911148	3.871161	1.59	2	28.2	PC
						28.3	PC
						28.4	PM
	29	50.913516	3.872813	5.63	1	29.2	PC
						29.4	PM

Forest	Plot	Latitude	Longitude	Surface area (ha)	tree diversity (spp.)	Nestbox	spp.
	30	50.91155	3.901021	12.04	1	30.1	PC
						30.3	PM
						30.4	PC
	31	50.973112	3.946005	9.21	1	31.3	PM
Nonnenbos (Serskamp)	32	50.985475	3.949129	32.69	2	32.2	PM
Serskamp	36	50.976824	3.926348	58.9	2	36.2	PC
						36.3	PM
						36.4	PC
	37	50.976977	3.9288	58.9	2	37.3	PM
						37.4	PM
Oud smetlede	38	50.978195	3.906263	47.77	1	38.1	PM
						38.3	PM
						38.4	PM
	39	50.976306	3.907334	47.77	1	39.4	PM
	40	50.975601	3.906863	47.77	2	40.1	PM
	41	50.976082	3.908319	47.77	3	41.3	PM
	43	50.970567	3.907196	47.77	1	43.1	PC
						43.2	PM
						43.3	PM
	44	50.971339	3.907868	47.77	1	44.1	PM
						44.3	PM
	45	50.982	3.914797	58.9	2	45.2	PM*
Hospicebossen (Nazareth)	46	50.99087	3.894436	18.73	1	46.3	PC
	47	50.98917	3.897568	18.73	2	47.1	PM
						47.2	PM
	48	50.988468	3.89644	18.73	1	48.1	PC
						48.2	PM
						48.4	PM
Oosterzele	49	50.962551	3.838403	30.65	1	49.1	PM
						49.3	PM
						49.4	PC
	50	50.96349	3.842156	30.65	3	50.1	PC
						50.2	PM
						50.3	PC
						50.4	PM
	51	50.964019	3.840559	30.65	1	51.1	PM
						51.2	PC
						51.4	PC
Ooidonk (Deinze)	52	50.996011	3.588524	46.16	1	52.2	PM
	53	50.997431	3.585583	46.16	3	53.1	PC
						53.4	PM

An asterisk (*) indicates a nest where brood parasitism was observed.

S2 Table: Summary of statistical analyses examining the difference between blue and great tits.

Factors	t/z/f-value	p-value
Log_{10} eggshell bacterial load	3.685	0.000373
Enterobacteriaceae	1.515	0.130
Lactobacillus spp.	1.456	0.145
Firmicutes	0.966	0.334
Bacteroidetes	1.356	0.175
Lysozyme	5.768	8.2×10^{-9}
Avidin	0.842	0.402
Ovotransferrin	1.131	0.261
IgY	4.598	1.26×10^{-5}
Hatching failure	-2.241	0.025

S3 Table: Summary of statistical analyses examining the driving factors for eggshell bacterial loads.

Factors	Great tit		Blue tit	
	z-value	p-value	z-value	p-value
Forest fragment surface area	-0.10	0.917	1.31	0.191
Egg volume	-0.04	0.970	-0.27	0.785
Clutch size	-0.07	0.940	-1.94	0.065
Laying date	0.50	0.614	1.31	0.191

S4 Table: Summary of statistical analyses examining the driving factors for lysozyme allocation.

Factors	t-value	p-value
Eggshell bacterial load	-0.418	0.676
Species	5.412	$6.3.x10^{-8}$

S5 Table: Summary of statistical analyses examining the driving factors for IgY allocation.

Factors	t-value	p-value
Eggshell bacterial load	-0.601	0.549
Species	4.058	0.000103

S6 Table: Summary of statistical analyses examining the driving factors for hatching failure.

Factors	Great tit		Blue tit	
	z-value	p-value	z-value	p-value
Forest fragment surface area	0.715	0.474	0.961	0.337
Eggshell bacterial load	-2.262	0.024	1.562	0.118
Enterobacteriaceae	-0.874	0.382	1.045	0.296
Lactobacillus spp.	0.686	0.493	-0.082	0.935
Firmicutes	1.39	0.165	1.729	0.839
Bacteroidetes	1.23	0.219	-0.846	0.398
Lysozyme	-1.47	0.143	-1.492	0.136
Avidin	0.037	0.970	0.170	0.865
Ovotransferrin	0.438	0.662	0.877	0.380
IgY	1.167	0.243	0.017	0.987
Clutch size	1.149	0.250	-0.232	0.817
Laying date	-0.299	0.767	0.350	0.727

GENERAL DISCUSSION

"We're the only species that have crapped up the planet and the only species that can clean it up"

In order to study the impacts of habitat fragmentation on microbial pressure and avian reproductive success, I have investigated the health status and microbial pressure of eggs and juveniles of great and blue tits along fragmentation gradients in Flanders. In the following section, we will discuss the major findings of this research, pinpoint the strengths and drawbacks of the thesis and formulate future perspectives.

Salmonella Typhimurium in tits

Three *Salmonella* Typhimurium DT99 and four *Salmonella* Typhimurium DT193 isolates were found on the eggs of great and blue tits inhabiting different forest fragments across our study area. From different nest boxes and study-plots, four fledglings harbored anti-*Salmonella* antibodies. The combination of these results suggests a low prevalence and widespread distribution of *Salmonella* Typhimurium in tit species without visible health related issues or reproductive problems (**Chapter I**), although this should be interpreted with care. When comparing to the prevalence of *Salmonella* Enteritidis, the most common serotype in laying hens in Europe (1.2%) (EFSA, 2016), the prevalence in the tit population is higher. This could be the result from preventive measure in laying hen flocks.

The use of isolation methods in combination with serology (detection of IgG in fledgling blood) provides a more complete view of the actual *Salmonella* Typhimurium presence, since the isolation of the bacterium will depend on the excretion (which has been shown to be intermittent for *Salmonella*) (Connolly et al., 2006) and the serology provides information regarding previous contact with the bacterial agents (Hassan et al., 1991; Barrow, 1992; Wobeser 2006). However, since the blood was collected from fledglings that were 14-15 days of age, it is unsure whether the detected immunoglobulins are antibodies which originated from maternal passive transfer (Hamal et al., 2006; King et al 2010) or were endogenously produced by the fledgling (King et al., 2010). Most avian studies performed on maternal transfer of antibodies have been performed in chickens (e.g. Hassan et al., 1991; Barrow, 1992; Hamal et al., 2006), and extrapolation to other bird species is not always correct, as was demonstrated by King et al. (2010). They showed that house sparrows' altricial young reached immunological independence earlier than precocial chicks, with maternal antibodies in altricial young having a shorter half-life (2,2 ± 0,25 days versus 3-7 days) and shorter persistence (8-9

days versus 14-21 days), although similar initiation for the de novo synthesis of immunoglobulins (3-6 days post hatching) was demonstrated for the chickens as well as the house sparrow offspring (King et al., 2010). Since great and blue tit nestlings are also altricial, it could be suggested that the detected antibodies in our study are endogenously produced antibodies. Nevertheless, species-specific tests should be performed to better understand the species differences in immunological investment, transfer of antibodies and start of de endogenous antibody production, which not only depend on the investigated species (King et al., 2010), but also on other factors such as the condition of the mother birds (Hargitai et al., 2006) and the type and characteristics of the pathogen of interest (Barrow et al., 1992; Hamal et al., 2006; Staszewski et al., 2007). Furthermore, since I only tested one egg of the clutch and did not test the faeces of the offspring or parental birds nor the nest material for the presence of *Salmonella*, I could have missed positive eggs, birds or nests, and as such this prevalence could be an underestimation of the real *Salmonella* prevalence.

In previous studies it has been suggested that environmental contamination with pathogenic bacteria, such as *Salmonella* Typhimurium, present a risk for wildlife for getting infected (Cízek et al., 1994; Andrés et al., 2013; Krawiec et al., 2015). Bird feeders in particular have been linked to the increased occurrence of *Salmonella* outbreaks in wild birds due to the aggregation of different bird species in high densities which enhances the faeco-oral contact (Brittingham and Temple, 1988; Pennycott et al., 2002 and 2010; Refsum et al., 2003; Hughes et al., 2010; Krawiec et al., 2015). Pigeons (*Columba livia*), also frequent visitors of bird feeders, have been shown to endemically carry *Salmonella* Typhimurium DT99, a host-adapted pigeon strain, in Belgium (Pasmans et al., 2003 and 2004). Since I cannot rule out that the tits in our study visited bird feeders in the neighboring environment (outside the forest fragments), it is possible that they came into contact with the host-adapted *Salmonella* Typhimurium DT99 isolates. Unfortunately, I was unable to compare our DT99 strains to those isolated from pigeons using MLVA, as such no epidemiological link could be ascertained. No visible adverse health effects were detected in the nests where DT99 was recovered from eggshells (**Chapter I**), although previous studies have demonstrated mortality linked to DT99 in passerines (Refsum et al., 2002a; Rouffaer et al., 2016).

Salmonella Typhimurium DT193, previously associated with salmonellosis in passerines (Lawson et al., 2011) could be hypothesized to have been acquired from the human waste. DT193 has previously been associated with human infections (Hampton et al., 1995; Hopkins et al., 2010; Brunelle et al., 2013; Wuyts et al., 2013), is present in pigs and poultry (Hopkins

et al., 2010; Parsons et al., 2013) and is one of the most encountered human phage types in Belgium (Wuyts et al., 2013). To test this assumption MLVA was performed, which demonstrated the distinctiveness between the human DT193 isolates and the ones from our tits, the latter clustering together and thus suggesting the presence of avian-adapted *Salmonella* Typhimurium in the tit species (**Chapter I**). In Belgium, only little research has focused on prevalence and effect of *Salmonella* on wild living birds (Pasmans et al., 2004; Haesendonck et al., 2016; Rouffaer et al., 2016). In tits, this is the first study demonstrating the presence of a possible host-adapted *Salmonella* Typhimurium in forest dwelling tit species (**Chapter I**). In Britain and other countries such as Norway, different studies have followed-up *Salmonella* Typhimurium outbreaks in passerines or have assessed the prevalence in apparently healthy passerines (Hughes et al., 2010; Lawson et al., 2010; Pennycott et al., 2002, 2006 and 2010; Refsum et al., 2002a and 2003). Some of the strains, which were isolated during these outbreaks, were considered passerine-adapted (DT40, DT56v) (Refsum et al., 2002b; Hughes et al., 2010; Lawson et al., 2011). Although adaptation of hosts and pathogens could reduce the overall pathogenicity of the pathogen through co-evolution (Anderson and May, 1982), host-adaptation has been associated with systemic disease in the respective hosts (Faddoul et al., 1965; Klemm et al., 2016), which have previously resulted in outbreaks in passerines (Hughes et al., 2010) and a declined reproductive success in pigeons (Faddoul et al., 1965). Despite the host-adaptive nature, spill-overs to humans have previously been observed with passerine-adapted strains (Lawson et al., 2014).

Microbial eggshell load and diversity - species specific maternal immune adaptations

In this thesis I found that, despite the rather similar ecology of great and blue tit species, the comparison of eggs laid in comparable forest environments and in previously unused nest boxes, the eggshell bacterial load of great tits was higher than the one on the eggshells of blue tits. The relative microbial eggshell composition, however, was similar for both passerine species (**Chapter II**). Since none of the included variables in our models could explain the observed difference between great and blue tit eggshell microbial load, the reason for this difference can only be speculated on. Life history traits (e.g. nest sanitization efforts, the incubation onset, the type of nest materials used) (e.g. Cook et al., 2005a; Godard et al., 2007; Mennerat et al., 2009a and 2009b; Shawkey et al., 2009; Peralta-Sánchez et al., 2012;

Goodenough and Stallwood, 2010; Wang et al., 2011; Walls et al., 2012), climate (Cook et al., 2003 and 2005b; Wang et al., 2011; Walls et al., 2012), nest orientation (e.g. Goodenough and Stallwoord, 2012) and individual differences in microbial communities of parental birds (e.g. Goodenough et al., 2017) could play a role in the bacterial exposure and community on the eggshells and subsequently the risk of trans-shell infections and reproductive success (Cook et al., 2003 and 2005a; Shawkey et al., 2009). When comparing blue and great tit nests, Goodenough and Stallwood (2010) also found a difference in microbial load as well as bacterial community between the two closely related species, although the bacterial load in the blue tit nests was found to be higher. Since the bacterial community of nests influences the eggshell bacterial load and diversity (Peralta-Sánchez et al., 2014; Ruiz-Castellano, 2016), I would have expected a higher bacterial load on the blue tit eggshells. This is in contrast with our results, although the use of culture methods (Goodenough and Stallwood, 2010) instead of molecular techniques (**Chapter II**) makes it difficult to properly compare the results since culture methods are known to highly underestimate the actual bacterial load and diversity (Amann et al., 1995). The use of aromatic plants with antimicrobial activities as nest lining material has previously (Mennerat et al., 2009a and 2009b; Goodenough and Stallwood, 2010) and in our study been observed in blue tit nests (**Chapter II**), nevertheless it has also been described in great tit nests although less well documented (Mainwaring, 2017). As such, it would be interesting to study the differences in behavioral adaptations between great and blue tits which they use to prevent microbial infections in detail.

Despite the exposure of great tit eggs to a higher infection pressure, as a risk factor for hatching success, only a small reduction in hatching success was noticed. The increased transfer of IgY and lysozyme from the mother great tit to the egg yolk and albumen respectively, compared to the blue tit, could have contributed to the protection of the embryos. This was supported by the statistical analyses which showed that IgY allocation to the eggs was species specific, although not correlated with bacterial load (**Chapter II**). Whether or not this differential immune-transfer to the eggs could have evolved through natural selection is a matter of debate and would be interesting to further investigate.

In our studies, I have investigated whether or not there were species-specific differences in immune-allocation of the best described and most abundant antimicrobial products in the egg content (lysozyme, avidin, ovotransferrin and immunoglobulin IgY (D'Alba and Shawkey, 2015)). However, many other proteins are present in the egg content, for which the function is not yet understood (Mann, 2007; Mann and Mann, 2011; D'Alba and Shawkey, 2015). As such,

other antimicrobial proteins could be present in the egg content and potentially be affected by the environment or the species. Besides the antibacterial products, also the thickness and antimicrobial properties of the eggshell, the membranes and cuticle could be different between the two tit species or be affected by forest fragment characteristics (e.g. reduced eggshell thickness due to acidification (Graveland, 1996; Mänd et al., 2000a and b; Tilgar et al., 2005; Wuyts et al., 2008), which could be interesting to incorporate in further studies.

Because of ethical considerations, only one egg (the fifth) per nest was investigated for the bacterial load, diversity, and presence of antimicrobial proteins and immunoglobulins. Since there are still many gaps in the knowledge on what influences the allocation of these antimicrobial products to the eggs and it is known that antimicrobial proteins and immunoglobulins can be distributed differentially within and between egg clutches (Blount et al., 2002; Saino et al., 2002; Hargitai et al., 2006; D'Alba et al., 2010), although this is not always the case and depends on the antimicrobial protein tested (Shawkey et al, 2008; D'Alba et al., 2010), I might have missed, over- or underestimated the correlation between the transfer of antibacterial products and the species specificity.

Interestingly, despite the higher bacterial load on the eggshells of great tits, the relative composition of some important phyla (*Firmicutes* and *Bacteroidetes*), the *Enterobacteriaceae* family and the genus *Lactobacillus*, on the eggshell microbiota was similar for blue and great tit eggs (**Chapter II**). Nevertheless, the abundance of only two phyla (*Firmicutes* and *Bacteroidetes* <10%) was assessed, disregarding other phyla that have found to be abundant in the cloacal microbiota of passerine birds (e.g. *Proteobacteria* and *Actinobacteria*) (Teyssier et al., 2017). Since it has been suggested that the eggshell bacteria originate from the female cloaca (Ruiz-de-Castañeda et al., 2011a and b), it would be interesting to compare the eggshell microbiota to the cloacal microbiota of the parental birds. As was observed in other studies, *Firmicutes* were abundant in the cloacal microbiota of passerines (Garcia-Mazcorro et al., 2017; Teyssier et al., 2018) and on the eggshells of the great and blue tits (**Chapter II**). *Bacteroidetes* have been shown to be one of the most important phyla in human microbiota (Arumugam et al., 2011), however they do not seem to be abundant in *Passerine* cloacal microbiota (<1%: Garcia-Mazcorro et al., 2017; Teyssier et al., 2018) or on the eggshells (<10%: **Chapter II**). The bacteria which represent this phylum and the function of this phylum in the avian microbiota is also not well understood (Garcia-Mazcorro et al., 2017). The family of the *Enterobacteriaceae* (Order: *Enterobateriales*; Class: *Gammaproteobacteria*; Phylum: *Proteobacteria*) and the genus *Lactobacillus* (Family: *Lactobacillaceae*; Order:

Lactobacillales; Class: *Bacilli*; Phylum: *Firmicutes*) have been chosen to be further investigated since many avian pathogenic bacteria belong to the former family (e.g. *Escherichia coli, Salmonella* spp., *Y. pseudotuberculosis*) (Pennycott et al., 2002; Cork et al., 1999), and *Lactobacillus* spp. have been shown to decrease the number of pathogenic bacteria (such as *Salmonella*) in the intestines through the stimulation of butyrate-producing bacteria (Higgins et al., 2008; Onrust et al., 2015). Despite the identification and enumeration of these bacterial phyla and families, recent studies have shown that besides the composition of the microbiota, the function of this microbiota is important to understand the impact of environmental effects on host health (Teyssier et al., 2018) and functional genes within the microbiota can be linked to certain host properties (Arumugam et al., 2011).

Impact of forest fragmentation and microbial pressure on avian reproductive success and juvenile health (and interactions)

Fragmentation of suitable habitat can have immediate and long term effects on animal populations (Wilcox and Murphy, 1985; Rolstad, 1991; Saunders et al., 1991; Fischer and Lindenmayer, 2007). The population declines and extinctions of some species are the most obvious consequence from fragmentation, as the result of a reduction in carrying capacity in fragmented habitats. However, effects on faunal and floral biodiversity and density (Saunders et al., 1991; Andrén, 1997), the reproductive success of populations living in these fragments, and the effect of fragmentation on host-pathogen dynamics are under-investigated, despite their important effect on population dynamics (Saunders et al., 1991; McCallum and Dobson, 2002; Keesing et al., 2006; Daszak et al., 2010; Becker et al., 2015).

Most research that investigates the effect of fragmentation on pathogen-prevalence in wild living avian populations has been performed on vector-borne diseases, and mostly in tropical zones (Ogrzewalska et al., 2010; Sehgal, 2010; Laurance et al., 2013). However, through the increasing human encroachment, pathogen pollution related to anthropogenic alterations is increasingly documented (Cunningham et al., 2003; Benskin et al., 2009; Andrés et al., 2013; Liang et al., 2015). For example, spreading of avian pathogens (e.g. enteropathogens, *Mycoplasma gallisepticum, Trichomonas gallinae*) through bird feeders are demonstrated to affect bird health and/or avian populations dynamics (Pennycott et al., 2002; Dhondt et al., 2007; Lawson et al., 2012).

In order to increase the knowledge on the effect of habitat fragmentation on host-pathogen interactions in temperate forests, I have assessed the effect of forest fragmentation on the presence of an important avian pathogen (*Salmonella* Typhimurium) and on the reproductive success in relation to bacterial eggshell load, diversity and immune-allocation to the eggs, in two closely related passerine birds inhabiting various fragmented forests in Flanders, Belgium. Contrary to our expectations, habitat fragmentation did not explain any of the investigated variables (**Chapter I** and **Chapter II**).

Following, I would like to discuss on the possible explanations for the lack of correlation and also on the interactions of the three factors.

- Since I only focused on fragment size, some aspects of the health parameters (SMI) and of the reproductive success (hatching success) and only studied the tits during one breeding season, I could have missed the actual effects. Long term studies, including for example the effect of genetic diversity and density of different trophic levels (e.g. insect prey items versus blue and great tits) (Matthysen et al., 1995; Matthysen et al., 2001; Van de Casteele et al., 2002), predators and stress (e.g. on health and immune-allocation) (Hargitai et al., 2006; Morosinotto et al., 2013), the accessibility of bird feeders (Benskin et al., 2009), presence of (non)infectious agents in the environment, decreased availability of essential micro-elements (Goosem, 2007; Wuyts et al., 2008), could show an effect on the egg- and offspring health, transfer of immunological substances to the eggs and/or reproductive success.

- In Flanders, all the forest-fragments, even the larger ones, suffer from anthropogenic disturbance (Maelfait and Hendrickx, 1997; Bossuyt et al., 1999; De Keersmaeker et al., 2015). As such, the difference between the smallest and largest fragment may not be distinctive enough to demonstrate the difference using the data I have collected. In addition, even with a clear difference in size between the smallest fragment (1,31ha) and the largest fragment (83,77ha), the shape of the fragments could increase the edge-area and thus increase the edge effect and the likelihood of increased contact with anthropogenic waste outside the forest fragments (Saunders et al., 1991; Haddad et al., 2015). It would be interesting to assess the home range of all the investigated tits and to check whether or not they forage exclusively within the forest fragments, or whether they (occasionally) forage outside the forest borders.

- The use of artificial nest may not reflect the natural level of nest predation and infection risks as they are considered to be safer and cleaner.

- Flanders is heavily fragmented and it is a process that has been going on for a long time. It is therefore possible that great and blue tits have been adapted to forest fragments.

- With respect to *Salmonella* Typhimurium, no significant correlations between fragment size and the presence of *Salmonella* on the eggshell nor between the presence of *Salmonella* and the reproductive success were found. Possibly, the prevalence was too low to perform statistical analyses with enough power. As such this should be followed up in order to determine whether or not the pathogen prevalence is affected by fragmentation in Flanders.

Future perspectives

In general, it is difficult to accurately assess the effects of these (host adapted) *Salmonella* Typhimurium strains at the individual level (avian health) and the population level, especially since I could only monitor the reproductive success and the health of the offspring during one breeding season. Furthermore, the potential risk the isolated phage types pose to humans and other animals is currently unknown. Long term studies, covering all the seasons over multiple years, investigating individuals' health, reproductive success and infection status (immunological and bacteriological) from all the age classes, using prevalence and incidence data are needed in order to more accurately assess the avian fitness and population effects in relation to *Salmonella* Typhimurium.

An increased transfer of maternal protective antibacterial proteins (lysozyme) and immunoglobulins (IgY) to the eggs of great tits was observed, which most likely decreased the negative effect of the higher eggshell bacterial load on the eggs (i.e. risk of hatching failure) of great tits. It would be interesting to know how the blue tits manage to have lower bacterial eggshell loads compared to the great tits (with the focus on differences between the life history traits of these species). Do other factors which defend the egg-content, such as eggshell/membrane/cuticle thickness or antibacterial mechanisms within these layers differ between closely related avian species living in the same fragmented habitat? Also the in depth analysis of the bacterial composition on the eggshells, including the investigation of the microbial function would be interesting to assess the impact of eggshell the microbial community on the reproductive success.

Although forest fragmentation did not seem to affect the health of the eggs and offspring or affect the reproductive success of the great and blue tits in our study area, our research was an

exploratory study and is a first step towards the understanding of the effect of forest fragmentation on avian health in temperate zones. The effects of habitat fragmentation are known to be complex and involve many factors. I only accounted for some characteristics of the fragments (fragment area-size), of the health parameters (SMI) and of the reproductive success (hatching success) and only performed a study covering one breeding season. Long term studies, including other factors such as the effect of the local and the surrounding environment (plant and animal diversity and density, intra- and interspecies interactions and the anthropogenic use of these environments), breeding close to or far from the fragment edge, plant and animal diversity in the fragment, access to bird feeders, density and diversity of insect prey items and/or predators, immigration and emigration of individuals, presence of (non)-infectious agents, could all have an effect on the egg- and offspring health and, if logistically possible, should be included. Only by doing this, population effects of fragmentation might be investigated and contribution can be made to improve conservation policies.

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SUMMARY

Human encroachment of natural environments is placing a major pressure on natural ecosystems. Habitat alteration and destruction are some of the most important factors of the increasing (native) species loss across the world. One of these habitat alterations is the fragmentation of landscapes (e.g. forest fragmentation), which has major implications on all the organisms inhabiting these environments. Entire ecosystems can get imbalanced and besides the direct effect of habitat fragmentation (e.g. direct species loss), many indirect effects can affect the continued existence and dynamics of populations and forest communities. For example, the alterations in climatic conditions and floristics, in foraging and nesting habitats, in biodiversity and species density can alter the inter- and intraspecific interactions and increases the stress level of the animals. This in combination with altered host-pathogen interactions can change the disease occurrence and outcome. The understanding of these host-pathogen interactions along a fragmentation gradient is of great importance to unravel the population dynamics in changing environments.

One of these long term effects, which can have devastating effects on population-level, is the effect of habitat fragmentation and host-pathogen dynamics on the reproductive success of animals. Nevertheless, there is a lack of studies trying to understand if and how habitat fragmentation (environmental factor) affect the infection pressure (pathogen effect) and the host health and reproductive effect (host parameter) and if this effect differs between various species.

In this thesis I have investigated the health status, reproductive success and infection pressure of apparently healthy great (*Parus major*) and blue (*Cyanistes caeruleus*) tits in 19 mature deciduous forest fragments of Flanders, Belgium which differ in fragment surface area (**Chapter I and II**). I used great and blue tits as model species, since they are resident birds that inhabit fragmented forests of different sizes and readily breed into nest boxes, which makes it easier to study their reproductive success.

During the first chapter I have focused on a well-known avian pathogen *Salmonella enterica* subspecies *enterica* serovar Typhimurium, which can be the cause of disease outbreaks and/or can have implications on the avian fitness and thus the reproductive success (e.g. increased hatching failure, retarded growth, reduced offspring survival). Therefore, I have sampled eggs for the presence of *Salmonella* Typhimurium, tested the fledglings for the presence of anti-*Salmonella* antibodies and estimated their health status using the scaled mass index, as a proxy for body condition (**Chapter I**).

Using isolation methods and seroprevalence data, a low prevalence (± 7%) of *Salmonella* Typhimurium DT99 and DT193 was detected in great and blue tit populations originating from different forest fragments, suggesting an endemic *Salmonella* presence within a wide distribution range. None of the fledglings, which originated from a nest in which eggs positive for *Salmonella* were found, were positive for anti-*Salmonella* antibodies and no significant association between *Salmonella* Typhimurium presence on the eggshell and fledgling SMI could be detected. As DT99 is usually considered a pigeon adapted *Salmonella* Typhimurium phage type and circulates endemically in pigeon populations in Belgium, these birds could have been the source of infection for our tit species. Since DT193 on the other hand is usually associated with human infections, I have assessed the potential epidemiological relationship between the tit and human DT193 isolates. Multi-locus variable number tandem repeat analysis, using these tit-DT193 isolates in comparison with human isolates, revealed a clear distinction between the human and tit isolates, the latter clustering together. It seems therefore likely that the DT193 isolates in this study represent avian-adapted strains with a limited impact on host health, which allows host-pathogen co-existence and pathogen population maintenance in the tit species. However, despite the lack of observed disease, mortality or reproductive effects in the different tit species, and the potentially low impact on humans, changes in pathogen virulence, in environmental characteristics or in the hosts susceptibility can alter the co-existence towards disease outbreaks that have been described before with *Salmonella* Typhimurium (**Chapter I**).

Besides the pathogenic bacteria, also opportunistic bacteria can affect the reproductive success. Microbial infections of the eggs (during the pre-hatching stage) and of the offspring (during the pre- and post-hatching stage) can lead to hatching failure, retarded growth of nestlings, mortality of the offspring, and thus lead to a reduced reproductive success. The infection risk has previously been linked to the bacterial load and/or the bacterial composition on the eggs, which can be influenced by life history traits of the species. Furthermore, female birds can allocate antibodies, and antibacterial proteins to the egg-content in order to protect the offspring against infections and to increase the overall reproductive success. In chapter II, I have compared great and blue tits and determined whether the bacterial load and microbial diversity, the immune investment and the hatching success differed between the two species. Furthermore, I have tested if the environmental factor “forest fragment size” is a driver for eggshell bacterial loads (**Chapter II**).

The eggshell bacterial load was found to be higher in eggs of great tit nests compared to blue tit nests. Despite this difference, the relative composition of the eggshell microbiota was the same between great and blue tits. The proportion of the family *Enterobacteriaceae*, the genus *Lactobacillus*, and the phyla *Firmicutes* and *Bacteroidetes* were similar for both tit species and are likely transmitted from the cloaca of the adult birds to the eggshells. Although, fragment area, egg volume, laying date and clutch size could not explain this species difference in microbial eggshell load, the use of different nest material (the use of aromatic plants with antimicrobial properties by blue tits) and differences in nest sanitization between the two species could explain the difference in microbial eggshell load (**Chapter II**).

Not only the bacterial pressure differed between the two tit species, also the immune-allocation to the eggs was found to be different between great and blue tits, with great tits significantly transferring higher concentrations of IgY and lysozyme to the egg yolk and albumen respectively. This immune-transfer was intrinsically linked to species of interest and not to the bacterial load as such, which indicates that the great tit embryos from our study are better protected against bacterial infection than the blue tit embryos (**Chapter II**).

The hatching success of great tit eggs, as a measure of reproductive success, declined when the bacterial eggshell load increased, which suggests the negative effect of microbial pressure on hatching success. No such effect was noticed for the blue tit eggs. Nevertheless, despite the exposure of great tit eggs to a higher infection pressure, only a small reduction in hatching success was noticed. This is most likely due to the increased allocation of immune factors to the great tit eggs (**Chapter II**).

Although our studies present interesting results related to potential host adaptation of *Salmonella* Typhimurium DT193 to tit species (**Chapter I**), the different microbial exposure between great and blue tit eggs and the discrepant transfer of maternal immunity to the tit eggs in order to increase the offspring survival (**Chapter II**), future research is necessary to better understand the life history traits driving these discrepancies and the long term population impact of host-adapted *Salmonella* Typhimurium strains in wild living birds.

SAMENVATTING

De mens oefent een steeds grotere druk uit op de natuur en heeft een grote invloed op natuurlijke ecosystemen en biodiversiteit. Veranderingen van de natuurlijke leefomgeving en habitat destructie zorgen voor afname van (inheemse) soortenaantallen of zelf het uitsterven van soorten wereldwijd. Een belangrijke verandering van de natuurlijke leefomgeving is landschapfragmentatie, zoals bosfragmentatie, wat een enorme impact heeft op alle organismen die in deze omgeving te vinden zijn. Het evenwicht in deze ecosystemen verdwijnt en naast de directe effecten van habitatfragmentie (bv. soortenverlies) zijn er tal van indirecte effecten die ook een invloed kunnen uitoefenen op het voortbestaan van populaties. Bijvoorbeeld klimaatveranderingen, veranderingen in flora, nestgelegenheid, foerageergebieden en soortendensiteit en het verlies van biodiversiteit kunnen de inter- en intraspecifieke interacties beïnvloeden en het stressgehalte van een dier verhogen. Dit op zijn beurt kan leiden tot veranderingen in kiem-gastheerinteracties, waardoor de ziektedynamiek beïnvloed kan worden. Om meer inzicht te krijgen in populatiedynamiek in veranderende omgevingen is het dus ook van groot belang om de invloed van habitatfragmentatie op kiem-gastheerinteracties na te gaan.

Eén van de langetermijneffecten die een zware impact kan hebben op populatieniveau is het effect van habitatfragmentatie en kiem-gastheerdynamiek op het reproductief succes van het dier. Tot op vandaag zijn er echter weinig studies die proberen na te gaan of, en hoe, habitatfragmentatie (omgevingsfactor) een invloed heeft op de infectiedruk (pathogeen effect) en de gezondheid van de gastheer (gastheerparameter) en of dit varieert tussen verschillende soorten.

In deze thesis hebben we de gezondheidsstatus, het reproductief succes en de infectiedruk van schijnbaar gezonde koolmezen (*Parus major*) en pimpelmezen (*Cyanistes caeruleus*) onderzocht in 19 mature loofbosfragmenten in Vlaanderen, België, die variëren in fragmentatie **(Hoofdstuk I en II)**. We hebben gekozen voor kool- en pimpelmezen als modelsoort omdat deze vogels vaak in onze gebieden terug te vinden zijn, ze voorkomen in gefragmenteerde bossen van verschillende groottes en ze gemakkelijk broeden in nestkasten, wat het voor ons gemakkelijker maakt om hun reproductief succes op te volgen.

In het eerst hoofdstuk van deze thesis hebben we ons gefocust op een gekend vogelpathogeen, namelijk *Salmonella enterica* subspecies *enterica* serovar Typhimurium, wat de oorzaak kan zijn van ziekte-uitbraken en/of een invloed kan hebben op de gezondheid van

het dier en ook het reproductief succes (bv. een verhoogde uitval van eieren, vertraagde groei en een daling in de overleving van nestjongen). Om de aanwezigheid en het effect van *Salmonella* Typhimurium na te gaan, werden eieren van kool-en pimpelmezen gesampled, werd het bloed van juveniele mezen onderzocht op de aanwezigheid van anti-*Salmonella* antilichamen en werd de "scaled mass index" bepaald als indicator voor de gezondheidsstatus van de dieren **(Hoofdstuk I)**.

Salmonella Typhimurium DT99 en DT193 werden met een lage prevalentie (± 7%) gedetecteerd in nesten van kool-en pimpelmezen afkomstig van verschillende bosfragmenten. Deze gegevens suggereren een endemische aanwezigheid van *Salmonella* en een groot verspreidingsgebied. De juveniele dieren waar anti-*Salmonella* antilichamen gedetecteerd werden, waren niet afkomstig van een nest waar *Salmonella* op de eischaal gedetecteerd werd. Daarenboven werd er geen significante associatie waargenomen tussen de aanwezigheid van *Salmonella* op de eischaal en de scaled mass index van de juveniele vogels. *Salmonella* Typhimurium faagtype DT99 wordt beschreven als een duif-geadapteerd faagtype en dit circuleert endemisch binnen duivenpopulaties in België. Het is dus ook hoogstwaarschijnlijk dat duiven een bron van infectie geweest zijn voor de kool-en pimpelmezen. *Salmonella* Typhimurium faagtype DT193 is een faagtype dat vaak geassocieerd wordt met humane infecties. Hierdoor hebben we de potentiële epidemiologische relatie tussen humane DT193 en onze mees isolaten onderzocht via "multi-locus variable number tandem repeat" analyse. Deze analyse toonde aan dat er een duidelijk verschil is tussen de humane isolaten en de mees isolaten, die duidelijk samen groeperen. Alles wijst er dus op dat de DT193 isolaten die in deze studie geïsoleerd werden, vogel-geadapteerde stammen zijn die geen risico vormen voor de mens, en die slechts een minimale impact hebben op de gezondheid van de kool- en pimpelmezen. Er ontstaat een kiem-gastheer co- existentie. Ondanks de lage impact op de mens en het gebrek aan duidelijke ziektebeelden, sterfte en een effect op het reproductief succes, kunnen veranderingen in pathogeniciteit, omgevingsfactoren of gastheergevoeligheid deze co-existentie beïnvloeden richting ziekte-uitbraken die in het verleden reeds beschreven zijn voor *Salmonella* Typhimurium (**Hoofdstuk I**).

Naast pathogene bacteriën, zoals *Salmonella*, kunnen ook opportunistische bacteriën een effect hebben op het reproductief succes van vogels. Infecties van de eieren (tijdens het uitbroeden) of van nestjongen (tijdens het uitbroeden en na het uitkomen van het ei) kunnen leiden tot uitval van eieren, vertraagde groei van de nestjongen, sterfte, en kunnen dus een effect hebben op de voortplanting van vogels. Het risico op infectie werd in het verleden gelinkt

aan de hoeveelheid bacteriën en/of de samenstelling van bacteriën aanwezig op eieren, wat beïnvloed kan worden door de levenswijze van de vogelsoort. Om de kans op infectie te reduceren, kunnen vrouwelijke vogels antilichamen en antibacteriële eiwitten transloceren naar de ei-inhoud. In **hoofdstuk II** hebben we de bacteriële hoeveelheid en samenstelling bepaald van eieren van kool-en pimpelmezen. Daarenboven werd nagegaan of de hoeveelheid antilichamen en antibacteriële eiwitten in de eieren en het broedsucces verschillen tussen kool- en pimpelmezen. Tevens werd nagegaan of bosfragmentatie leidt tot hogere bacteriële aantallen.

De hoeveelheid bacteriën aanwezig op de eischaal bleek significant hoger te zijn in koolmezen t.o.v. pimpelmezen. Ondanks het verschil in hoeveelheid, bleek de relatieve compositie van de eischaal microbiota gelijk te zijn tussen deze twee vogelsoorten. De proportie van de *Enterobacteriaceae* familie, het genus *Lactobacillus*, en de phyla *Firmicutes* en *Bacteroidetes* was gelijk en de bacteriën die hiertoe behoren worden heel waarschijnlijk getransloceerd vanuit de cloaca van de volwassen vogel naar de eischaal. Het verschil in de hoeveelheid bacteriën kon niet verklaard worden door bosfragmentatie, ei volume, legdatum en nestgrootte. Het gebruik van verschillend nestmateriaal (aromatische planten met antimicrobiële eigenschappen bij pimpelmezen) en een verschillend schoonmaakgedrag kunnen (deels en suggestief) het verschil in microbiële lading van het ei verklaren **(Hoofdstuk II)**.

Niet alleen de bacteriële druk, maar ook de verdeling van immuunfactoren verschilde tussen de twee vogelsoorten. Koolmezen transloceren significant meer IgY en lysozyme naar de eidooier en het eiwit, respectievelijk. Deze immuunoverdracht bleek intrinsiek gelinkt aan de vogelsoort, wat erop wijst dat embryo's van koolmezen beter beschermd zijn dan deze van pimpelmezen **(Hoofdstuk II)**.

Het broedsucces van koolmezen, als een indicator voor reproductief succes, daalde wanneer de microbiële lading op de eischaal groter werd. Dit suggereert een negatief effect van de microbiële druk op het broedsucces. Dit werd niet waargenomen bij pimpelmezen. De daling in het broedsucces bleek echter minimaal te zijn, mogelijks doordat de eieren van koolmezen sterker beschermd zijn door immuunfactoren **(Hoofdstuk II)**.

Samengevat tonen deze studies aan dat *Salmonella* Typhimurium DT193 potentieel gastheer-geadapteerd is voor mezensoorten **(Hoofdstuk I)** en dat ondanks het feit dat kool- en pimpelmezen heel erg gelijkend zijn op elkaar, er toch verschillen zijn in pathogeen druk. De

microbiële lading op eieren van koolmezen is hoger, maar ze zijn meer beschermd met IgY en lysozyme, waardoor er slechts een minimaal effect op het reproductief succes wordt waargenomen **(Hoofdstuk II)**. Er is echter verder onderzoek nodig om meer inzicht te krijgen in de kenmerken (levenswijze) die deze verschillen veroorzaken en om de langetermijnimpact van gastheer-geadapteerde *Salmonella* Typhimurium stammen te bepalen op populaties van wilde vogels.

CURRICULUM VITAE

Roschong Boonyarittichaikij was born on 28 January 1980, in Chiang Mai, Thailand. She studied at Regina Coeli School and then The Prince Royal's College for her primary and secondary education. From 1998, she studied Veterinary Medicine at Chiang Mai University, and obtained a doctor of Veterinary Medicine degree in 2004.

After graduation, she worked as a lecturer and a staff of The Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University, Nakorn pathom, Thailand.

During 2009-2010, she studied a master of science in Veterinary Epidemiology and Economics at the faculty of Veterinary Medicine, Utrecht University, The Netherlands, under the supervision of Dr. Monique Paris.

In August 2014, she started this doctoral study at the Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Belgium. The doctoral research was funded by a Thai Government Scholarship (Higher Educational Strategic Scholarships for Frontier Research Network). Under the guidance of Prof. An Martel, Prof. Luc Lens and Dr. Elin Verbrugghe, she performed 4 years of research on health and reproductive success of blue and great tits in forest fragments of Flanders. The project is under the UGent GOA project Scaling up Functional Biodiversity Research: from Individuals to Landscapes and Back (TREEWEB). Her research resulted in several scientific publications in international journals and several presentations at academic conferences.

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3. Oral presentations at (inter)national conferences

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