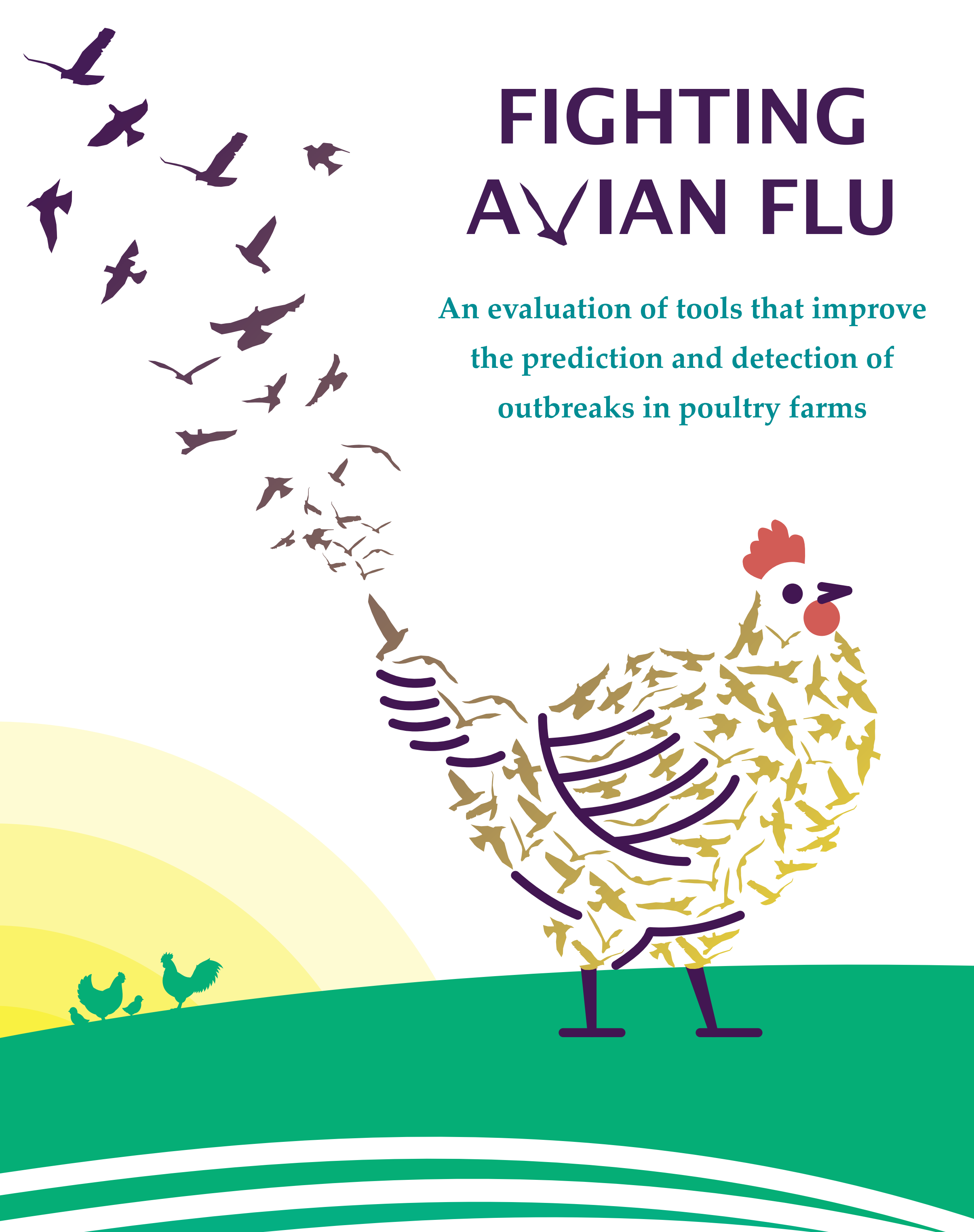
FIGHTING AVIAN FLU

An evaluation of tools that improve the prediction and detection of outbreaks in poultry farms



Janneke Schreuder

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Janneke Schreuder
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FIGHTING AVIAN FLU

An evaluation of tools that improve the prediction and detection of outbreaks in poultry farms

Bestrijding van vogelgriep
Een evaluatie van methoden om het voorspellen en opsporen van uitbraken op pluimveebedrijven te verbeteren
(met een samenvatting in het Nederlands)

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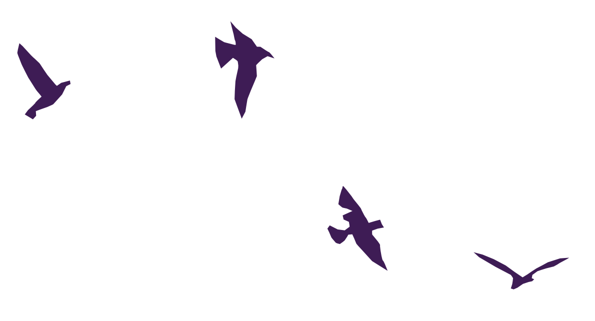
1

General introduction



In the current society, humans heavily rely on livestock production as a source for food, and other products. To feed the seven billion people on this planet, the livestock industry has evolved through the years and has become more intensified to increase efficiency and production. Smaller farms were up-scaled to larger farms with higher feed efficiency and increased production (Thornton, 2010; Guyomard et al., 2013). In recent years, especially in developed countries, another trend is observed, where the growing consumer concern for animal welfare has led to an increase in free-range and organic farming, in which the animals have more space and spend part of their day outside (Thornton, 2010). While the access to an outdoor range is believed to be beneficial for the animal's health (Bestman and Wagenaar, 2003), contact with an outdoor environment increases the likelihood for direct or indirect exposure to wild animals, thus increasing the risk of transmission of infections along the wildlife – livestock interface (Gortázar et al., 2007).

Chicken meat and eggs are an efficient and affordable source of high quality protein and other nutrients, that can help feed many millions of people worldwide (Scanes, 2007). Feed efficiency and high performance of the birds are therefore crucial goals in poultry production. Currently one of the major concerns for the poultry industry are the global epidemics of highly pathogenic avian influenza. Widespread outbreaks among poultry in the last two decades have caused enormous economic losses and are a concern for the poultry's welfare and the poultry industry in general (Figueroa et al., 2021).

Avian influenza viruses (AIV) are categorized as low pathogenic avian influenza viruses (LPAIV) or highly pathogenic avian influenza viruses (HPAIV), based on the pathobiological effects of the virus in chickens: in general LPAIV infections may be asymptomatic or may produce only mild disease in chickens (Gonzales and Elbers, 2018), while HPAIV infections produce high morbidity and mortality in poultry (Pantin-Jackwood and Swayne, 2009; Beerens et al., 2020; Schreuder et al., 2020). Influenza viruses carry two glycoproteins on their surface: haemagglutinin (HA) and neuraminidase (NA), and on the basis of these glycoproteins are

divided into subtypes (Webster et al., 1992). Among the known HA subtypes affecting birds (H1–H16), H5 and H7 virus subtypes can be either LPAIV or HPAIV and infections with HPAIV are notifiable to the World Organization for Animal Health (OIE). Moreover, as HPAIVs have the potential to cause zoonotic infections and to acquire human-to-human transmissibility, the recurrent outbreaks of HPAIVs across the globe are also a concern for public health (Pohlmann et al., 2019; Chen et al., 2020).

Over the past two decades, several outbreaks occurred in the Netherlands. The largest HPAI epidemic in 2003 most likely originated from an LPAIV infection, followed by massive between-farm transmission in a poultry-dense areas (Elbers et al., 2006). In more recent outbreaks, between 2014-2018 and currently in the autumn-winter period of 2020-2021, separate introductions with HPAIVs of clade 2.3.4.4 occurred on poultry farms, which were in most cases related to indirect contact with HPAIV infected wild birds (Beerens et al., 2019; Adlhoch et al., 2020). It is known that free-range layer farms have a higher risk of introduction of LPAIV and HPAIV compared to indoor layer farms (Terregino et al., 2007; Gonzales et al., 2013; Kirunda et al., 2014; Bouwstra et al., 2017), and most LPAIV introductions in the Netherlands occur on free-range layer farms (Gonzales et al., 2013; Bouwstra et al., 2017). Aquatic birds, especially wild waterfowl, are the natural reservoir of AIV (Webster et al., 1992; Hill et al., 2019), and infected waterbirds excrete high amounts of AIVs in their feces, infecting other species via the fecal-oral route (França et al., 2012). Although wild birds may not frequently visit the outdoor range during the day, and direct contact with the chickens is limited, known carriers of AIV, i.e. several species of gulls and dabbling ducks, do visit the outdoor range regularly during the night or between sunrise and the time the chickens enter the range (Elbers and Gonzales, 2020). As infectious agents like AIVs can survive in the environment for a long time under favourable conditions (Brown et al., 2007), indirect transmission via 'same-place different-time' spatial coincidences becomes possible (Richardson and Gorochowski, 2015). This means that although the same environment, like the outdoor range, is visited at different times, AIVs shed by an infected wild bird, can still result in an infection of chickens when

they pass the range at a different time. Transmission of AIV to poultry farms therefore likely occurs via (in)direct contact of poultry with infected birds or via an AIV contaminated environmental virus reservoir, such as water, soil, vectors or fomites (Breban et al., 2009; Rohani et al., 2009; Velkers et al., 2017; Elbers and Gonzales, 2020).

In the Netherlands, an housing order is often issued after the first cases of HPAI infections in wild birds have been detected. Also, poultry farmers are urged to follow strict biosecurity protocols to reduce the risk of incursion of the virus in the poultry houses, and subsequent spread to other farms. Although these measures have most likely contributed to minimizing between farm transmission, primary introductions of HPAIVs still regularly occur on poultry farms. The recurrence of HPAI outbreaks and their economic and social impact underline the need for improved control strategies. Therefore, government, industry and knowledge institutes are collaborating to improve current and develop new control strategies to combat HPAI outbreaks.

An approach that has been proposed to improve current control strategies is to improve the prediction of HPAI outbreak risk across the Netherlands to be able to take appropriate measures in these specific areas. A better prediction of the HPAI risk could be used to support timely decisions on a preventive housing order and increased biosecurity measures in specific areas. Also, it could facilitate prioritization of areas for increased surveillance for AIV infection and the identification of unfavourable areas to start new poultry farms. As wild birds play a key role in the transmission of AIV to farms, we looked into several possible strategies to identify and predict the risk for exposure of chickens to infections from wild waterbirds.

The microbiome as potential proxy for contact between wild birds and poultry

Microbiome research is a rapidly expanding field, and increasing knowledge of the relationship between microbiomes and host health has grown over the past two decades. Gut microbiota are influenced by their direct and indirect environment (Kers et al., 2018, 2019; Hubert et al., 2019). The direct

environment is shaped by interactions among gut-residing microbiota, interactions with their host, and the feed of the host. The indirect environment is mostly determined by the living conditions of the host, which in the case of poultry includes the environment of the poultry house and outdoor range, biosecurity level, litter and climatic conditions. Social interactions among hosts play a role in transmitting pathogens and parasites between individuals in a contaminated environment (Rohani et al., 2009; Richardson and Gorochowski, 2015; Woodroffe et al., 2016; Colenutt et al., 2020), and these social interactions can also alter and influence the composition of commensal microbiota in several species (VanderWaal et al., 2014; Archie and Tung, 2015; Tung et al., 2015; Antwis et al., 2018). Furthermore, spatial proximity between individuals has been shown to facilitate exchange of microbiota even when direct social interactions are minimal (Antwis et al., 2018). These phenomena may also be relevant in relation to exchange of potential pathogens in the wildlife – livestock interface, such as AIV. Wild waterbirds may visit the outdoor range of layers during the night (Elbers and Gonzales, 2020), which means that although wild birds and chickens are not likely to have direct contact, they do share an environment, and can therefore exchange microbiota with one another via the fecal-oral route, according to the 'same-place, different-time' principle as described in the previous section. As the role of waterfowl in the transmission of infectious agents to poultry is linked to fecal contamination (Swayne and Pantin-Jackwood, 2006), studying transmission of fecal microbiota between waterfowl and chickens may reveal proxies for contact between them. Previously, the genetic subtypes of gut residing *Escherichia coli* served as a proxy for contact between different giraffes (*Giraffa camelopardalis*) and different wild primates (VanderWaal et al., 2014; Springer et al., 2016), as well as for pathogen transmission between mountain brushtail possums (*Trichosurus vulpecula*) (Blyton et al., 2014). Similarly, the fecal microbiota of chickens may be affected by the presence of waterfowl, and thus, the chicken's fecal microbiome could be useful to assess whether contact with waterfowl feces has occurred. It was hypothesized that if changes in the fecal microbiome can be determined in chickens, then this may serve as a proxy

for the risk of pathogen transmission from the environment, e.g. AIV, prior to actual outbreaks, and may be used for risk assessment purposes.

Spatial modeling of wild bird densities

As described above, it is known that wild waterbirds play an important role in dissemination of HPAIVs across the globe (Verhagen et al., 2015; The Global Consortium for H5N8 and Related Influenza Viruses, 2016), as well as introduction of HPAIV on poultry farms (Beerens et al., 2019). Several wild bird species have been identified as high risk HPAI bird species, of which most belong to the order of Anseriformes (mainly duck, geese, swans) and Charadriiformes (gulls, turns, shorebirds) (Animal and Plant Health Agency (UK) et al., 2017; Hill et al., 2019). Research has shown that HPAI outbreaks on poultry farms are spatially associated with the proximity of waterbodies or the presence of wild birds (Belkhiria et al., 2018; Napp et al., 2018; Velkers et al., 2020). The density of HPAI high-risk bird species around infected poultry farms in wetlands was significantly higher than around non-infected farms in non-water-rich areas in the Netherlands (Velkers et al., 2020), and wild bird densities have been used previously to quantify risk of HPAIV introduction on poultry farms in Great-Britain (Hill et al., 2019). Furthermore, disease distribution models also showed that land cover, particularly the presence of wetlands, were highly predictive for the HPAI risk in California (Belkhiria et al., 2018), and land cover and environmental variables were used to successfully predict outbreak risk for H5N1 in Europe (Si et al., 2013). This suggests that wild bird presence and abundance, as well as land cover data could be used as predictors in identifying HPAI high risk areas, which could help in prioritization of areas for surveillance and biosecurity measures.

Increased knowledge on how to predict introduction of HPAIV into poultry farms is important and will likely reduce HPAIV introduction risk, it is however unlikely that this will completely prevent all primary introductions, and subsequent between farm transmissions between poultry farms. Therefore, it remains important to detect infection as early as possible to control the spread of HPAIV to other farms and minimize

socio-economic impact of the disease (Elbers et al., 2004; Backer et al., 2015). For HPAI outbreaks, sudden increase in mortality has prompted farmers and veterinarians to raise suspicion of infection (Elbers et al., 2007; Malladi et al., 2011; Gonzales and Elbers, 2018; Ssematimba et al., 2019), as has the sudden onset of clinical signs (Elbers et al., 2004, 2005; Velkers et al., 2006) in different poultry types. However, it is yet unknown whether combining mortality rate and clinical signs can further enhance early detection in different poultry types.

THESIS AIMS AND OUTLINE

The main aim of the research described in this thesis is to evaluate tools that can be used to predict and detect HPAI outbreaks on poultry farms in order to reduce risks of HPAIV introductions and prevent further spread to other farms via appropriate control measures. The first part of the thesis explores if exposure of layers to an outdoor environment results in detectable changes in the gut microbiota community, and if these changes might be used as a proxy for contact of layers with wild birds. The second part of the thesis focuses on the identification of HPAI high risk areas using wild bird density data and the timely diagnosis of possible HPAI outbreaks on poultry farms. Both are important steps to reduce the risk of HPAIV introductions on farms on the one hand and to control the spread of HPAIV to other farms, in order to reduce the impact on animal welfare, public health and poultry production. The three main research questions that are addressed are:

1. Can the gut microbiome of layers be used as a proxy for contact with wild birds or the outdoor environment?
2. Can wild bird density data and land cover variables be used to predict HPAI outbreak risk?
3. What are the clinical signs and mortality rates at the onset of HPAI infection and can they be used for early diagnosis of HPAI infection on poultry farms?

The first Chapters of this thesis address whether exposure to an outdoor environment results in detectable changes in the microbiota community in

laying chickens, and whether these changes might be used as an indicator for indirect contact of layers with wild birds. As layers use the outdoor range, they are exposed to an environment that might be contaminated with fecal droppings of HPAIV-infected wild birds that visited or passed the range, and the gut microbiome of layers might hold clues that relate to this exposure and the level of biosecurity on the farm. This was investigated in three steps. In Chapter 2, as a proof of principle, we orally inoculated laying hens with wild duck feces to determine if the microbiota in the feces could be transferred to laying hens via oral intake. In Chapter 3, we performed an observational field study, and studied the differences in the cloacal microbiota between indoor- and outdoor-housed layers. Layers from four indoor- and four outdoor flocks were sampled and cloacal community composition was compared to determine if there are differences between these two types of flocks in a commercial setting that can be detected with 16S rRNA sequencing. In Chapter 4, we performed a longitudinal study over a period of 16 weeks in four commercial layer flocks. Here, two flocks were given access to an outdoor range and two flocks remained indoors. The community dynamics of the cloacal microbiota of the flocks were studied after they were exposed to this new environment to determine if community dynamics between both groups differed over time.

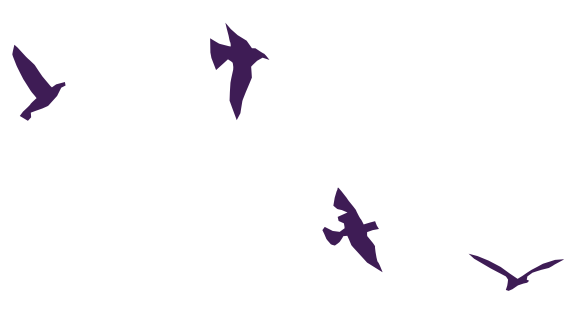
The second part of the thesis focuses on identification of HPAI high risk areas and the timely diagnosis of possible HPAI outbreaks. In Chapter 5 the locations of HPAI outbreaks in the Netherlands between 2014-2018 were used to spatially model the HPAI infection probability in relation to landscape variables and wild bird densities of high risk HPAI bird species. This model was then used to generate a risk map for HPAI infection probability across the Netherlands. In addition to mapping the risk, early warning of an outbreak is important to control the spread of HPAIV to other farms and reduce the impact on animal welfare, public health and poultry production. In Chapter 6, we give a detailed description of species-specific clinical signs in the early stages of HPAI infection on poultry farms in the Netherlands between 2014-2018 and describe how these developed over time in the days before notification, as observed by poultry farmers and

veterinarians. For each outbreak we compared the onset of observed clinical signs, with the onset of increased mortality (as calculated via a mortality ratio), and describe how they facilitate early diagnosis of HPAIV infections.

Finally, Chapter 7 concludes with a general discussion of the results in this thesis in a broader context and additional analyses of ten new HPAI outbreaks on poultry farms in the Netherlands which occurred in the autumn and winter months of 2020-2021.

2

Limited changes in the fecal microbiome composition of laying hens after oral inoculation with wild duck feces



Janneke Schreuder, Francisca C. Velkers, Ruth J. Bouwstra, Nancy Beerens, J. Arjan Stegeman, Willem F. de Boer, Armin R. W. Elbers, Pim van Hooft, Anneke Feberwee, Alex Bossers, and Stephanie D. Jurburg

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ABSTRACT

Interspecies transmission of fecal microbiota can serve as an indicator for (indirect) contact between domestic and wild animals to assess risks of pathogen transmission, e.g., avian influenza. Here, we investigated whether oral inoculation of laying hens with feces of wild ducks (mallards, *Anas platyrhynchos*) resulted in a hen fecal microbiome that was detectably altered on community parameters or relative abundances of individual genera. To distinguish between effects of the duck inoculum and effects of the inoculation procedure, we compared the fecal microbiomes of adult laying hens resulting from 3 treatments: inoculation with wild duck feces (duck), inoculation with chicken feces (auto), and a negative control group with no treatment. We collected cloacal swabs from 7 hens per treatment before (day 0), and 2 and 7 D after inoculation, and performed 16S rRNA amplicon sequencing. No distinguishable effect of inoculation with duck feces on microbiome community (alpha and beta diversity) was found compared to auto or control treatments. At the individual taxonomic level, the relative abundance of the genus *Alistipes* (phylum *Bacteroidetes*) was significantly higher in the inoculated treatments (auto and duck) compared to the control 2 D after inoculation. Seven days after inoculation, the relative abundance of *Alistipes* had increased in the control and no effect was found anymore across treatments. These effects might be explained by the perturbation of the hen's microbiome caused by the inoculation procedure itself, or by intrinsic temporal variation in the hen's microbiome. This experiment shows that a single inoculation of fecal microbiota from duck feces to laying hens did not cause a measurable alteration of the gut microbiome community. Furthermore, the temporary change in relative abundance for *Alistipes* could not be attributed to the duck feces inoculation. These outcomes suggest that the fecal microbiome of adult laying hens may not be a useful indicator for detection of single oral exposure to wild duck feces.

Keywords: laying hen, fecal microbiota, wild duck, 16S rRNA gene sequencing, inoculation

INTRODUCTION

Contact between wildlife and domestic animals can lead to transmission of pathogens, as wildlife can serve as a reservoir host (Gortázar et al., 2007). The interaction between wild birds and poultry has become more important in recent years, because of the increased demand for free-range poultry products whereby outdoor access for poultry increases the risk to pathogen exposure originating from wild birds (Koch and Elbers, 2006). It is therefore important to have alternative methods available to study transmission of infectious agents between wild birds and poultry to facilitate risk assessment and develop preventive measures to reduce potential risks for transmission of infectious diseases.

The most striking example of potential risks associated with the wild bird-poultry interface is avian influenza virus (AIV) outbreaks in poultry farms. Wild migratory birds play an important role in the spread of both low pathogenic AIV and highly pathogenic AIV across continents (Lycett et al., 2016). The close genetic relationship between AIV in waterfowl and domestic poultry in several outbreaks supports the role of wild waterfowl in outbreaks (Munster et al., 2005; Berhane et al., 2009; Lebarbenchon and Stallknecht, 2011; Beerens et al., 2018).

In waterfowl, low pathogenic AIV is most often detected in mallards (*Anas platyrhynchos*) (Verhagen et al., 2017). Moreover, video-camera monitoring at a Dutch poultry farm showed that mallards were frequent occupants of the outdoor range at night between November and March (Elbers, 2017). As AIV is shed in high concentrations in feces of infected birds (França et al., 2012), infected waterfowl in the vicinity of outdoor ranges can contaminate the farm environment. Depending on environmental conditions, the virus may persist in the environment for many months (Brown et al., 2007; Stallknecht and Brown, 2017). Chickens can become infected directly via coprophagic behavior (Hyun and Sakaguchi, 1989; von Waldburg-Zeil et al., 2019), or indirectly via contact with an environmental virus reservoir (Brown et al., 2007; Rohani et al., 2009).

As the role of waterfowl in the transmission of infectious agents to poultry is mainly linked to fecal contamination (Swayne and Pantin-

Jackwood, 2006), studying transmission of fecal microbiota between waterfowl and chickens may reveal proxies for contact between them. Previously, the genetic subtypes of gut-residing *Escherichia coli* served as a proxy for contact between giraffes and wild primates (VanderWaal et al., 2014; Springer et al., 2016) or for pathogen transmission between individuals (Blyton et al., 2014). Song et al. (2013) showed that humans in the same household shared fecal microbiota. If dogs were present in the household, humans also shared certain skin microbiota with the dogs. In wild baboons, social group membership and social network relationships predicted the taxonomic structure of the gut microbiome, and rates of social interaction directly explained variation in the gut microbiome (Tung et al., 2015). Similarly, the fecal microbiota of chickens may be affected by the presence of waterfowl. Thus, the chicken's fecal microbiome may be used to assess whether contact with waterfowl feces has occurred. If changes in the fecal microbiome can be determined in chickens, then this may serve as a proxy for the risk of pathogen transmission, e.g., AIV, prior to actual outbreaks, and can be used for risk assessment purposes.

In this study, we investigated the transmissibility of fecal microbiota from wild mallard feces (further referred to as duck) to antibiotic-free recipient laying hens in the week following an oral inoculation with these duck feces. In medicine, fecal microbiota transplants (FMT) are applied to human subjects as a treatment for gut dysbiosis, for instance in patients with *Clostridium difficile* infection (Hamilton et al., 2012; Cammarota et al., 2017). Although a treatment with antibiotics is often applied before FMT, Li et al. (2016) showed that FMT could also be successful in antibiotic-free patients with metabolic syndrome. We hypothesized that duck fecal microbiota can be transmitted to laying hens via oral inoculation with duck feces, causing detectable shifts in the fecal microbiome composition of laying hens by altering the whole microbial community or the relative abundance of specific bacterial taxa. To distinguish between the effects of the feces inoculation process and the specific effects of the duck feces inoculation, we compared the fecal microbiomes resulting from duck feces inoculation to those resulting from an inoculation with chicken feces (auto inoculation) and a negative control group. In particular, we expected to detect novel taxa

in the hen feces after inoculation which were present in the duck inoculum, or altered dominance patterns resulting in an altered community in the hen microbiome after inoculation.

MATERIALS AND METHODS

Ethics

The study protocol was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee of Wageningen University and Research, the Netherlands. The animal experiments were executed at the Dutch Animal Health Service (GD Deventer, the Netherlands) and were done in full compliance with all relevant legislation. The capture of free-living birds was approved by the Dutch Ministry of Economic Affairs based on the Flora and Fauna Act (permit number FF/75A/2014/054).

Hens, Management, and Experimental Design

A total of 54 Bovans Brown laying hens of 19 weeks of age were obtained from a commercial pullet-rearing farm and transported to the experimental facility. Upon arrival, the hens were placed in a 3-tiered aviary system. The tiers were divided by plastic partitions, and cages on the same tier were separated by wire fences, with wood shavings covering the ground. The hens had a habituation period of 12 weeks prior to the start of the experiment and were subjected to a standard light regime for laying hens. A commercial layer feed (ABZ Diervoeding, Nijkerk) without antibiotics and water was supplied ad libitum. The animals were observed daily and the presence of clinical signs or abnormal behavior, and mortality was recorded.

To study the transmission of fecal microbiota from wild ducks to laying hens, we subjected the hens to one of 3 treatments: a single oral inoculation with an inoculum made from wild duck feces (duck treatment); a single oral inoculation with an inoculum made from the feces of the recipient laying hens (auto treatment); and a negative control without any treatment (control treatment). Each treatment group consisted of 18 laying hens.

Inoculum preparation, inoculation, and sample collection

Fresh fecal droppings of mallards were collected opportunistically during avian influenza surveillance activities in wild birds in the Netherlands, as routinely performed by Erasmus Medical Center (Rotterdam, the Netherlands). Fecal droppings of a maximum of 3 wild ducks were pooled (a batch). In total, 39 batches with fecal droppings from 104 wild ducks were collected over 2 sampling days. The fecal batches were immediately stored on ice, and processed on the day of collection. The batches were processed separately to prevent cross contamination between batches with AIV. Prior to further processing, all batches were tested by PCR on AIV (Bouwstra et al., 2015) and *Salmonella* (Halatsi et al., 2006) at GD Deventer (the Netherlands). Batches which tested positive for AIV or *Salmonella* (18 batches in total) were excluded from further processing to prevent introduction of these pathogens into the experimental facilities. Fresh fecal droppings of all chickens in the research facilities were collected and processed as a single pool.

The pool of chicken feces and the duck fecal batches were prepared according to the protocol described by Youngster et al. (2014) with slight modifications: batches with duck fecal droppings were diluted 1:1 and pooled chicken fecal droppings were diluted 1:2 with sterile PBS (DPBS, Gibco, ThermoFisher Scientific, the Netherlands). The mixtures were thoroughly homogenized, and large particles were removed by passing through a sterile 0.7 mm sieve. Of these fecal suspensions, 5 samples of the chicken inoculum and 5 samples of each duck batch were stored in −80°C for DNA extraction and 16S rRNA gene sequencing at a later stage. The suspension was centrifuged at 3,000 rpm for 30 min, and the obtained pellet was suspended in sterile PBS with 20% glycerol (BioXtra >99% GC, Sigma Aldrich, the Netherlands). The final fecal concentration in all inocula was approximately 1 g of pooled feces in 1 mL of PBS + 20% glycerol. Bacterial viability was checked for all inocula by quantifying colony-forming units using blood agar plates (Supplemental Table S1). All inocula were stored at −80°C and were thawed at 4°C for 12 h prior to further processing at day of inoculation.

At the day of inoculation (day 0), 10 batches with fecal duck inocula

were combined and homogenized to form 1 duck inoculum. Subsequently, the chickens (31 weeks of age) were inoculated with 6 mL of either the duck or auto inoculum via oral gavage. The negative control group remained untreated. Two cloacal swabs per hen were collected daily for all chickens from day 0 (prior to inoculation) until the end of the experiment at day 13. Cloacal swabs were stored on ice upon collection and stored at −80°C within 2 h. On day 13, all chickens were euthanized by intravenous injection with a 20% pentobarbital-sodium solution.

DNA extraction and 16S rRNA gene amplicon sequencing

Per treatment, 7 chickens, from a total of 18 chickens, were selected for further analysis of samples taken on a subset of timepoints, i.e., 0, 2, and 7 D after inoculation. Cloacal swabs of 7 chickens were selected and visually assessed to ensure that sufficient fecal material for DNA extraction was available on the swabs on all 3 selected timepoints. Based on microbiota studies in laying hens and broilers, a sample size of 7 cloacal swabs per treatment group was expected to be large enough to detect differences in microbiota composition with sufficient statistical power (Videnska et al., 2014b; Jurburg et al., 2019). Day 0 was chosen as a reference baseline, and we expected to measure the first shift in the fecal microbiome 2 D after inoculation. The last timepoint chosen for analyses was day 7 after inoculation was included to determine if shifts in the fecal microbiota composition found on day 2 were still detectable. For each time × chicken combination, the duplicate swab samples were used for DNA extraction to ensure sufficient DNA was obtained for sequencing. Five of the duck and chicken fecal suspensions (Inoduck and Inochicken respectively), which were stored at −80°C during inoculum preparation, were used for DNA isolation. Swabs were thawed at room temperature, diluted in 1 mL of sterile PBS, and vortexed for 15 s. DNA was extracted from 200 μL of these diluted fecal suspension or cloacal swab samples using the Qiagen QIAamp Fast DNA stool mini kit (Qiagen, Hilden, Germany) and processed according to the manufacturer's instructions, with an additional bead-beating step. DNA extracts were quantified with Invitrogen Qubit 3.0 Fluorometer and stored at −20°C for further processing. DNA from duplicate

swab samples was pooled after extraction.

The V3–4 region of the 16S rRNA gene was amplified in a PCR with the primers CVI_V3-forw CCTACGGGAGGCAGCAG and CVI_V4-rev GGACTACHVGGGTWTCT. The following amplification conditions were used: step 1: 98°C for 2 min, step 2: 98°C for 10 s, step 3: 55°C for 30 s, and step 4: 72°C for 10 s, step 5: 72°C for 7 min. Steps 2 to 4 were repeated 25 times. PCR products were checked with gel electrophoresis, and PE300 sequencing was performed using a MiSeq sequencer (Illumina Inc., San Diego, CA).

Processing of sequencing data

All sequence processing and statistical analyses were performed in R 3.5.1 (R Core Team, 2013). The sequenced reads were filtered, trimmed, dereplicated, chimera-checked, and merged using the dada2 package (Callahan et al., 2016) using standard parameters (TruncLength = 240, 210), and reads were assigned with the SILVA v.132 classifier (Quast et al., 2012). Downstream analyses were performed with the phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen et al., 2007) R packages. Good's coverage was >0.999. Prior to all analyses, the data were rarefied to 2,658 reads per sample (rarefy_even_depth, seed = 1), to standardize the number of reads while preserving all samples. The final dataset contained 1,193 amplicon sequence variants.

Statistical analysis

The number of amplicon sequence variants per sample was used as a measure of observed richness (alpha diversity). To test for effects of inoculation on richness, Kruskal–Wallis tests were performed per time x treatment. To evaluate whether the duck inoculation had an effect on the bacterial community composition, principal coordinate analysis of Bray–Curtis distances was used to visualize differences in microbiome community structure across treatments and over time. Clustering patterns of samples were assessed visually, and the statistical significance was confirmed with a PERMANOVA-like adonis on Bray–Curtis distances from the vegan package. Homogeneity of variances in microbial communities between samples from the same time x treatment combination was measured with

betadisper from the vegan package. To examine if inoculation with duck feces had an effect on the relative abundance of specific genera compared to the auto inoculation and control on the samples taken 2 and 7 D after inoculation, we performed Kruskal–Wallis tests on genera with an average relative abundance of at least 0.5%. Genera for which P < 0.05 were selected for further analysis. To further disentangle effects of the inoculation procedure itself vs. actual inoculation with duck feces, we checked for significant differences in selected genera between inoculated (duck + auto) vs. control and between duck and auto treatments with Wilcoxon rank-sum tests. These genera were plotted in ternary plots per timepoint using R ggtern package (Hamilton and Ferry, 2018). Relative abundances in taxa over time are reported throughout the manuscript as mean ± SD.

RESULTS

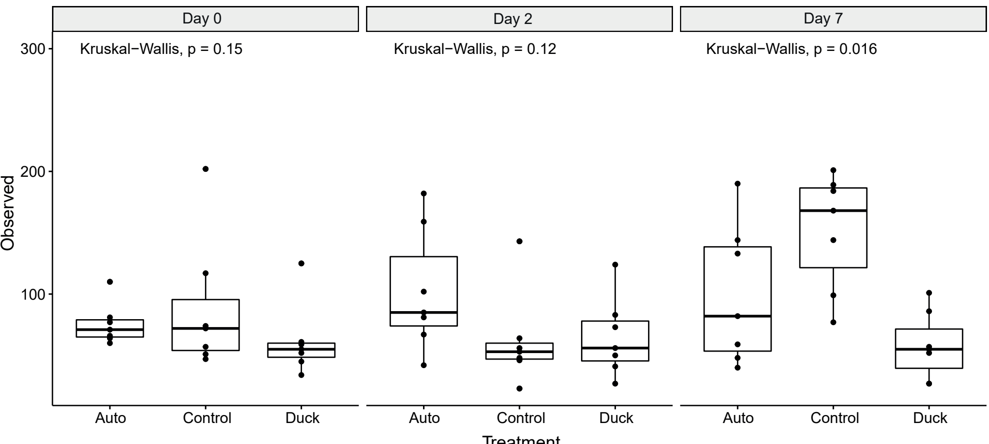

Figure 1: Observed species richness in all 3 treatments and grouped per day. Each dot represents an individual laying hen. Kruskal–Wallis test was used to detect significant differences in alpha diversity across treatment groups per day. The chicken inoculum (InoChicken; n = 5) exhibited mean observed species richness of 166 ± 6.2, and the duck inoculum (InoDuck; n = 5) exhibited mean observed species richness of 112.2 ± 36.44 (results are not shown). Control: no treatment (n = 7). Auto: inoculation with own chicken feces (n = 7). Duck: inoculation with duck feces (n = 7).

Community level changes

To characterize the microbial community of the laying hen's fecal microbiome, we first explored community diversity. Observed species richness (alpha diversity) exhibited no significant differences across treatments on days 0 and 2 (Figure 1). On day 7 after inoculation, there was a lower diversity in the inoculated hens (auto and duck) compared to the control hens (Wilcoxon rank-sum, P = 0.011). No significant difference was found between the auto and duck treatments on day 7 (Wilcoxon rank-sum test, P = 0.20). There were also no significant differences within treatments over time (Kruskal–Wallis test, P > 0.05). A principal coordinate analysis of the Bray–Curtis distances (beta diversity) was used to evaluate the changes in community structure across treatments and over time (Figure 2). Samples did not show any significant clustering (P > 0.05) of hen samples according to their treatment on day 2 or 7 after inoculation (Supplemental Table S2). Prior to inoculation on day 0, a significant difference in community structure was detected between the control and duck treatments (PERMANOVA-like Adonis, P = 0.048). No significant clusters were observed within treatments over time (P > 0.05).

Bacterial composition and temporal dynamics

In order to examine the dynamics in specific phyla and genera between treatment groups and over time, we selected the 10 most abundant phyla and 15 most abundant genera among all samples collected in the study on average. Average relative abundances are ± standard deviation. At the phylum level, the relative abundance of inocula consisted mainly of the phyla *Firmicutes* (Inoduck 49.2 ± 5.3%; Inochicken 40.0 ± 0.9%) and *Bacteroidetes* (Inoduck 27.6 ± 3.8%; Inochicken 45.5 ± 0.9%). At the genus level, the relative abundance of Inoduck was dominated by *Megamonas* (15.7 ± 2.4%, phylum *Firmicutes*) and *Bacteroides* (14.2 ± 4.1%, phylum *Bacteroidetes*) (Supplemental Figure S1). Inochicken was dominated by *Rikenellaceae_RC9_gut_group* (13.0 ± 1.1%) followed by *Bacteroidales* (7.2 ± 0.6%), *Alistipes* (7.0 ± 0.8%), and *Bacteroides* (6.7 ± 0.6%), all belonging to the phylum of *Bacteroidetes* (Supplemental Figure S1). All other genera showed relative abundances below 6% in the inocula.

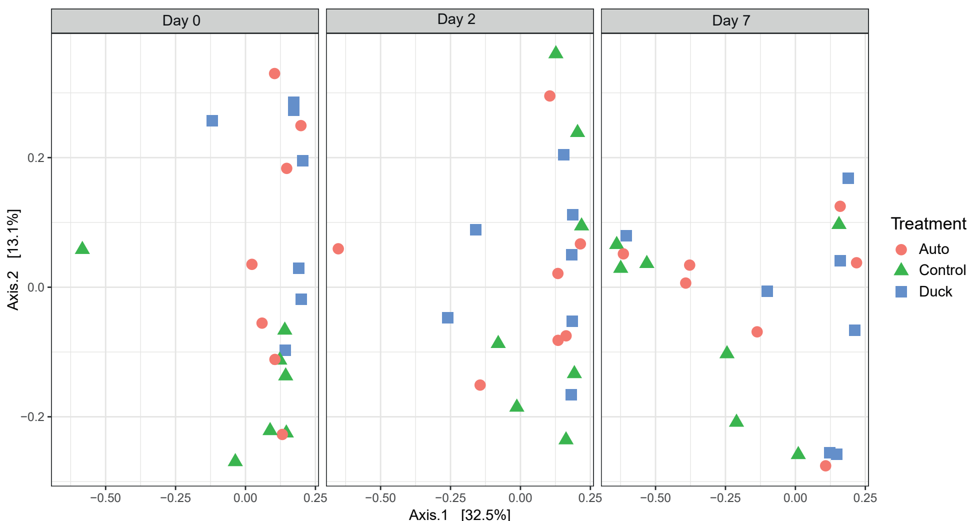

Figure 2: Change in community composition of the fecal microbiomes in hens visualized in a single PCoA plot of Bray–Curtis distances per treatment group and faceted per day. Control: no treatment (n = 7). Auto: inoculation with own chicken feces (n = 7). Duck: inoculation with duck feces (n = 7).

In the fecal samples of the hens, *Firmicutes* was dominant across all treatment groups and timepoints (66.4 ± 12.8%). *Fusobacteria* had a much lower abundance (10.5 ± 12.7%). All other phyla exhibited relative abundances <10% (Figure 3). At the genus level (Figure 4), *Romboutsia* (19.8 ± 12.3%, phylum *Firmicutes*) and *Fusobacterium* (10.5 ± 12.6%, phylum *Fusobacteria*) were most abundant across all treatments and timepoints (Figure 4). Although highly present in the duck inoculum (15.7 ± 2.4%), *Megamonas* was not observed in the fecal samples of the hens.

To further explore phyla and genera which showed consistent differences across treatment groups (Kruskal–Wallis test, $P < 0.05$), we selected the 10 most abundant phyla, and genera with an average relative abundance of > 0.5% at 2 and 7 D after inoculation, resulting in 1 phylum and 7 genera for further analyses. The phylum *Bacteroidetes* had a higher relative abundance in the inoculated treatments (duck and auto) compared to the control (Wilcoxon rank-sum test, $P = 0.028$ and $P = 0.014$ respectively) 2 D after inoculation. Of the 7 genera, 5 were present in the duck inoculum, and 4 of these (*Alistipes*, *Bacteroides*, *Faecalibacterium*, and *Ruminiclostridium 9*)

had a lower relative abundance ($P < 0.05$) prior to inoculation (Supplemental Table S3). *Alistipes* exhibited a higher (Wilcoxon rank-sum test, $P = 0.009$) relative abundance in samples from the inoculated treatments (auto and duck) compared to the control 2 D after inoculation, with no difference between the duck and auto treatments. The relative abundance of *Alistipes* was higher (Wilcoxon rank-sum test, $P = 0.035$) 2 D after inoculation than before inoculation in both the auto and duck treatments. However, 7 D after inoculation, the relative abundance of *Alistipes* in the auto and duck treatments was similar to the control (Wilcoxon rank-sum test, $P = 0.12$). No significant changes in the relative abundances of *Bacteroides*, *Faecalibacterium*, and *Ruminiclostridium 9* were detected in the inoculated hens (duck and auto) over time, nor were there significant differences in the relative abundances of these genera between auto and duck treatments either 2 or 7 D after inoculation ($P > 0.05$). Although not present in the duck inoculum, *Enterococcus* (phylum *Firmicutes*) exhibited higher relative abundances in the duck treatment compared to the auto treatment 2 D after inoculation (Wilcoxon rank-sum test, $P = 0.03$), but not compared to the control ($P = 0.44$). Seven days after inoculation, the relative abundance of *Enterococcus* was higher (Wilcoxon rank-sum test, $P = 0.03$) in the duck treatment compared to the control, but there was no significant difference in relative abundance of *Enterococcus* between duck and auto treatments (Wilcoxon rank-sum test, $P = 0.074$). Although not significant, the relative abundance of *Enterococcus* increased over time in the duck treatment (Kruskal–Wallis test, $P = 0.068$), but decreased significantly over time in the control (Kruskal–Wallis test, $P < 0.01$).

DISCUSSION

Identification of a proxy for the direct or indirect contact between domestic and wild animals may provide more insight into potential effects of these interactions and shed light on the mechanisms of pathogen transmission. This proxy could be used for risk assessment and identification of potential preventive measures to help reduce risks for disease outbreaks. In the present

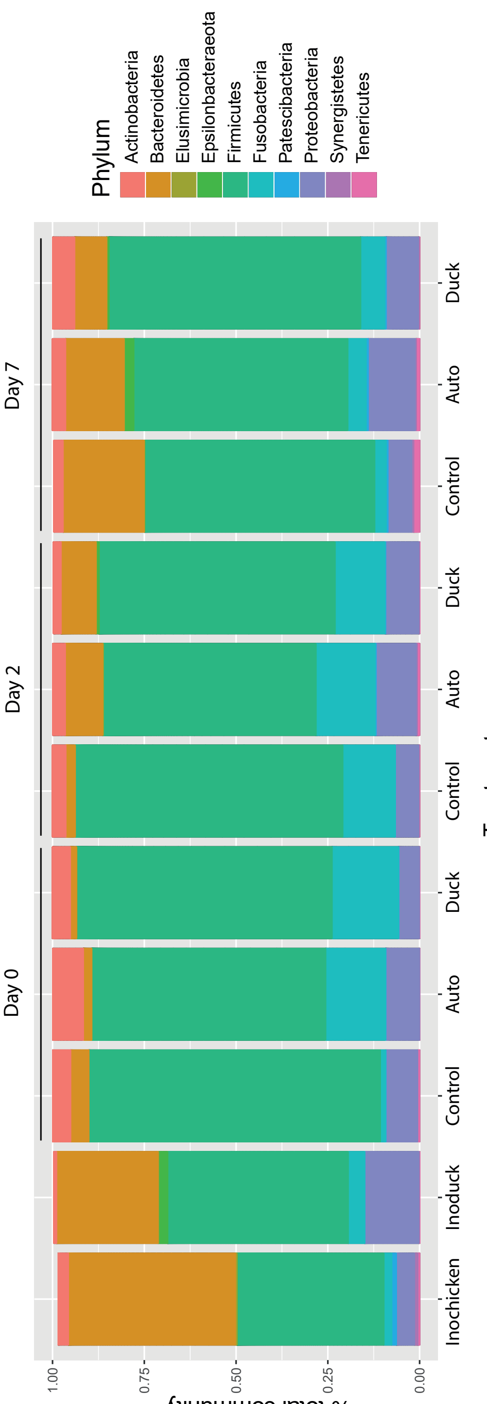

Figure 3: Relative abundance (%) of ten most abundant phyla displayed per treatment group and faceted per timepoint. Average values per inoculum, and treatment and timepoint are displayed. Inochicken: chicken inoculum (n = 5). Inoduck: duck inoculum (n = 5). Control: no treatment (n = 7). Auto: inoculation with own chicken feces (n = 7). Duck: inoculation with duck feces (n = 7).

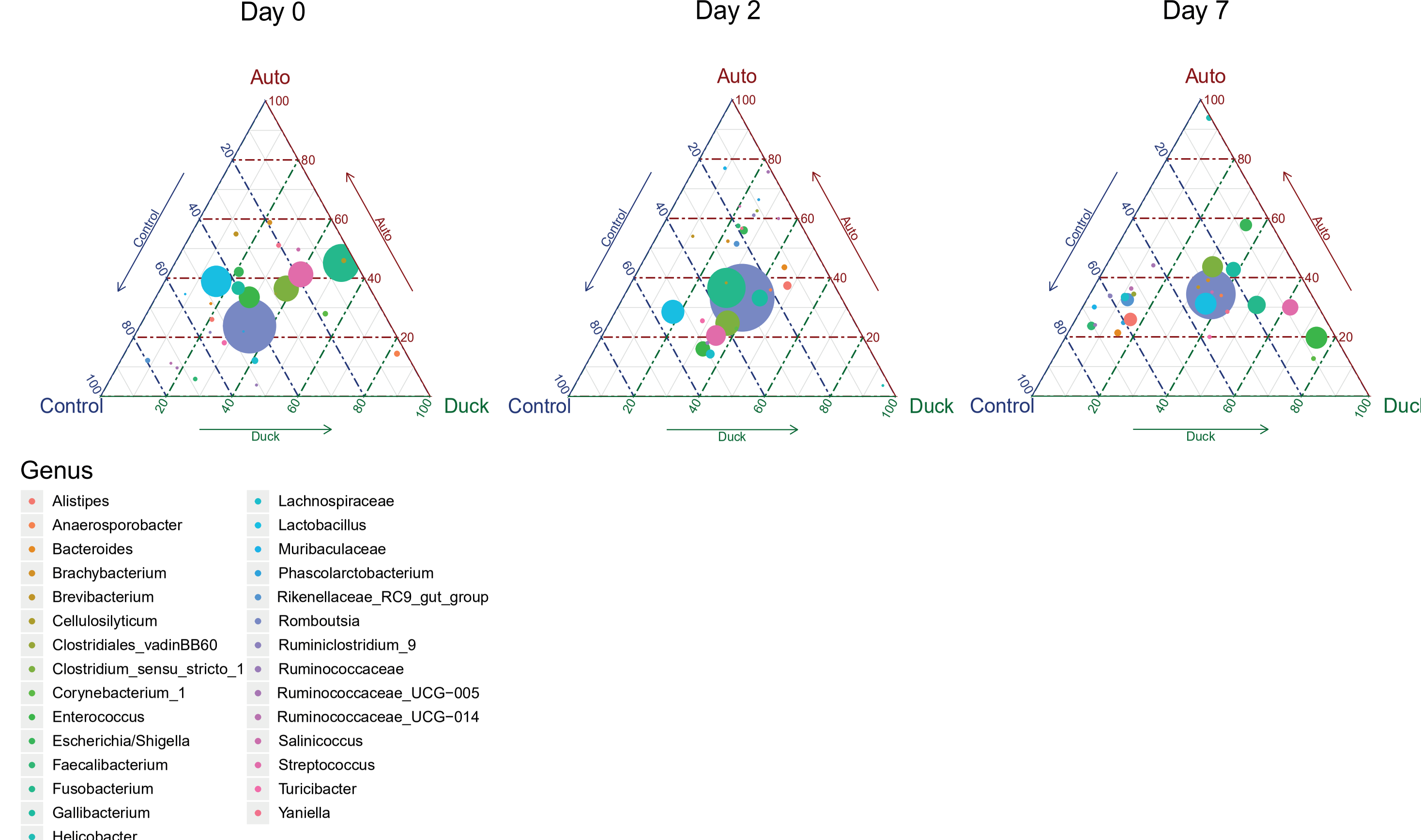

Figure 4: Ternary plot of genera with average relative abundance >0.5% per timepoint. The plot shows the proportion of the abundance of the genera per treatment group as positions in the triangle using barycentric coordinates. The 3 treatment groups are displayed on the 3 axes of the plot: control in blue, auto in red, and duck in green. The size of the circle indicates the relative abundance for that genus. Control: no treatment (n = 7). Auto: inoculation with own chicken feces (n = 7). Duck: inoculation with duck feces (n = 7).

study, we investigated whether an oral inoculation of laying hens with duck fecal microbiota resulted in a hen fecal microbiome that was detectably altered. We hypothesized that the inoculation would result in changes in microbial community parameters community diversity, community structure) as well as changes in the relative abundance of individual genera that might serve as an indicator for contact between ducks and laying hens.

The microbiome composition of the fecal and inoculum samples was markedly different. However, this was to be expected as fecal swabs and inocula are different matrixes, and the collection and processing after collection differed. Therefore, the samples are not directly comparable. Rather the inocula samples were meant to serve as a general reference for the types of shift that we could expect.

We were not able to detect significant differences in community diversity in the fecal microbiomes of hens inoculated with duck feces compared to hens inoculated with auto treatment or controls. However, we found that the relative abundance of the genus *Alistipes* (phylum *Bacteroidetes*) was significantly higher in the inoculated treatments (auto and duck) compared to the control at 2 D after inoculation. Previous studies also reported an increase in relative abundance of *Alistipes* after FMT in humans (Shahinas et al., 2012; Hamilton et al., 2013; Lee et al., 2017b), which was thought to be associated with colonization properties of bacteria from the order *Bacteroidales* (Lee et al., 2017b). However, 7 D after inoculation, the relative abundance of *Alistipes* had also increased in the control group, and no significant differences were detected between any of the treatments. Thus, it is also possible that the significant difference 2 D after inoculation was a result of the intrinsic temporal variation of the microbiome (Li et al., 2017; Fu et al., 2019). Alternatively, the patterns observed for *Alistipes* may have been a result of the inoculation and sampling procedures, which may have been stressful, and thus affected the microbiome composition (De Palma et al., 2014; Li et al., 2016). It has been shown that *Alistipes* was higher in fecal samples of mice that were exposed to daily stress compared to a non-stressed control group (Li et al., 2017). In addition, the increase in the relative

abundance of *Alistipes* in the control treatment may have been a result of transmission of *Alistipes* from inoculated hens. Humans and animals that live together are known to exchange microbiota (Song et al., 2013; Schloss et al., 2014). In animal studies, a cage effect is especially likely to occur for animals that are coprophagic such as mice (McCafferty et al., 2013; Laukens et al., 2016) and chickens (Kers et al., 2018; von Waldburg-Zeil et al., 2019). To avoid cage effects in chickens and to prevent the intake of particles and feathers containing potential intestinal microbiota "contaminants" (Meyer et al., 2012), studies have previously used individual housing of chickens (Zhao et al., 2013). For the purpose of this experiment, we decided not to house animals separately because this would be an additional stress factor for the birds, and would not be representative for the field situation. Therefore, all treatment groups were housed and handled in the same research unit. Consequently, transmission of *Alistipes* (and potentially other genera) from inoculated to control hens cannot be ruled out.

Curiously, the change in the relative abundance of *Alistipes* was the only significant alteration. Numerous studies have been published about the successful colonization of donor microbiota in recipients after FMT in humans (Hamilton et al., 2013; Broecker et al., 2016; Li et al., 2016; Lee et al., 2017b; Moss et al., 2017) and other animals (Diao et al., 2016; De Palma et al., 2017; Siegerstetter et al., 2018). In humans, FMTs can be administered orally (Youngster et al., 2014), but are often preceded by preparatory antibiotic treatment or bowel cleansing, which means that the gut microbiome at the time of FMT was disturbed (Manichanh et al., 2010; Dethlefsen and Relman, 2011), making it difficult to disentangle effects of FMT vs. preparatory treatments (Schmidt et al., 2018). In animal studies, young (Volf et al., 2016; Hu et al., 2018; Siegerstetter et al., 2018) or germ-free animals (Diao et al., 2016; De Palma et al., 2017) are often used for FMT. Volf et al. (2016) showed that a single inoculation of newly hatched ISA Brown pullets with cecal contents from donor hens of different ages could establish long-lasting measurable shifts in the cecal microbiota composition. However, in all these studies, the animals and chickens did not have fully developed gut microbiota. As we attempted to find a proxy for contact between wild ducks and adult laying

hens with outdoor range, we did not want to use younger hens as recipients of the duck inoculum nor did we want to use a preparatory treatment.

In a previous study, Videnska et al. (2014b) found that the cecal microbiome of laying hens underwent several successional changes in the process of aging. The age of the hens used in this study was 31 weeks, which is categorized as the fourth stage (28 to 52 weeks). At this stage, the gut microbiome has reached an adult microbial equilibrium (Videnska et al., 2014b). A stable microbiome forms a complex ecosystem and is characterized by a capacity for self-regeneration after an external perturbation (Lozupone et al., 2012; Lahti et al., 2014; Sommer et al., 2017). The single oral inoculation of healthy adult laying hens in our experiment may therefore have been insufficient to result in a perturbation that could cause a detectable shift in the established gut microbiome of the hens.

Previous studies have also described that colonization after FMT is more successful for genera which were already present in the recipient before FMT, and that rare genera are less likely to colonize (Li et al., 2016; Schmidt et al., 2018). This may explain why the genus *Megamonas* (phylum *Firmicutes*), which was found with a high relative abundance in the duck inoculum, was not detected in any of the hen samples, even though *Megamonas* has been reported to be present in the cecum and feces of laying hens (Videnska et al., 2014a; Polansky et al., 2016b). Clearly, *Megamonas* can inhabit the chicken gut, but as it was also absent in the auto inoculum, the gut conditions in the chickens of this study may not have been favorable for *Megamonas*.

We collected cloacal swabs from the chickens because our daily sampling scheme and longitudinal follow-up of the same individual laying hens required a rapid and accurate sampling methodology, without sacrificing the birds. The cloacal swabs were inserted deeply into the cloacal opening to enter the last part of the colon and to ensure the cloacal swabs contained enough fecal material for DNA extraction, we visually assessed the swabs prior to DNA extraction. It has been found that fecal microbiota of chickens were qualitatively similar to the cecal microbiota, but that they differed quantitatively (Stanley et al., 2015) and that the fecal microbiome is more variable than the cecal microbiome (Oakley and Kogut, 2016).

Collection of cecal droppings might have therefore been preferable over collection of cloacal swabs but was not feasible in our experimental design. However, we anticipated that major shifts would have also been picked up by sampling of the fecal microbiome, which has been demonstrated before (Oakley and Kogut, 2016; Jurburg et al., 2019).

Furthermore, it has been proposed that to accurately determine the fate of donor microbiota after FMT, it is necessary to track the microbiota at the resolution of strains rather than at the level of genera or species as is done with 16S rRNA gene amplicon sequencing (Li et al., 2016; Schmidt et al., 2018). For example, Li et al. (2016) demonstrated that single nucleotide variant analysis was able to detect donor strains colonizing the recipient after FMT, where 16S ribosomal RNA gene-based profiling was not sensitive enough to distinguish colonization of donor species from the temporal fluctuations of new species in the recipient. Therefore, it might be possible that certain strains of microbiota were transmitted with the inoculation, but not detected with our method of analysis. Also, we chose to analyze samples of 2 D after inoculation and not to analyze samples collected 1 D after inoculation. This was decided because we expected that samples collected 1 D after inoculation would detect the inoculum after passing through the intestinal tract rather than shifts in the fecal microbiome composition. However, we cannot rule out that minor changes in the fecal microbiome due to inoculation had occurred before 2 D after inoculation.

In conclusion, our findings show that a single oral inoculation of adult laying hens with duck feces in an experimental set-up results in limited effects at the genus level in the gut microbiome of the hens. We detected an increase of *Alistipes* across all treatments, but this may have been an effect of intrinsic temporal fluctuation or of the inoculation procedure itself and could not be attributed to the inoculation with duck feces. Further studies are needed to determine whether repeated exposure of adult chickens to duck feces, which are common in the field, may result in different outcomes, or whether other proxies can be identified that could serve as a measure for contact between ducks and laying hens.

ACKNOWLEDGMENTS

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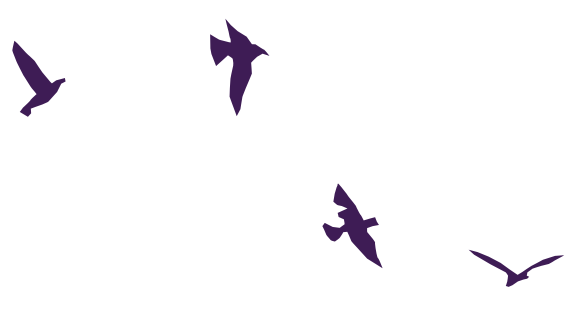
SUPPLEMENTARY DATA

Supplementary data are available online at Poultry Science https://www.sciencedirect.com/science/article/pii/S0032579119579627#cesec1701

3

An observational field study of the cloacal microbiota in adult laying hens with and without access to an outdoor range



Janneke Schreuder, Francisca C. Velkers, Ruth J. Bouwstra, Nancy Beerens, J. Arjan Stegeman, Willem F. de Boer, P. van Hooft, Armin R. W. Elbers, Alex Bossers, and Stephanie D. Jurburg

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ABSTRACT

Background: Laying hens with access to outdoor ranges are exposed to additional environmental factors and microorganisms, including potential pathogens. Differences in composition of the cloacal microbial community between indoor- and outdoor-housed layers may serve as an indicator for exposure to the outdoor environment, including its pathogens, and may yield insights into factors affecting the chickens' microbiota community dynamics. However, little is known about the influence of outdoor housing on microbiota community composition in commercial layer flocks. We performed a cross-sectional field study to evaluate differences in the cloacal microbiota of indoor- vs outdoor-layers across farms.

Eight layer flocks (four indoor, four outdoor) from five commercial poultry farms were sampled. Indoor and outdoor flocks with the same rearing flock of origin, age, and breed were selected. In each flock, cloacal swabs were taken from ten layers, and microbiota were analysed with 16S rRNA gene amplicon sequencing.

Results: Housing type (indoor vs outdoor), rearing farm, farm and poultry house within the farm all significantly contributed to bacterial community composition. Poultry house explained most of the variation (20.9%), while housing type only explained 0.2% of the variation in community composition. Bacterial diversity was higher in indoor-layers than in outdoor-layers, and indoor-layers also had more variation in their bacterial community composition. No phyla or genera were found to be differentially abundant between indoor and outdoor poultry houses. One amplicon sequence variant was exclusively present in outdoor-layers across all outdoor poultry houses, and was identified as *Dietzia maris*.

Conclusions: This study shows that exposure to an outdoor environment is responsible for a relatively small proportion of the community variation in the microbiota of layers. The poultry house, farm, and rearing flock play a much greater role in determining the cloacal microbiota composition of adult laying hens. Overall, measuring differences in cloacal microbiota of layers as an indicator for the level of exposure to potential pathogens and biosecurity seems of limited practical use. To gain more insight into

environmental drivers of the gut microbiota, future research should aim at investigating community composition of commercial layer flocks over time.

Keywords: microbiota, 16S rRNA, poultry, laying hen, outdoor range

INTRODUCTION

In recent years the demand for free-range poultry products has increased. Free-range housing for commercial laying chickens allows laying hens to access an outdoor range during the day, which is believed to benefit hens welfare (Green et al., 2000; Bestman and Wagenaar, 2003). Access to an outdoor range leaves layers exposed to more environmental factors, including weather and soil and environmental micro-organisms, including potential pathogens (Miao et al., 2005), one of which is the avian influenza virus (AIV) (Koch and Elbers, 2006). Layers with access to an outdoor range have an increased risk of low pathogenic AIV introduction (Bouwstra et al., 2017) via oral ingestion of infected wild bird feces directly or indirectly via an environmental virus reservoir (Brown et al., 2007; Rohani et al., 2009). These environmental factors may also affect the gut microbiota of the layers, and altered cloacal bacterial communities may therefore indicate exposure to the outdoor environment, which may potentially serve as an indicator for the level of biosecurity and exposure to pathogens. Furthermore, understanding the interactions between the gut microbiota in layers and other environmental factors in a commercial setting may yield insights into important drivers of microbiota community composition in layers. This could contribute to better understanding of ways to modulate the microbiota in favour of chicken health and production.

A review by Kers et al. (2018) on specific factors that affect the composition of the intestinal microbiota in poultry revealed that in addition to host-related factors like age and breed, environmental factors including housing, litter, feed and climate also affect the composition of intestinal microbiota. Other studies in poultry species have demonstrated that husbandry systems affect the microbiota composition of Pekin ducks (Best et al., 2017) and broilers (Ocejo et al., 2019). In layers, access to an outdoor range may result in altered gut microbiota due to exposure to environmental factors including soil, vegetation, natural lighting and rainfall (Hubert et al., 2019). Additionally, it has been shown that chickens housed in a free-range environment have different microbial community compositions, and increased diversity compared to indoor-housed or caged chickens (Xu et al.,

2016; Chen et al., 2018; Hubert et al., 2019). Xu et al. (2016) also reported increased relative abundance of *Bacteroidetes* in free-range chickens, and Hubert et al. (2019) reported a higher similarity among the microbiota of free-range chickens compared to caged chickens.

Although in previous studies differences in the microbiota of caged and free-range chickens have been described (Best et al., 2017; Hubert et al., 2019; Ocejo et al., 2019), these effects were most likely confounded by the effects of caged compared to non-caged chickens, the breed of the chicken or the age, and did not truly measure the effect of the access to the range. The aim of our study was to determine if there are differences in the composition of the cloacal microbiota in indoor- and outdoor-housed chickens under field conditions. We selected indoor and outdoor flocks based on breed and rearing flock to minimize the effect of other factors than the outdoor range. Cloacal swabs of laying hens from eight commercial layer flocks (four indoor and four outdoor flocks), were analysed to characterize differences in the cloacal microbiota of adult layers with and without access to an outdoor range.

To our knowledge, this is the first report of a cross-sectional study comparing the cloacal microbiota of indoor-and outdoor-housed commercial laying hens. We hypothesized that diversity parameters (i.e., community richness and structure) in outdoor-layers will be higher compared to indoor-layers, because of greater substrate diversity and exposure of the layers to more diverse microbiota in the outdoor environment. Furthermore, we anticipated distinct clustering of the community composition of outdoor-layers compared to indoor-layers due to specific alteration in the community as a result of outdoor exposure.

RESULTS

We amplified and sequenced the V3-V4 hypervariable region of the 16S rRNA gene. After quality control of all samples with qPCR, samples with low 16S DNA concentration or bad melting curves were removed from further analysis (14 samples in total; 4 negative controls and 10 chicken samples). The final dataset contained 70 samples (7–10 samples per poultry house),

with 35 indoor-layer and 35 outdoor-layer samples (Table S2). Each sample was rarefied to 13,154 reads per sample, which was the number of reads in the lowest sample. The final dataset contained a total of 3037 amplicon sequence variants (ASVs).

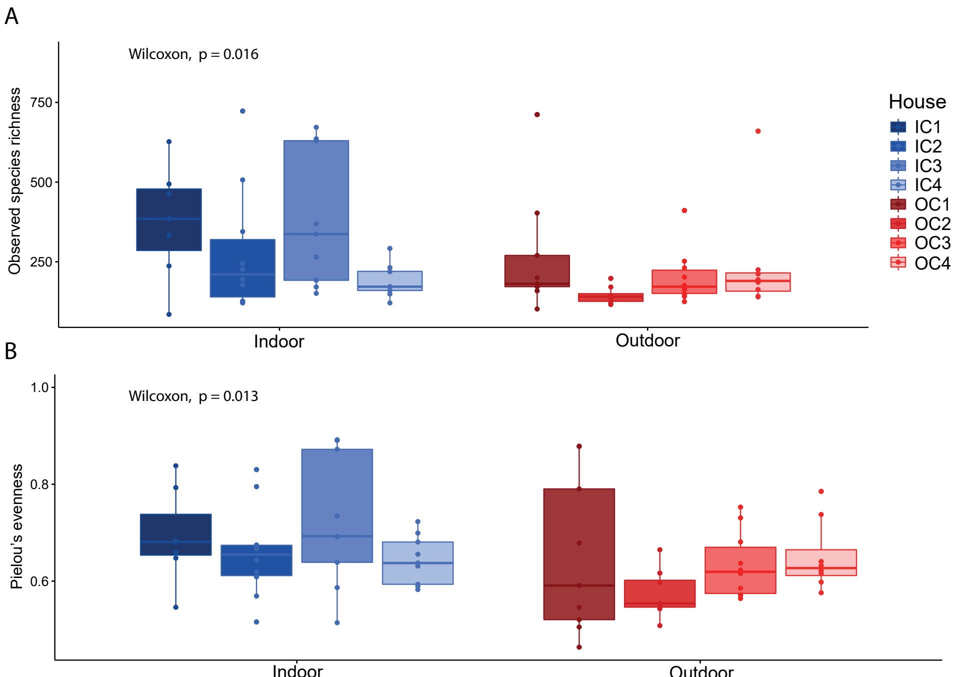

Figure 1: Comparison of observed species richness (a) and Pielou's evenness (b) between indoor- (blue) and outdoor-layers (red). Each box contains samples from a single poultry house. Each dot represents an individual chicken. Wilcoxon-Rank-Sum test were performed between indoor- and outdoor-layers. A lower value for Pielou's evenness indicates less evenness in the microbial community of a sample

Microbial community composition

We evaluated the overall composition of the microbial community in the cloacal samples of all layers. At the phylum level, we observed similarities between the microbiotas of indoor- and outdoor-layers (Figure S1), and no significant differences in the relative abundances of the ten most abundant phyla were found between indoor- and outdoor-layers. These ten phyla constituted 99.4% ± 1.3 (unless otherwise indicated, results are expressed as mean ± SD) of the community, across all samples. The microbiota in both

groups were dominated by *Firmicutes* (54.0% ± 17.3), *Proteobacteria* (15.2% ± 10.2) and *Fusobacteria* (13.6% ± 17.3; Figure S1). At the genus level, members of the genera *Romboutsia* (22.8% ± 16.0) and *Fusobacterium* (13.5% ± 17.7) were most dominant in both indoor- and outdoor-layers (Figure S2). *Escherichia/Shigella* were significantly more abundant in outdoor- than indoor-layers (Wilcoxon Rank-Sum test, $p < 0.005$).

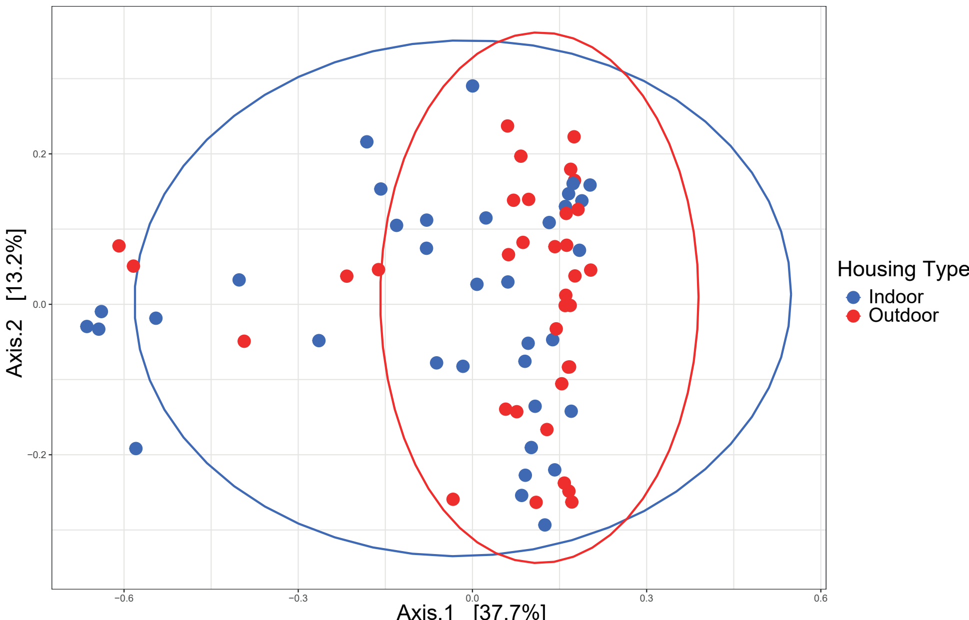

Figure 2: Principal coordinate analysis of Bray-Curtis dissimilarity between samples. Color indicates poultry farm and the ellipses the housing type encompassing the 95% CI range of each housing type. Each dot represents an individual sample. Housing type explained 5.6% (R^2, adonis, $p = 0.0025$) of the variation. Poultry house explained 32.8% (R^2, adonis, $p = 0.0001$) of the variation

Differences in community structure

To evaluate the microbial community composition of the layers, we first explored community diversity. Observed species richness (number of ASVs) was significantly higher (Wilcoxon Rank-Sum test, $p = 0.016$) in indoor-layers (302 ± 182 ASVs) compared to outdoor-layers (213 ± 136 ASVs; Figure 1). Pielou's evenness was also higher in indoor-layers compared to outdoor-layers (Wilcoxon Rank-Sum test, $p = 0.013$). To evaluate the differences

in community structure between indoor- and outdoor-layers, we used a principal coordinate analysis (PCoA) of Bray-Curtis dissimilarities. We found a modest, but significant clustering of microbial communities according to housing type (indoor vs outdoor), explaining 5.6% of the variance in community structure (R^2; adonis, p = 0.0025; Figure 2). The poultry house where the layers were kept was a much stronger driver of community structure, explaining 32.8% of the variance (R^2; adonis $p < 0.001$). When ASVs were clustered at the phylum level, no differences between the community composition of different housing types were found (data not shown).

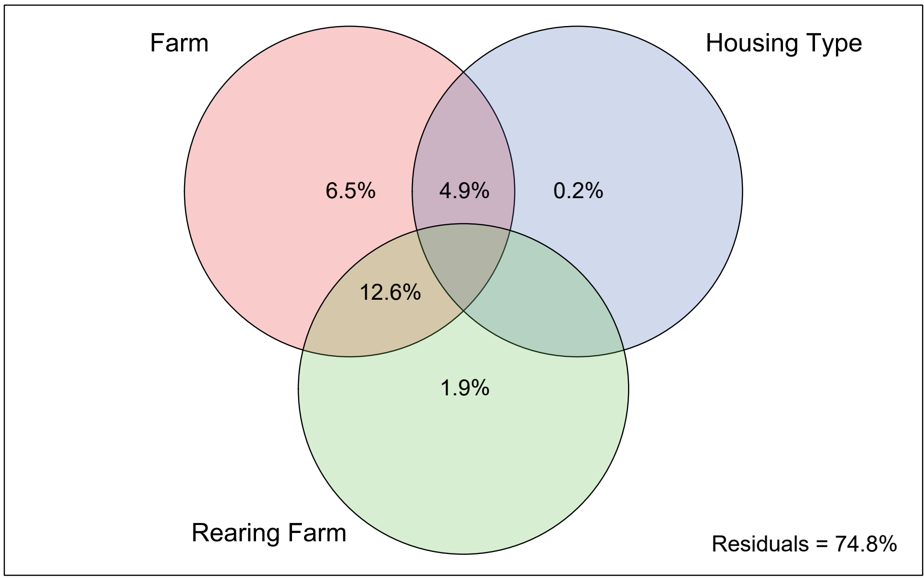

Figure 3: Venn diagram depicting distance-based variation partitioning using Bray-Curtis dissimilarity. The contribution of rearing farm of origin (green, 6.5%), the farm the poultry houses were located in (red), and housing type (indoor or outdoor, blue) to the microbiota composition of layers is shown

To better explain the effect of access to an outdoor range on the variation in microbial community composition, we performed a variation partitioning analysis using Bray-Curtis dissimilarity, with factors housing type (indoor vs outdoor), rearing farm and farm (Figure 3). Poultry house was excluded from the variation partitioning, as this factor explained most (20.9% R^2 adj) of the observed variation and its influence could not be

disentangled from other factors due to collinearity with the other factors in our study. Housing type explained the smallest part of the variation (0.2% R^2 adj; Figure 3), whereas the interaction of farm and rearing flock explained most of the variation (12.6% R^2 adj). This was followed by farm (6.5% R^2 adj), the interaction of housing type and farm (4.9% R^2 adj), and rearing farm (1.6% R^2 adj). To examine the variation in microbiota among chickens of the same poultry house, we calculated Bray-Curtis dissimilarities between layers of the same poultry house. Notably, the community composition in the indoor poultry houses was significantly more variable than in outdoor poultry houses (Wilcoxon Rank-Sum test, $p = 0.03$; Figure 4a). In addition, dissimilarities between outdoor-layers, excluding within house comparisons, were significantly lower than dissimilarities between indoor-layers (Wilcoxon Rank-Sum test, $p = 0.002$; Figure S4). Overall, the cloacal microbial communities of chickens within a poultry house were more similar to each other than to those within a housing type (Wilcoxon Rank-Sum test, $p < 0.001$; Figure 4b).

Differential abundance of individual taxa

We found five genera which were differentially abundant between indoor- and outdoor-layers (Wilcoxon Rank-Sum test, $p < 0.005$): *Porphyromonas*, *Escherichia/Shigella*, *Sutterella*, *Campylobacter* and *Faecalibacterium* (Figure S5). Of these, *Escherichia/Shigella* (7.2% contribution to overall Bray-Curtis dissimilarity, $p = 0.003$) and *Porphyromonas* (1.3% contribution to overall Bray-Curtis dissimilarity, $p = 0.001$) contributed most to the dissimilarity between the housing types according to a SIMPER analysis. However, *Porphyromonas* was found in only one outdoor poultry house (OC3) and was absent in samples from all other poultry houses (Figure S5). *Escherichia/Shigella* was found to have a higher relative abundance in the outdoor flocks, but this increase was specific to poultry houses OC2 and OC3 and not to outdoor flocks OC1 and OC4 (Figure S5).

Faecalibacterium was more abundant in indoor-layers (1.16% ± 2.06) compared to outdoor-layers (0.7% ± 1.86), as well as *Sutterella* (indoor 0.76% ± 1.25; outdoor 0.37% ± 1.09). *Campylobacter* was higher in outdoor-

layers (indoor 0.41% ± 0.78; outdoor 1.10% ± 2.55). However, this pattern was specific to individual poultry houses, and none of the genera had a consistently higher or lower relative abundance across all poultry houses of one housing type (Figure S5).

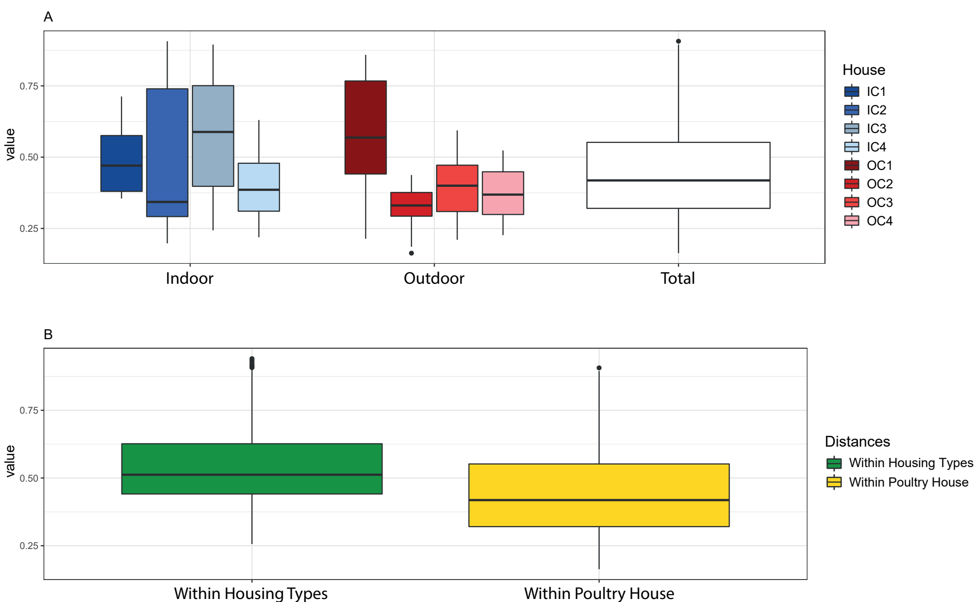

Figure 4: **A.** Pairwise Bray-Curtis dissimilarity between the cloacal microbiota of layers from each poultry house. Greater values indicate higher dissimilarity. 'Total' contains all possible pairwise comparisons, for reference. Community composition in the indoor poultry houses was more variable than in outdoor poultry houses (Wilcoxon-Rank-Sum test, $p = 0.03$). **B.** Bray-Curtis dissimilarities between the cloacal microbiota of layers within a poultry house (Within Poultry House) compared to dissimilarities between cloacal microbiota of layers within a housing type (indoor vs outdoor), excluding within poultry house comparisons (Within Housing Types). The cloacal microbial communities of layers within a poultry house (0.46 ± 0.18, mean ± SD) were more similar to each other than to those within a housing type (mean 0.55 ± 0.15, Wilcoxon-Rank-Sum test, $p < 0.001$)

In contrast, we identified a single ASV (Wilcoxon Rank-Sum test, $p < 0.0001$; Figure 5), which was present in 20 outdoor-layers (57%) across all outdoor poultry houses, with a mean relative abundance of 0.05% ± 0.07% in these 20 layers. This ASV was not present in any of the indoor-layers. A BLAST search (Basic Local Alingment Search Tool) of this ASV classified it as a *Dietzia maris* (99.74% identity to strain DSM 43672), which is associated with soil (Rainey et al., 1995).

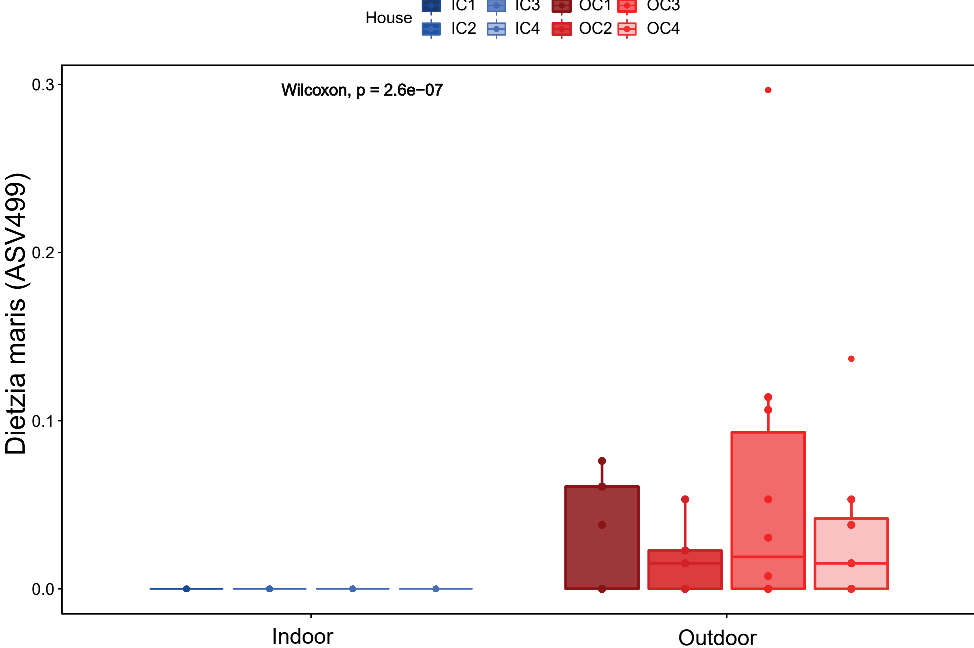

Figure 5: Relative abundance (%) of *Dietzia maris* (ASV499). *Dietzia maris* was present in 57% of the outdoor-layers assessed, and was detected in all outdoor poultry houses. It was not present in any of the indoor-layer samples. Each dot represents an individual chicken. Colors intensity indicates which poultry house the chickens originated from. Wilcoxon-Rank-Sum test was performed between all indoor-layers and all outdoor-layers ($p < 0.001$)

DISCUSSION

The evaluation of differences between the cloacal microbiota of indoor- and outdoor-layers in commercial flocks may contribute to an increased understanding of interactions between gut microbiota, housing conditions, and other environmental factors, and help to determine whether the microbiota composition might be used as an indicator of the risk of potential pathogen exposure from the farms' outdoor environments. Furthermore, understanding the dynamics in microbiota community composition of adult layers in a field setting is relevant, as it may contribute to the insights needed to develop ways to modulate the chickens' microbiota in favour of health and increased production performance. Although we previously found limited change in the hens fecal microbiota after a single oral inoculation with wild duck feces (Schreuder et al., 2019), we hypothesized that continued exposure

of laying hens to an outdoor environment would be more likely to result in detectable alterations in the fecal microbiota of outdoor-layers.

In this study we found that access to an outdoor range only explained a small proportion (0.2% R^2 adj) of the total variation in the cloacal microbiota of layers. Instead, poultry house was found to be the most important driver of community composition (20.9% R^2 adj). When poultry house was excluded from further analysis to more precisely estimate the effect of the outdoor range, the farm where the poultry houses were located (6.5% R^2 adj), the rearing farm the chickens originated from (1.9% R^2 adj), and the interaction between these two factors (12.6% R^2 adj) explained most of the variation. The relatively high R^2 for this interaction is to be expected, considering the overlap between the factors rearing farm and layer farm in our study design (Figure 6). We also found that the diversity and evenness in indoor-layers were slightly higher compared to outdoor-layers, suggesting the presence of more dominant species in outdoor-layers. This contrasts with previous studies, which found higher microbial diversity in outdoor-layers (Xu et al., 2016; Chen et al., 2018; Hubert et al., 2019; Ocejo et al., 2019). Differences in diversity and community composition in previous studies has been related to greater substrate diversity and intake of fibrous feedstuff (Xu et al., 2016), as well as exposure of the chickens to more abundant microbiota in the outdoor environment (Hubert et al., 2019). Our findings may deviate from those of previous studies due to several reasons.

In the first place, we selected indoor and outdoor poultry flocks of the same breed (Dekalb White) and based on the rearing farm of origin to minimize variation due to host genetics and rearing conditions. Also, the in-house environment of both indoor- and outdoor-layers was similar in our study. Chickens were housed in cage-free aviary systems, with the same stocking density, feed, minimum number of perches, similar litter etc. This is in contrast with previous research where a comparison was made between either free-range poultry with access to an outdoor range and caged layers (Xu et al., 2016; Chen et al., 2018; Hubert et al., 2019), between fast- and slow-growing broilers (Ocejo et al., 2019), or in a semiexperimental set-up (Xu et al., 2016). Effects found in these previous studies are likely confounded by

the effects of caged vs. non-caged chickens or the breed of the chicken, and not truly measure the effect of the access to the range. Additionally, two studies were performed on either broiler chicks of 42 days of age (Ocejo et al., 2019) or indigenous Chinese Dagu chickens, a dual purpose breed which produces both meat and eggs, of only 12 to 18 weeks of age (Xu et al., 2016). The microbiota of adult layers develops over time to a stable equilibrium (Videnska et al., 2014b), which is less sensitive to external perturbations (Schreuder et al., 2019) and hence, may explain the unanticipated limited effect of the outdoor environment in our field study. The timing of access to the range may also be of importance. In the study by Xu et al. (2016), Dagu chickens had access to the outdoor range from the beginning of the experiment when the chickens were 6 weeks of age. In contrast, when access to the outdoor range occurred in the last 4 weeks of the cycle in broiler chicks, no change in the richness of the broiler intestinal microbiota was found (Gong et al., 2008). The hens in our study were only able to access the range from 19 to 20 weeks of age, after transport from the rearing farm, which means that their microbiotas were almost fully developed and had likely reached a stable equilibrium prior to given access to the range. It is known that a well-developed normal gut microbiota protects the host through creating gastrointestinal resistant environments, which prevent external (pathogenic) bacteria from colonizing the gut (Lawley and Walker, 2013; Han et al., 2017).

Moreover, it is likely that only a small proportion of the hens in the outdoor flocks of our study visited the outdoor range. Although limited information is available about actual range usage of layers, in the Netherlands it has been estimated that in large flocks (> 10.000 layers) only 3–15% of the hens use the outdoor range at a certain timepoint which is partly dependent on the degree of cover provided by trees or artificial structures in the range (Bestman and Wagenaar, 2003; van Niekerk and Leenstra, 2016b). This is supported by Hegelund et al. (2005) who found that in commercial layer flocks with access to a range, on average only 9% of the chickens used the range area. In contrast, Gebhardt-Henrich et al. (2014) reported that 47–90% of chickens in outdoor flocks were registered in the outdoor range at least

once over a period of approximately 3 weeks; the individual hens used the range differently, and many of them did not enter the free-range every day. Furthermore, chickens tended to only use the area immediately outside the hen house (Hegelund et al., 2005), which has also been observed in the Netherlands, resulting in trampled vegetation closer to the hen house and hence lower availability of fibrous feedstuff for the hens (Bestman, 2017). The outdoor ranges in the Netherlands in general consist of open fields with some tree coverage and bare soil close to the poultry house (van Niekerk and Leenstra, 2016b). Both the limited use of the outdoor range by the hens, together with the low availability of fibrous feedstuff in the range, may explain why we only found limited effects of the outdoor range on the microbial community composition of layers.

We hypothesized that the microbiota of outdoor-layers would be more variable due to their exposure to the outdoor environment and the fact that not all layers use the outdoor range. However, we found more variability in microbiota of indoor-layers compared to the outdoor-layers. Previous research has shown that microbiota of free-range layers contained a greater variability of bacterial species compared to caged layers (Nordentoft et al., 2011; Cui et al., 2017). The greater variability in the bacterial community composition of the chickens with only indoor housing in our study could be a result of the spatial distribution of the hens within the poultry house. In all flocks in this study, compartments were present in the indoor area of both the flocks with indoor housing and those with access to the outdoor range. This is according to Dutch regulations, that stipulate that poultry houses need to be divided into compartments which contain no more than 6000 hens (Schouwenburg, 2019a). Although outdoor poultry houses also have these compartments, layers are able to move freely between these compartments because they have access to the range and can enter another compartment from the outdoor range. This means that the outdoor-layers are more evenly distributed across the poultry house, whereas strictly indoor-layers stay in the same separate compartment within the poultry house all the time. Consequently, this increased level of compartmentalization in indoor-layers can cause a so called cage-effect, which has been reported in

several animal studies (Nordentoft et al., 2011; Laukens et al., 2016), and could explain the higher variation between layers from indoor houses. Unfortunately, we were not able to adequately measure this effect, because we did not take the compartmentalization into account when sampling the flocks. In future studies where different housing types are being compared with regard to microbiota composition, potential impact of differences in compartmentalization should be taken into account in the study design.

In order to sample commercial flocks, we opted for cloacal swabs, which served as a rapid and accurate sampling methodology that did not entail sacrificing the birds. The fecal microbiota of chickens are qualitatively similar to the cecal microbiota (Stanley et al., 2015), but more variable (Oakley and Kogut, 2016). While our sampling technique may explain why we found a high degree of variation between individual chickens, it does not explain the differences in variability in the microbiota of layers of different housing types, as the same sampling technique was used across the study. We found one specific ASV, *Dietzia maris*, that was only found in outdoor-layers and is related to soil (Rainey et al., 1995). However, this was a lowly abundant taxon, and we did not detect differences between indoor- and outdoor-layers when we looked at the all ASVs in the genus *Dietzia* jointly. Moreover, no other genera were found to be differentially abundant across all indoor and outdoor poultry houses. This suggests that although the chickens can pick up some specific taxa from the range, access to an outdoor range does not cause a distinct shift in the microbial community of layers. Therefore, we cannot use community-wide microbiota assessments as a measure for biosecurity or exposure to pathogens from the outdoor environment of a farm.

This study furthermore emphasises the importance of the environment of the poultry house, and the likely influence of daily management on the fecal microbiota, which was also found in broilers (Kers et al., 2019) and several murine models (Parker et al., 2018). In the study by Kers et al. (2019), broiler chicks were raised in different housing environments, and were given two diets. These feed interventions alone explained 10% (R^2) of the variation in microbiota composition between the broilers, whereas

housing condition alone explained 28% (R^2). The effect of the poultry house environment explained a similar amount of variation in our study. Future research should aim at better understanding the interactions between the gut microbiota in layers and environmental factors at the level of the poultry house over time. This may shed light on important drivers of microbiota community composition in commercial layers and could contribute to better understanding of ways to modulate the microbiota in favour of chicken health and production.

CONCLUSIONS

This cross-sectional field study shows that exposure to an outdoor environment is responsible for a relatively small proportion of the community variation in the microbiota of layers. We did not detect unique patterns in the community composition of outdoor-layers compared to indoor-layers or detect specific microbiota that could be related to contact with an environment contaminated by wild birds. Overall, measuring differences in cloacal microbiota of layers as an indicator for the level of exposure to potential pathogens and biosecurity seems of limited practical use. To be able to gain more insight into environmental drivers of the gut microbiota that may be associated with pathogen exposure, and hence performance, future research should aim at investigating community composition of commercial layer flocks over time.

MATERIALS AND METHODS

Study design and sample collection

Eight commercial flocks of laying hens (Dekalb White) were selected for cloacal sampling: four layer flocks with access to an outdoor range (outdoor flocks) and four flocks without access to an outdoor range (indoor flocks). To minimize potential variation in the microbiota composition due to rearing and other environmental factors, outdoor and indoor flocks were selected based on the rearing farm of origin as well as on age (Figure 6, Table S1). All flocks consisted of layers between 27 and 40 weeks of age, which are

assumed to have matured to a stable gut microbiota composition (Videnska et al., 2014b). Flocks from the same rearing farm, were of the same age, and were sampled within the same week. All flocks were sampled within the same month (October 2017) to avoid short term weather and seasonal effects. The sampled flocks were kept in separate poultry houses, which were located on five different poultry farms: two indoor and outdoor flocks were kept in poultry houses at the same farm, two indoor flocks were located in poultry houses at the same farm, and two outdoor flocks were housed at two separate farms (Figure 6). All flocks were healthy at the time of sampling, and had not been treated with antibiotics on the layer farm. Both indoor and outdoor-layer flocks were kept in a cage-free aviary system with a maximum stocking density of nine chickens per m^2, with one flock per poultry house (Schouwenburg, 2019a). The laying hens of the outdoor flocks had access to an outdoor range during the day with at least 4 m^2 per hen according to standards of the Dutch quality assurance scheme, i.e. the Integrated Chain Control program, 'IKB Egg' (Schouwenburg, 2019b). The chickens had access to the outdoor range for 8 h a day on average (van Niekerk and Leenstra, 2016b). Outdoor ranges were mostly open grass field with some trees, and bare soil directly around the poultry house and drainage systems to prevent formation of rain puddles (van Niekerk and Leenstra, 2016b). Outdoor ranges were all fenced off with chicken wire.

In each flock, two cloacal dry swabs were collected from 50 laying hens. The poultry houses contained several subsections, and an equal number of birds was randomly selected from each subsection of each flock. Samples were placed on ice immediately after collection and stored at −80°C within 5 h after collection.

DNA extraction and 16S rRNA gene amplicon sequencing

Per flock, one cloacal swab of a selection of ten chickens, was chosen for further analysis. Swabs of chickens were selected based on equal distribution across the farm and visual assessment of the swab to ensure that sufficient fecal material was present for DNA extraction. DNA extraction was performed according to the protocol in Schreuder et al. (2019). In each

DNA isolation round a negative control sample containing PBS was added to identify possible contamination from reagents. Following extraction, the DNA extracts were quantified with Invitrogen ™ Qubit™ 3.0 Fluorometer and stored at −20°C for further processing. The V3–V4 region of the 16S rRNA gene was amplified in a PCR with the primers CVI_V3-forw CCTACGGGAGGCAGCAG and CVI_V4-rev GGACTACHVGGGTWTCT. The following amplification conditions were used as previously described (Schreuder et al., 2019): step 1: 98°C for 2 min, step 2: 98°C for 10 s, step 3: 55°C for 30 s, and step 4: 72°C for 10 s, step 5: 72°C for 7 min. Steps 2 to 4 were repeated 25 times. Negative controls were included at each amplification round to confirm sterility of PCR reagents. PCR products were checked with gel electrophoresis, and PE300 sequencing was performed using a MiSeq sequencer (Illumina Inc., San Diego, CA). An additional 16S rRNA gene qPCR was performed on the DNA samples, to quantify the amount of 16S rRNA gene DNA and identify samples of poor quality (Table S2). An additional two samples did not have good quality melting curves, and these samples were discarded from further analyses. The qPCR consisted of 40 cycles with the same primers and protocol as for the PCR.

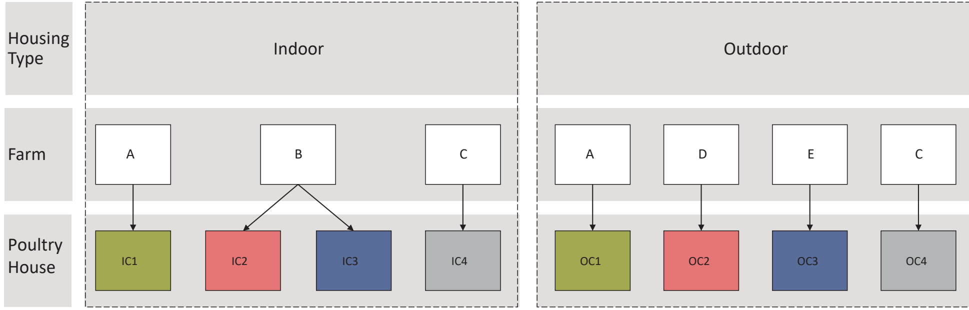

Figure 6: Overview of the study design. Four indoor-laying hen flocks (indoor cross-sectional = IC) and four outdoor flocks (outdoor cross-sectional = OC), each kept in an individual poultry house, were sampled. Chickens from all flocks were of the same breed. Indoor and outdoor flocks that had the same rearing flock of origin were selected, which is indicated with numbers 1, 2, 3 or 4, and colors at the poultry house level. Some poultry houses were situated at the same farm. Hens originating from rearing farm 1 and 4 were placed in the layer farm houses that were situated on the same farm, A and C (Table S1). Farm B housed two flocks (IC2 and IC3) that came from different rearing farms (2 and 3)

Processing of sequencing data

All sequence processing was performed in R 3.5.1 (R Core Team, 2013). The sequence reads were filtered, primer-trimmed (35 nucleotides), dereplicated, chimera-checked, and merged using the dada2 package (Callahan et al., 2016) using standard parameters (TruncLength = 240,210, MinOverlap = 1 and maxEE = (2,2)). Reads were assigned with the SILVA v.132 classifier (Quast et al., 2012). Negative controls from the DNA extraction did not contain any sequences above detection level and were discarded (n = 4). Some of the samples (n = 10) contained very low 16S DNA concentration after the qPCR or gave poor quality melting curves, and were discarded after sequencing. The final dataset contained 70 samples. In the final dataset the number of samples per poultry house ranged between seven and ten samples, with 35 indoor-layer and 35 outdoor-layer samples (Table S2).

Statistical analyses

All downstream analyses were performed in R (version 3.5.1) with the phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen et al., 2007) R packages. We measured diversity as the number of observed ASVs in a sample, and evaluated species evenness within samples with Pielou's index J (Pielou, 1966) at the species level. Bray-Curtis dissimilarity measure was used to evaluate differences in community structure between the layers on Hellinger transformed ASV abundances in phyloseq (Bray and Curtis, 1957), and selected ASVs with a total sum value of greater than 1. Factors that were included in further analysis were housing type (indoor- and outdoor-layers), poultry house (stable in which flocks were housed), rearing flock (rearing flock where layers from a flock originated from), and farm (farm where poultry houses were based, i.e. some farms had multiple houses, Figure 6). Feed was not included in the analyses as this could not be disentangled from the effect of the poultry house. Differences between the microbiota composition of layers were examined for each factor using the adonis function on Bray-Curtis dissimilarity (Anderson, 2001). To further assess the contribution of each factor to the observed variation in the microbiota composition, we performed distance-based (Bray-Curtis)

redundancy analysis (Anderson, 2001). A model with housing type, and poultry house (Figure S3) was most parsimonious, explaining 31.8% of the variation. As poultry house explained most of the variation in the microbiota composition, we further disentangled the contribution of the factors farm, rearing farm, and housing type with distance-based variation partitioning, leaving poultry house as a factor out of the model (Borcard et al., 1992). To test how well samples from individual layers within one poultry house represented the microbiota of that house, we calculated community Bray-Curtis dissimilarity between layers within each poultry house. Additionally, we calculated community Bray-Curtis dissimilarity between layers of the same housing type, excluding the comparisons between layers of the same poultry house, to evaluate how well samples from individual layers of one housing type represented the microbiota of that housing type.

We used two approaches, Wilcoxon Rank-Sum tests and DESeq2 (Love et al., 2014), to check for differences in relative abundances of the ten most abundant phyla, 0.5% most abundant genera and 0.01% most abundant ASVs. We present only the result of the Wilcoxon Rank-Sum test, as this non-parametric test is most suitable for high variability between samples, and only this approach identified taxa which were consistently higher in one condition. Taxa for which Wilcoxon Rank-Sum test resulted in $p < 0.01$ were selected for further analyses. We used a SIMPER analysis to identify which of the genera contributed most to the beta diversity (Warton et al., 2012). With Wilcoxon Rank-Sum test we identified if specific ASV were consistently increased or decreased in either of the two housing types. The figures from ggplot2 and ggpubr were further refined in Adobe Illustrator CC (version 21.0.2.).

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AUTHORS' CONTRIBUTIONS

RJB, NB, WFB, JAS, JS initiated this project. RJB, NB, JAS, WFB, PH, ARWE and FCV contributed to the design of the experiment. JS performed sample collection and manuscript writing. JS, AB and SDJ did data processing and analysis. SDJ, FCV, JAS, RJB, NB, AB, PH, ARWE and WFB contributed to the development of the manuscript by giving constructive feedback on the manuscript during its preparation. The authors gave approval of the manuscript for publication.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This field study did not require ethical approval by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee as only non-experimental procedures were used.

COMPETING INTERESTS

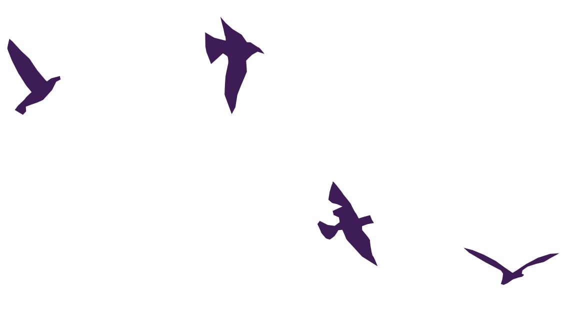
The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

SUPPLEMENTARY INFORMATION

Supplementary information accompanies this paper at https://doi.org/10.1186/s42523-020-00044-6.

4

Temporal dynamics of cloacal microbiota in adult laying chickens with and without access to an outdoor range



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ABSTRACT

Associations between animal health and performance, and the host's microbiota have been recently established. In poultry, changes in the intestinal microbiota have been linked to housing conditions and host development, but how the intestinal microbiota respond to environmental changes under farm conditions is less well understood. To gain insight into the microbial responses following a change in the host's immediate environment, we monitored four indoor flocks of adult laying chickens three times over 16 weeks, during which two flocks were given access to an outdoor range, and two were kept indoors. To assess changes in the chickens' microbiota over time, we collected cloacal swabs of 10 hens per flock and performed 16S rRNA gene amplicon sequencing. The poultry house (i.e., the stable in which flocks were housed) and sampling time explained 9.2 and 4.4% of the variation in the microbial community composition of the flocks, respectively. Remarkably, access to an outdoor range had no detectable effect on microbial community composition, the variability of microbiota among chickens of the same flock, or microbiota richness, but the microbiota of outdoor flocks became more even over time. Fluctuations in the composition of the microbiota over time within each poultry house were mainly driven by turnover in rare, rather than dominant, taxa and were unique for each flock. We identified 16 amplicon sequence variants that were differentially abundant over time between indoor and outdoor housed chickens, however none were consistently higher or lower across all chickens of one housing type over time. Our study shows that cloacal microbiota community composition in adult layers is stable following a sudden change in environment, and that temporal fluctuations are unique to each flock. By exploring microbiota of adult poultry flocks within commercial settings, our study sheds light on how the chickens' immediate environment affects the microbiota composition.

Keywords: poultry (chicken), cloacal microbiota, 16S rRNA gene amplicon sequencing, temporal dynamics, host microbiome, outdoor range

INTRODUCTION

The digestive tract of chickens is colonized by complex microbial communities, which play important roles in their overall health and performance (Yegani and Korver, 2008; Ducatelle et al., 2015; Kogut, 2019). Changes in the chickens' microbiota have been linked to many factors (Kers et al., 2018), including host related factors such as age (Cox et al., 2014; Videnska et al., 2014b; Jurburg et al., 2019; Ngunjiri et al., 2019) and breed (Schokker et al., 2015; Richards et al., 2019). Outside of the host, differences in climate, soil, litter, and feed affect the host's exposure to other microbes, which may colonize the animal's intestinal tract (Björk et al., 2019). Indeed, under controlled settings, housing conditions have been found to modulate the chickens' microbiota (Hubert et al., 2019; Kers et al., 2019). How the gut microbiota responds over time to changes in the housing environment under standard farm conditions is less well understood, however.

In commercial settings, layers may be restricted to indoor housing, or may have access to an outdoor range. Layers housed in free-range environments have different microbial community compositions and higher diversity than indoor housed layers (Xu et al., 2016; Chen et al., 2018; Hubert et al., 2019). However, in these studies the effect of access to a free range was compared between caged and free-range chickens in semi-experimental setups (Xu et al., 2016; Chen et al., 2018; Hubert et al., 2019). Furthermore, chickens were given access to the outdoor range during the rearing period (6–11 weeks of age, Xu et al., 2016; Chen et al., 2018). An increasing amount of commercial layer flocks are kept in aviary systems rather than in cages (Miao et al., 2005), in which the layers in free-range or organic systems are given access to an outdoor range after the rearing period (approximately 17 weeks of age). Previous research showed that access to an outdoor range only explained limited variation in the community composition in a cross-sectional study (Schreuder et al., 2020). However, this study sampled animals only once after long-term acclimation and it was not possible to determine whether the microbiota had been affected by outdoor exposure and recovered to their original composition over time, i.e., resilient, or whether the microbiota were resistant to outdoor range exposure, i.e.,

resistant (Sommer et al., 2017). These temporal dynamics and the immediate effects of exposure to a new environment remain poorly understood.

Microbial communities exhibit complex, non-linear temporal dynamics, especially during host development (Jurburg et al., 2019; Kers et al., 2020). Understanding how the hosts' microbiota respond to environmental fluctuations requires temporal monitoring in order to detect changes in the microbial community over time, following exposure to new conditions. To date, a limited number of studies have explored the temporal dynamics in the gut microbiota of layers (Videnska et al., 2014b; Ballou et al., 2016; Han et al., 2016; Polansky et al., 2016a; Ngunjiri et al., 2019). Most studies focus on young layers (aged 0–8 weeks), and are performed under experimental conditions (Ballou et al., 2016; Polansky et al., 2016a; Han et al., 2017). However, adult chickens have fully developed microbiota, which are more stable than microbial communities of young layers (Videnska et al., 2014b; Ngunjiri et al., 2019). It has been proposed that as an animal ages, the host's influence on microbial selection increases due to physiochemical maturation of the gut and the ability of the host to curate its microbiota (Björk et al., 2019), likely making the microbiota of adult layers less prone to external perturbations or changes (Schreuder et al., 2019).

It is essential to understand how the gut microbiota of commercial animals respond to environmental changes to guarantee their health in the face of unforeseen events, such as disease outbreaks. To examine the extent to which sudden environmental changes affect the gut microbiota of adult layers in commercial setting, we monitored the cloacal microbiota of four flocks of laying hens over 4 months following the lifting of mandatory indoor confinement regulations, which was a unique opportunity to study the effects of the outdoor range access on the microbiota of commercial chickens over time. Over a 16 weeks period, we sampled 10 chickens per flock three times in 8 weeks intervals. We hypothesized that as layers accessed an outdoor range, they would be more exposed to alternative food sources and novel environmental microbes, and microbial richness would increase over time in outdoor chickens. If the colonization of novel microbes occurred stochastically (i.e., randomly), we also expected the microbiota

of outdoor flocks to become more variable between outdoor chickens than indoor chickens.

MATERIALS AND METHODS

Study design

Four commercial flocks of laying hens (Dekalb White) were selected for this study: two layer flocks with access to an outdoor range and two flocks without access to an outdoor range (Figure 1). To minimize potential variation in the microbiota composition due to rearing and other environmental factors, outdoor and indoor flocks were selected based on the rearing farm of origin, numbers 1 and 2, respectively (Figure 1). The sampled flocks were kept in separate poultry houses, which were located on three different poultry farms: indoor (IA1) and outdoor flock 1 (OA1) were located on the same farm, indoor (IB2), and outdoor flock 2 (OC2) were located on two different farms (Figure 1). Flocks IA1 and OA1 were 33 weeks old at the start of the sampling, and flocks IB2 and OC2 were 24 weeks old. All flocks were well-producing and healthy based on regular veterinary inspections during the study, and had not been treated with antibiotics on the layer farm.

None of the layers from the indoor or outdoor flocks had access to the outdoor range prior to the start of this study due to the mandatory indoor confinement measures, which were instated because of HPAI outbreaks in the winter of 2016– 2017. All flocks were sampled three times in 2017: the first sampling took place 1–2 days after the lift of mandatory indoor confinement at the end of April 2017; and the second and third sampling rounds took place 8 and 16 weeks after the lifting date, respectively (Figure 1). During each sampling round, all flocks were sampled in the same week, to avoid short term weather effects. We did not sample flock IB2 on the third sampling round, because these chickens were in the process of forced molting at that time, in order to reduce fipronil contamination during the fipronil affair in the Netherlands (Sok et al., 2020). Molting was induced by feed deprivation, and feed deprivation has major impact on the gut microbiota composition (Dunkley et al., 2007), making the samples of the chickens of flock IB2 unsuitable for our study at the time of the third sampling.

Flock	Rearing Farm	Farm	0 weeks	8 weeks	16 weeks
IA1	1	A			
IB2	2	B			
OA1	1	A			
OC2	2	C			

Figure 1: Study design. Four flocks were sampled three times (0, 8, and 16 weeks after the study began) each. Two indoor flocks (IA and IB) and two outdoor flocks (OA and OC) were sampled. Flock IB2 was not sampled on week 16. Week 0 began 1–2 days after the lift of mandatory indoor housing ban of all layer flocks and none of the layer flocks had access to the outdoor range prior to that moment. Flocks IA1 and OA1 were located on the same farm (1), originated from the same rearing flock (A) and were of the same age (33 weeks at the start of sampling). Flocks IB2 and OC2 were located on two different farms (2 and 3), originated from the same rearing flock (B) and were of the same age (24 weeks at start of sampling). Cloacal swabs of 10 chickens per flock were collected at each sampling time.

Both indoor and outdoor layer flocks were kept in cage-free aviary systems with a maximum stocking density of nine chickens per m^2 with one flock per house (Schouwenburg, 2019a). The hens of each flock were placed in the poultry house on the layer farm around 17 weeks of age. Flock IB2 had a different feed supplier than OA1, IA1, and OC2, but all flocks received a similar standard commercial feed for layers according to their age with a similar regime across farms, and no changes in the feed composition occurred during the period of the study. The laying hens in outdoor flocks had access to an outdoor range during the day with at least 4 m^2 per hen according to standards of the Dutch quality assurance scheme, i.e., the Integrated Chain

Control program, “IKB Egg” (Schouwenburg, 2019b). The hens had access to the outdoor range for 8 h per day on average (van Niekerk and Leenstra, 2016a). Outdoor ranges were mostly open grass field with some trees, and bare soil directly around the poultry house, with drainage systems to prevent formation of rain puddles (van Niekerk and Leenstra, 2016a).

DNA sampling, extraction, and 16S rRNA gene amplicon sequencing

At each sampling time, two cloacal dry swabs per chicken were collected from 50 laying hens per flock. The swabs were inserted deep into the cloaca to ensure we would collect enough fecal material. Wired panels, dividing the house in multiple subsections, were present in all houses, and an equal number of birds was randomly selected from each subsection within each flock. Samples were placed on ice immediately after collection and stored dry at -80°C within 5 h after collection.

Per flock and sampling time, swabs of 10 of the 50 sampled chickens were selected based on equal distribution across the poultry house. One swab of each chicken was used for analysis. Prior to analysis, each swab was visually assessed to ensure that sufficient fecal material was present for DNA extraction. DNA extraction and subsequent 16S rRNA gene amplicon sequencing were performed according to the protocol described in Schreuder et al. (2020). In each DNA isolation round, a negative control sample containing PBS was added to identify possible contamination from reagents, and DNA extracts were quantified with InvitrogenTM QubitTM 3.0 Fluorometer and stored at 20°C for further processing. The V3–V4 region of the 16S rRNA gene was amplified in a PCR with the primers CVI_V3-forw CCTACGGGAGGCAGCAG and CVI_V4-rev GGACTACHVGGGTWTCT and amplified as previously described (Schreuder et al., 2019): step 1: 98 °C for 2 min, step 2: 98°C for 10 s, step 3: 55°C for 30 s, and step 4: 72°C for 10 s, step 5: 72°C for 7 min. Steps 2–4 were repeated 25 times. Negative controls were included at each amplification round to confirm sterility of PCR reagents. PCR products were checked with gel electrophoresis, and PE300 sequencing was performed using a MiSeq sequencer (Illumina Inc., San Diego, CA). Negative controls from the DNA extraction did not contain

any sequences and were discarded (n = 6). An additional 16S rRNA gene qPCR was performed on theDNA samples to quantify the amount of 16S rRNA gene DNA and identify samples of poor quality (Supplementary Table S1). The qPCR consisted of 40 runs with the same primers and protocol as for the 16S barcoding PCR. Samples which contained very low 16S rRNA gene DNA concentrations or low quality melting curves were excluded from the analysis (n = 10). The final dataset contained 100 samples. The number of samples per house for each sampling time ranged between 7 and 10 samples at each timepoint (Supplementary Table S1).

Processing of sequencing data

All sequence data processing was performed in R 3.6.3 (R Core Team, 2013). The sequence reads were quality-filtered, primer-trimmed (35 nucleotides), error-corrected, dereplicated, merged into pseudoreads and chimera-filtered using the dada2 package (Callahan et al., 2016) using standard parameters [TruncLength = (240, 210), MinOverlap = 10 and maxEE = (2,2)], and reads were assigned with the SILVA v.132 classifier (Quast et al., 2012). The final dataset contained 100 samples, which were rarefied to 8,170 reads per sample (rarefy_even_depth, seed = 1) and a total of 2,839 amplicon sequence variants (ASVs) distributed over 347 genera.

Statistical analyses

All downstream analyses were performed in R (version 3.6.3) with the phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen et al., 2007) packages. We measured diversity as the number of observed ASVs in the rarefied samples and taxon evenness with Pielou's index (Pielou, 1966). A linear mixed effects model was fitted to both diversity measures, with poultry house as a random effect and sampling time and housing type as fixed effects using the lme4 package (Bates et al., 2015). Bray and Curtis (1957) and Sørensen (1948) dissimilarities were used to evaluate differences in community structure between the layers on Hellinger-transformed abundances. Community composition was visualized with principal coordinates analyses (PCoA) of Bray-Curtis and Sørensen dissimilarities.

Differences between the microbiota composition of layers were examined for each factor using the adonis function. Variance in community composition within a flock was evaluated as the Bray–Curtis and Sørensen pairwise distances between flock members. To assess the contribution of each factor to the observed variation in the microbiota composition, we performed a distance-based variation partitioning (Borcard et al., 1992) and distance-based redundancy analysis (dbRDA) using Bray–Curtis dissimilarities (Anderson, 2001). We included housing type (indoor and outdoor layers, HousingType), poultry house (stable in which flocks were housed) and sampling time (SamplingTime) as explanatory variables. Feed, age, farm, and rearing farm were nested within poultry house (Figure 1 and Supplementary Table S1), and thus were not included. Model selection for dbRDA was performed with forward selection based on Akaike's Information Criterion (AIC), with the lowest AIC indicating the best fit (Blanchet et al., 2008).

To visualize the number of taxa that were shared between poultry houses across sampling times, we used Venn-diagrams on rarefied data. In the Venn-diagrams, taxa were considered as rare when the relative abundance was < 0.01% across all samples. We used Wilcoxon rank-sum tests to check for differences in relative abundances of the 10 most abundant phyla and of genera with a relative abundance of at least 0.5% over time within each housing type. Unless otherwise indicated, results are expressed as mean ± SD throughout the manuscript. We used DESeq2 analysis (Love et al., 2014) on non-rarefied data to detect ASVs that were differentially abundant over time between indoor and outdoor housed chickens.

Figures made with ggplot2 and ggpubr packages.

RESULTS

Microbial community composition

We evaluated the composition of the microbial community in the cloacal samples of all layers. At the phylum level, we observed similarities between the microbiotas of indoor and outdoor layers (Supplementary Figure S1), and no significant differences in the relative abundances of the 10 most

abundant phyla were found. These 10 phyla constituted 99.8% of the community, across all samples. On average across all samples, the microbial communities were dominated by *Firmicutes* (63.7 ± 17.3%), *Proteobacteria* (13.4 ± 14.3%), and *Fusobacteria* (9.0 ± 15.4%; Supplementary Figure S1). At genus level, members of the genera *Romboutsia* (31.4 ± 22.3%), *Gallibacterium* (9.5 ± 12.1%), and *Fusobacterium* (8.9 ± 15.2%) were most abundant across all samples (Supplementary Figure S2).

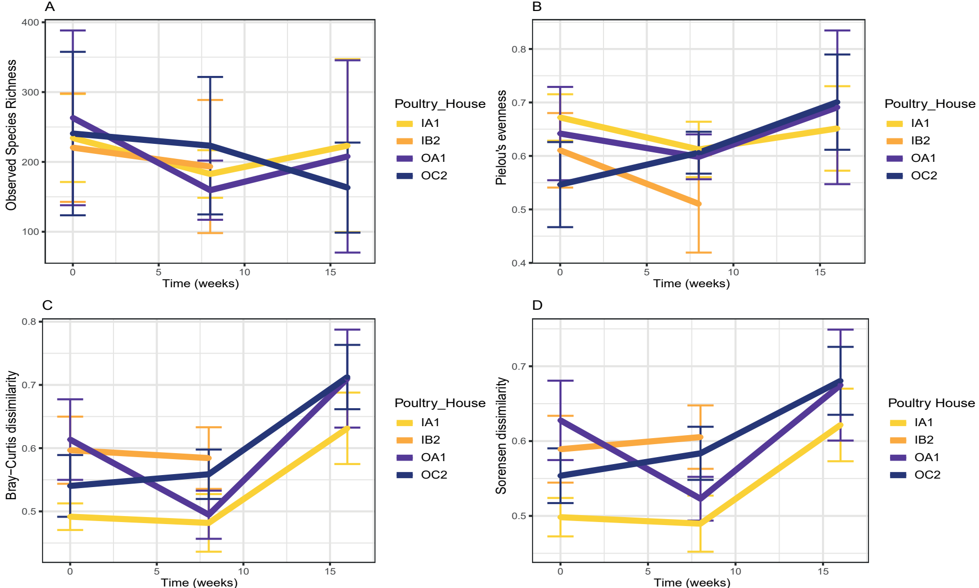

Figure 2: Temporal trends in observed species richness (A) and Pielou's evenness (B) per poultry house at each sampling time. Pairwise Bray–Curtis (C) and Sørensen (D) dissimilarities between the cloacal microbiota of layers from poultry house at each sampling time. In C and D, greater values indicate higher dissimilarity. Means ± confidence interval are shown. Time is shown as weeks since first sampling.

We did not find temporal patterns in species richness in both indoor and outdoor housed chickens (Figure 2A). A modest, but significant increase in evenness was detected in chickens from outdoor houses over time (from 0.59 ± 0.12 at 0 weeks to 0.70 ± 0.13 at 16 weeks; $p < 0.001$; Figure 2B).

To analyze changes in community composition over time, we evaluated Bray–Curtis and Sørensen dissimilarities between chickens of each flock at each sampling time (Figures 2C,D). Although microbiota of chickens in outdoor flocks were more variable than those of indoor flocks

on average, this was not significant, and the variation did not significantly increase over time (Figures 2C,D). Across all samples, variation between chickens from each poultry house had increased at 16 weeks compared to the first sampling ($p < 0.001$).

For both dissimilarity measures, microbial communities clustered according to poultry house (Figure 3), which explained most of the variance in the community (Bray–Curtis R^2 = 14.5%, adonis, $p < 0.001$; Sørensen R^2 = 14.5%, adonis, $p < 0.001$; Table 1). Sampling time (Bray–Curtis R^2 = 2.97%, adonis, $p = 0.013$, Sørensen R^2 = 3.75%, adonis, $p < 0.001$) and housing type (Bray–Curtis R^2 = 2.91%, adonis, $p = 0.001$; Sørensen R^2 = 3.41%, adonis, $p < 0.001$) explained limited variation, but were significant for both dissimilarity measures (Table 1).

Table 1: Overview of explained variation in community composition by individual factor as tested with a PERMANOVA-like adonis.

Factor	Dissimilarity measure	R^2 (adonis)	F.Model	*p*	FDis
Time	BC	2.97	2.98	0.0013	0.01
	Sørensen	3.75	3.82	<0.001	0.11
Housing type	BC	2.91	2.94	0.001	0.78
	Sørensen	3.41	3.46	<0.001	0.19
Poultry House	BC	14.49	5.42	<0.001	0.68
	Sørensen	14.48	5.42	<0.001	0.26
Farm	BC	11.81	6.50	<0.001	0.65
	Sørensen	11.81	6.50	<0.001	0.48
Rearing farm	BC	8.42	9.01	<0.001	0.46
	Sørensen	7.43	7.87	<0.001	0.14

Both Bray–Curtis and Sørensen dissimilarity were used. R^2 = Percentage of the variation between chickens explained. Significance was tested with 9,999 permutations.

To further disentangle the effects of poultry house, sampling time and housing type, we performed a distance-based variance partitioning using Bray–Curtis dissimilarities (Figure 4). Poultry house explained most of the variation in community composition (9.2% R^2 adj) and sampling time explained 4.4% of the variation (R^2 adj). In contrast, housing type alone did not explain any variation. This was further supported by a dbRDA (Supplementary Figure S2). Model selection supported a model with both poultry house and sampling time (AIC = 301.38) compared to a full model,

with housing type and the interaction between housing type and sampling time (AIC = 304.64). Poultry house ($p = 0.005$) and sampling time ($p = 0.005$) were both significant in this most parsimonious model (Supplementary Figure S2).

Differential abundance of individual taxa over time

We identified ASVs that were shared by the different poultry houses over time (Figure 4). ASVs that were shared between all poultry houses had a lower percentage of rare taxa (40.1%) than ASVs that were unique to a poultry house (between 94.1 and 98.0%, Figure 4). Each poultry house had a similar number of shared taxa between all sampling times (between 257 and 322 ASVs, with 9.3–14.9% rare ASVs), whereas the amount of unique taxa to a sampling time varied between 103 and 437 ASVs, but the percentage of rare ASVs was similar at each sampling time ranging between 66.3 and 93.8% (Figures 5A–C). Most rare ASVs belonged to the phyla *Firmicutes* (54.6%) and *Bacteroidetes* (25.9%, Figure 5D).

DESeq2 analysis was performed to determine if specific ASVs were differentially abundant over time between indoor and outdoor housed chickens. We compared a full model with factors: HousingType + SamplingTime + HousingType:SamplingTime to a reduced model with factors HousingType + SamplingTime, and identified 16 ASVs with differential responses (Supplementary Figure S4). These 16 ASVs belonged to nine genera in two phyla, *Firmicutes* and *Actinobacteria*. Most ASVs ($n = 8$) belonged to the genus *Lactobacillus* (Figure 6). The 16 ASVs had an average relative abundance of $0.60 \pm 0.65\%$ across all samples, but none of the ASVs showed a consistent increase or decrease in all chickens of one housing type over time (Figure 5 and Supplementary Table S2). The genus *Lactobacillus* also fluctuated significantly over time in outdoor housed chickens ($p < 0.001$, Kruskal–Wallis test), but not in samples from indoor housed chickens ($p > 0.001$, Kruskal–Wallis test). Furthermore, genera *Akkermansia* and *Aeriscardovia* ($p < 0.001$, Kruskal–Wallis test both) fluctuated significantly over time in outdoor chickens, but not in indoor housed chickens (Supplementary Table S3).

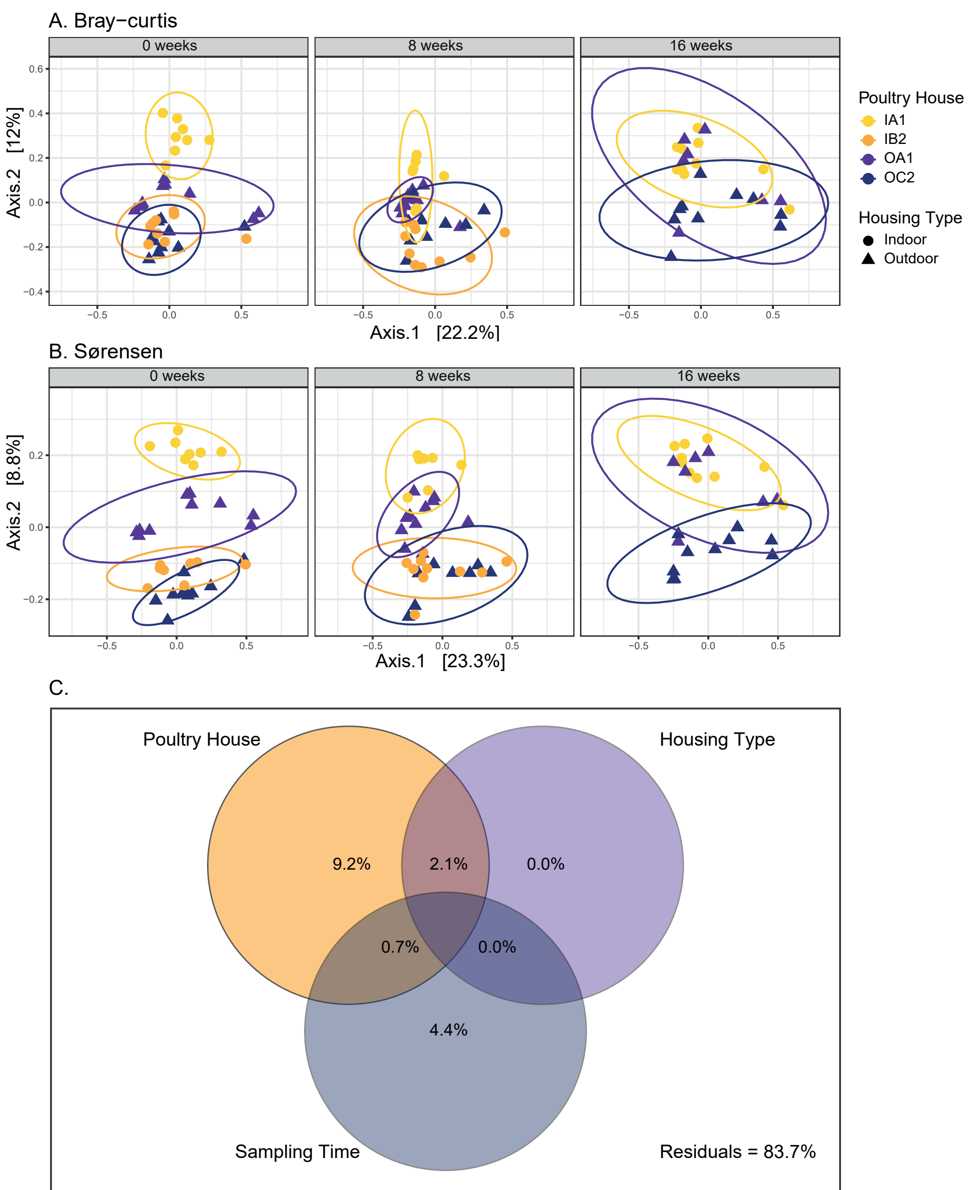

Figure 3: Principal coordinate analysis (PCoA) of Bray–Curtis (A) and Sørensen (B) dissimilarities. Each PCoA is faceted per sampling time, with ellipses encircling poultry houses. Each symbol represents an individual chicken. Time is shown as weeks since first sampling. (C) Distance-based partitioning of Bray–Curtis dissimilarities with poultry house, housing type and sampling time as explanatory variables. Adjusted R^2 are shown in each circle. The model explains 14.8% of variation in community composition overall.

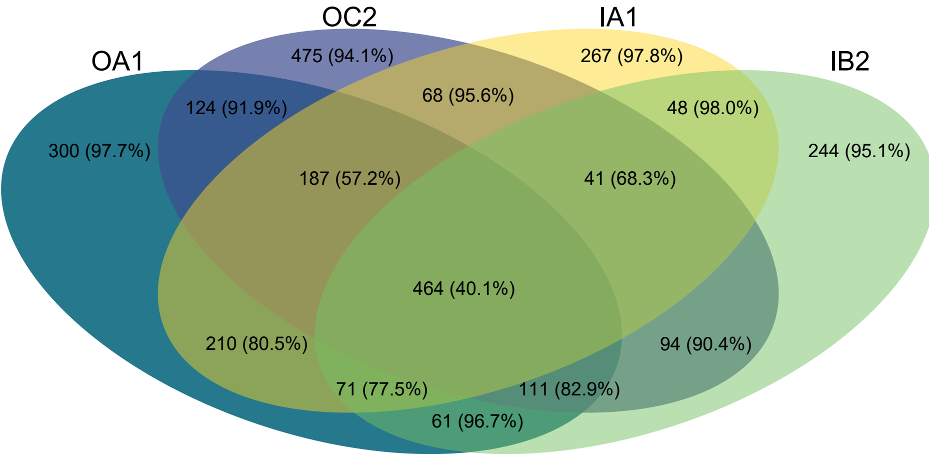

Figure 4: Venn diagram depicting the number of shared ASVs per poultry house across all timepoints. In parentheses, the percentage of these ASVs which are rare (relative abundance of < 0.01%) are shown.

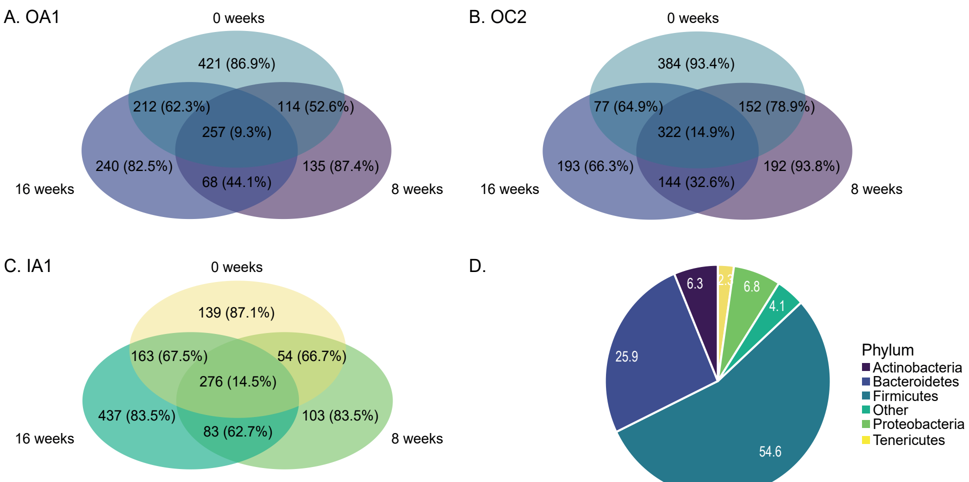

Figure 5: Venn diagrams of each poultry house [(A) OA1; (B) OC2; (C) IA1] showing the number of ASVs shared across all timepoints or unique to a single sampling time. Poultry house IB2 was excluded. Number of ASVs are shown for each compartment, and the percentage of rare taxa (ASVs with relative abundance of < 0.01%) are shown in parentheses. (D) Relative abundances (%) of five most abundant phyla within the subset of rare ASVs (relative abundance < 0.01% across all samples).

DISCUSSION

Many factors in the immediate environment of the chicken can influence the microbiota community composition (Kers et al., 2018). In this temporal study in commercial laying hens, we found that of the variables measured, poultry house explained most variation in community composition in the flocks' microbiota (9.2%), whereas access to an outdoor range (housing type) did not explain any of variation in the microbial community. Some temporal effects were found, but the proportion of variation explained by time of sampling (4.4%) was comparatively smaller than that of poultry house. At the level of community diversity, flocks which were allowed into the outdoor range did not become more variable or more species-rich over time, and the chickens' microbiota showed a modest but significant increase in evenness over time in outdoor flocks, but not in indoor flocks. The latter was not accompanied by changes in species richness over time, which indicates that the increase in evenness over time in outdoor layers did not result from the colonization of more species in the chickens' microbiota, but rather from a shift in abundances. Abundances of several ASVs were found to fluctuate differently between indoor and outdoor layers over time. However, none of the ASVs showed a consistent increase or decrease in all chickens of one housing type over time. Previous research found a slightly higher variation in community composition in indoor flocks relative to outdoor flocks, but also found large differences in variation between poultry houses from the same housing type (Schreuder et al., 2020). In this study, the poultry house also was the most important driver of community composition, and outdoor range access only had a modest effect on the microbiota community of chickens across eight separate flocks (Schreuder et al., 2020). The results of the current study further highlight that the environment of the poultry house is an important driver for community composition, even over time.

We found that differences in microbial communities over time between layers within each flock were most likely driven by the replacement of rare taxa between sampling times within a poultry house. Indeed, most of the taxa, between 66.3 and 93.8%, at each sampling time were rare, and 94.1–97.8% of taxa that were unique to a poultry house, were also rare. Moreover,

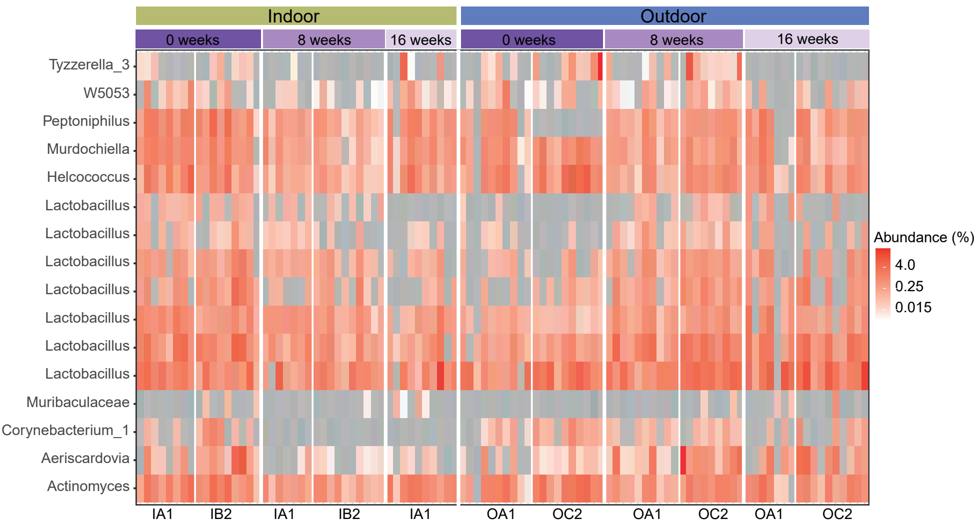

Figure 6: Heatmap of the relative abundance of 16 differentially abundant ASVs that were significantly different over time between indoor and outdoor housed chickens (DESeq2, fdr $p < 0.01\%$). Samples are ordered by poultry house for each sampling time and divided by housing type (indoor vs. outdoor). Each box represents the relative abundance of an ASV in an individual chicken. 0 values are shown as gray boxes.

no difference in explained variation was found when communities were weighted by their relative abundances (Bray-Curtis dissimilarity) compared to using presence/absence data (Sørensen dissimilarity), indicating that taxon abundance was likely less relevant in differentiating these communities. Costa et al., (2017) also found that treatment with different antimicrobials resulted in changes in community membership of cecal microbiota of broilers, but not in community structure, suggesting that the antimicrobials had a greater impact on rare taxa, rather than on dominant ones. These findings indicate that temporal fluctuations are unique to each flock within each poultry house and support the need to learn more about the functional role of rare bacteria, and the need for techniques which focus on analyses of active bacteria (i.e., metatranscriptomics).

The strong influence of poultry house on the microbiota suggests that the living environment of the chicken is important in shaping the hens' microbiota, however we found no effect of moving outdoors. One explanation for this phenomenon and the relatively small effect of sampling time on the

community composition compared to previous research (Jurburg et al., 2019; Kers et al., 2019), is the developmental stage of the chickens studied. Layers of flocks in this study were adult chickens of either 24 or 33 weeks old at the first time of sampling. To date, most temporal studies in chickens looked at the temporal dynamics of young chickens and thus at changes in the primary environment of the host as a result of the host's development (Cox et al., 2014; Oakley and Kogut, 2016; Jurburg et al., 2019; Kers et al., 2019; Richards et al., 2019). Here, we studied the effect of temporal changes in a secondary environment in adult layers (i.e., indoor or outdoor range), where the effect of the outdoor range was likely dampened by the adults' host homeostatic responses. As an animal host ages, its influence on microbial selection in the development of the intestinal microbiota increases (Björk et al., 2019). Indeed, layers above the age of 25 weeks (Ngunjiri et al., 2019) or 28 weeks (Videnska et al., 2014b) reach an adult microbial equilibrium (Videnska et al., 2014b). It is likely that in our case the chicken microbiota was more plastic at an earlier stage, as we still see a strong effect of the rearing farm on the chickens microbiota in this study (Table 1). In the Dutch table egg production system, groups of laying hens reared together in one rearing farm are transported to the poultry houses of the final layer farm at the age of 17–18 weeks. By the time the layers were allowed outside in our study, the layers' intestinal microbiota had likely already reached a stable equilibrium, which is less prone to perturbations (Schreuder et al., 2019). A well-developed intestinal microbiota community protects the host by creating gastrointestinal resistant environments, which help prevent external microbiota from colonizing, i.e., resistant (Lawley and Walker, 2013; Han et al., 2017), and is characterized by a capacity for self-regeneration after an external perturbation, i.e., resilience (Lozupone et al., 2012; Sommer et al., 2017). In previous research, it was not possible to determine whether the microbiota of adult layers were resistant or resilient after exposure to an outdoor range, because the temporal changes weren't taken into account. The current study indicates that the microbiota of these adults layers was likely resistant rather than resilient.

Alternatively, the limited effect of the outdoor range on the chickens' microbiota may occur if the chickens only made limited use of the outdoor

range, despite having access. The effects of access to an outdoor range in previous studies (Xu et al., 2016; Chen et al., 2018; Hubert et al., 2019; Ocejo et al., 2019) have been related to greater substrate diversity and intake of fibrous feedstuff (Xu et al., 2016), as well as exposure to more abundant microbiota from the outdoor environment (Hubert et al., 2019). However, likely only a small proportion of the hens in the outdoor flocks of our study used the outdoor range extensively. Previous research estimated that only 3–15% of layers in large commercial flocks (> 10,000 layers) used the outdoor range (Bestman and Wagenaar, 2003; Hegelund et al., 2005), with individual hens using the range differently, of which many did not enter the free-range every day (Gebhardt-Henrich et al., 2014). Nevertheless, chickens that do not go outdoors themselves could indirectly become affected by the altered microbiota of their flock mates that do go outside, as these also defecate indoors. Humans and animals that are housed together are known to exchange microbiota (Song et al., 2013; Schloss et al., 2014), and this effect may be enhanced for coprophagic animals, including chickens (Kers et al., 2018; von Waldburg-Zeil et al., 2019). However, with a rather stable microbiota community, the small changes in the chickens that go outdoors are also less likely to affect the stable microbiota community of the chickens remaining indoors. Furthermore, other studies have shown that chickens tend to use the area immediately outside the poultry house most (Hegelund et al., 2005; Bestman, 2017), resulting in trampled vegetation and hence, lower availability of fibrous feedstuff. Both the limited use of the outdoor range by the hens, and the low availability of fibrous feedstuff in the most frequently used part of the range, together with the age of the animals, may explain why we found no effect of access to an outdoor range on the microbial community of these adult layers.

In order to sample commercial layer flocks, we collected cloacal swabs because the longitudinal follow-up required a rapid and minimally invasive sampling methodology, without sacrificing the birds. To ensure the cloacal swabs contained enough fecal material, the cloacal swabs were inserted deeply into the cloacal opening to enter the last part of the colon and the swabs were visually assessed prior to DNA extraction. Although

research has shown that cloacal and fecal microbiota of chickens might not be an accurate representative of the cecal composition and are more variable (Williams and Athrey, 2020), it has also been shown that fecal samples are qualitatively similar to the cecal microbiota (Stanley et al., 2015) and non-shared taxa between cloacal and cecal samples accounted for a very low percentage of the diversity: 0.49% in one case (Andreani et al., 2020) and 0.75% in another (Stanley et al., 2015). Furthermore, it has been reported that cloacal swabs are similar to fecal samples (Videvall et al., 2018), and shifts in microbiota composition have been detected successfully using fecal samples (Oakley and Kogut, 2016; Jurburg et al., 2019). Therefore, we anticipated that major shifts in community composition would have been detected by our way of sampling. Nevertheless, future studies should carefully consider the trade-off between applicability of a sampling technique in commercial practice vs. the quality of the taken sample.

CONCLUSION

In conclusion, our study gives insight into the temporal dynamics of the cloacal microbiota of adult layer flocks exposed to environmental change. We find that cloacal community composition in adult layers is rather stable, even after a sudden environmental change, illustrating the layers' ability to maintain their own microbiota. Furthermore, we show the strong influence of poultry house on the microbiota composition of these layers, and that temporal dynamics are unique to each poultry house. Our study thus sheds light into the drivers of the poultry microbiota, and the stability of the adult chicken microbiota to environmental change, however our understanding of the temporal dynamics of adult animal microbiota remains limited. Future research should consider the influence of a host's immediate environment (i.e., poultry house) and the animals' previous exposure to environmental change (i.e., rearing farm). Furthermore, the stability of adult poultry microbiota should be tested in both healthy and diseased flocks, with shorter sampling intervals and larger sample sizes across multiple commercial flocks.

AUTHOR CONTRIBUTIONS

RB, WdB, JS, AS, and JS initiated this project. RB, JS, WdB, PvH, SJ, AS, and FV contributed to the design of the experiment. JS performed sample collection and manuscript writing. JS, AB, and SJ did data processing and analysis. SJ, AS, FV, JS, RB, AB, PvH, and WdB contributed to the development of the manuscript by giving constructive feedback on the manuscript during its preparation. AS and SJ contributed equally and are both considered last author. All authors gave approval of the manuscript for publication.

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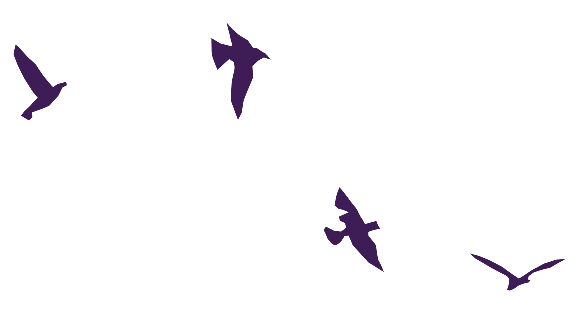
SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.626713/full#supplementary-material

5

Wild bird densities predict spatial HPAI outbreak risk across the Netherlands using data from 2014-2018



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In submission

ABSTRACT

Introduction: Introduction of highly pathogenic avian influenza viruses (HPAIVs) from infected wild birds in poultry farms occurs on a large global scale, especially in the migratory season. To map HPAI outbreak risk in relation to spatial differences in wild bird densities and land cover variables, a retrospective case control study, using locations of 16 HPAI outbreaks (i.e. cases) on poultry farms and 10 control farms per case was performed.
Methods: HPAI risk was modelled in relation to bird density and land cover variables in the Netherlands. A random forest model was used in a leave-one-group-out cross validation approach to predict outbreak risk based on densities of 54 wild bird species and five land cover categories analyzed at different spatial scales.
Results: Spatial differences in the densities of 17 waterbird species, of which 11 of the family Anatidae, and two raptor species, were most important for predicting HPAI outbreaks. Land cover variables had no added value and were excluded from the model. The model had an average precision of 88%, and was used to construct a HPAI outbreak risk map for the Netherlands.
Discussion: Despite the limited number of cases, HPAI risk areas were accurately predicted. A similar modelling approach can be used elsewhere, to generate region-specific predictions, which may include land cover data in addition to bird data, depending on the local situation. Risk maps can help in prioritization of areas for surveillance and biosecurity measures, and support decisions on establishments of new poultry farms to reduce HPAI outbreak risks.

Keywords: avian influenza, influenza A virus, poultry, disease outbreaks, wild birds, spatial modelling, random forest

INTRODUCTION

Highly pathogenic avian influenza A viruses (HPAIVs) of clade 2.3.4.4 have spread globally causing massive outbreaks in commercial poultry farms (Verhagen et al., 2015b; Adlhoch et al., 2020), especially from 2014 onwards. The migratory movements of wild birds were shown to play an important role for the global spread of HPAI H5N8 in 2014, as spatial and temporal patterns of outbreaks coincided with migratory flyways and the timing of autumn migration (Verhagen et al., 2015b; Lycett et al., 2016). H5N8 (2014 and 2016) and H5N6 (2017) have caused outbreaks in Europe on commercial poultry farms, as well as massive mortality in wild birds (Bouwstra et al., 2015a; Verhagen et al., 2015a; Beerens et al., 2017; Kleyheeg et al., 2017). Currently, HPAI outbreaks have been reported in 18 EU countries since the autumn of 2020 of which most are of subtype H5N8. Until 8 February 2021, 1527 HPAI outbreaks were reported, of which most cases in wild birds (n = 948), primarily in waterbirds such as barnacle goose (*Branta leucopsis*), greylag goose (*Anser anser*) and Eurasian wigeon (*Mareca penelope*), and numerous poultry (n = 548) (Adlhoch et al., 2020; ADNS, 2021).

HPAI outbreaks on poultry farms are spatially associated with the proximity of waterbodies or the presence of wild birds (Belkhiria et al., 2018; Napp et al., 2018; Velkers et al., 2020). For example, Velkers et al. (2020) showed that the density of HPAI high-risk bird species around infected poultry farms in wetlands was significantly higher than around non-infected farms in non-water-rich areas. This was especially true for the Eurasian wigeon, which was one of the species with massive mortality due to HPAI in 2016-2017 (Kleyheeg et al., 2017). Also, dead wild birds found at sites in the vicinity of HPAIV infected poultry farms had phylogenetically related viruses, suggesting that HPAIV on these farms originated from infected wild birds (Beerens et al., 2017). HPAIV introduction into poultry houses most likely results from indirect contact with wild birds, and it is hypothesized that the virus enters the poultry house via vectors or fomites contaminated with wild bird feces (Beerens et al., 2019; Elbers and Gonzales, 2020). This suggests that wild bird presence and abundance can be used as predictor in identifying HPAI high risk areas.

The recurrent outbreaks of HPAI underline the need for better prediction of HPAI risk areas to reduce outbreak risk and take appropriate measures. Previously, disease distribution models showed that land cover, particularly the presence of wetlands, were highly predictive for the HPAI risk in California (Belkhiria et al., 2018), and land cover and environmental variables were used to successfully predict HPAI outbreak risk (Si et al., 2013). However, landscape variables are merely a proxy for presence of wild birds, and we therefore hypothesize that wild bird densities can more accurately predict HPAI outbreak risk compared to landscape variables only. The aim of this study was to model HPAI outbreak risk across the Netherlands in relation to wild bird density data and landcover features in the Netherlands, to generate a HPAI risk map, and identify wild bird species and land cover variables that are associated with HPAI outbreak risk on poultry farms.

MATERIALS AND METHODS

Study design

Case farms

A case control study was performed retrospectively, using all 16 diagnosed HPAIV H5N8 and H5N6 infections on poultry farms in the Netherlands between the autumn-winter periods of 2014/2015, 2016/2017 and 2017/2018. Six outbreaks were on layer farms, seven in Pekin duck farms, and three on broiler breeder farms. Some farms were affected repeatedly in different years (Table 1). The multiple outbreaks on these farms were all primary introductions and occurred in different years (Table 1).

Control farms

We randomly selected 10 unique uninfected poultry farms from the Netherlands Food and Consumer Product Safety Authority (NVWA) database for each HPAI case farm in every year. Control farms were selected based on similar poultry type to the infected case farm (i.e. broiler breeder, layer, or Pekin duck farm), registration as an active poultry farm in the same year as the outbreak year in the case farm, and located within a 100 km

radius of the case farm. Farms that had been a case in any of the years were excluded for control selection and controls could only be selected for one case farm. We were unable to select ten controls for cases on Pekin duck farms due to the high number of affected Pekin duck farms relative to their overall abundance in the Netherlands. For these cases, we selected four to eight controls per case. In total, we included 132 control farms consisting of 60 layer, 30 broiler breeder and 42 Pekin duck farms (Table S1).

Table 1: Overview of highly pathogenic avian influenza (HPAI) cases in the Netherlands on individual farms (ID 1 to 12) with confirmed HPAIV infection between 2014-2018. Poultry type indicates the type of farm that was affected. For each year the month when the HPAIV infection was diagnosed on a poultry farm, is shown. For poultry farms 1 and 6, two HPAI outbreaks were diagnosed between 2014-2018, and on one poultry farm (CaseID 4), three outbreaks were diagnosed between 2014-2018.

Case-ID	Poultry Type	2014-H5N8	2016-H5N8	2017-H5N6	2018-H5N6
1	Layer	11	12		
2	Layer	11			
3	Layer	11			
4	Pekin Duck	11	12		3
5	Broiler Breeder	11			
6	Pekin Duck		12	12	
7	Pekin Duck		11		
8	Pekin Duck		11		
9	Layer		12		
10	Layer		12		
11	Broiler Breeder		12		
12	Broiler Breeder				2

Selection of wild bird density data

We reviewed literature of wild bird associations with HPAIV infection from the Netherlands, but also from other countries (Bouwstra et al., 2015b; Verhagen et al., 2015b; Animal and Plant Health Agency (UK) et al., 2017; Kleyheeg et al., 2017; Beerens et al., 2018; Poen et al., 2018), to compile a list with species that had a known association with HPAIV infection (Table S2). Bird species taxonomically close to species that had been associated with HPAIV infection were also included. Bird species that were rare (<500 individuals in the winter counts across the Netherlands), had a small geographical range (e.g. only present in the Wadden Sea) or only present in

summer months, were excluded to prevent spurious negative associations.

Bird count data

Dutch bird count data collected by Sovon, the Dutch Center for Field Ornithology Nijmegen, the Netherlands), and published in the bird atlas (Sovon, 2018), were used for the analyses. The bird atlas was compiled through, among other things, structured bird counts across the whole of the Netherlands by (largely voluntary) observers in three winter seasons from December – February 2012/2013 – 2014/2015. We only used winter bird density data, as HPAI outbreaks only occurred between November and March. In short, for organizing the field work and processing the data the whole of the Netherlands was divided in 5x5 km squares. Each square was assigned to an observer who performed bird counts in pre-defined months. Sovon used the obtained data to construct maps with estimated numbers of wild birds per species per square (Supplement S1). For 54 of the 58 bird species of interest the estimates per square were available. For four bird species (i.e. mallard, Eurasian magpie, carrion crow and western jackdaw) the maps with estimates per square did not pass the internal review process. For these species, we transformed relative abundances maps with 1x1 km resolution, into maps with the estimated number of the particular species per 5x5 km square.

Land cover data

Land cover data (LGN7) was available as a geographic information system (GIS) raster layer with 25-m resolution, and resampled (i.e. averaged) to obtain a 5-km resolution that aligned with the 5x5 km square bird density abundance. We selected land cover features based on relevance for distribution of high risk HPAI bird species and HPAI risk on poultry farms (Si et al., 2013; Belkhiria et al., 2018). These land cover classes were aggregated in five major classes (agriculture, freshwater systems, grasslands, swamps and peats area, and saltwater systems) to reduce the number of features to be used for further analyses (Table S3).

Spatial scaling of environmental context

All downstream analyses were performed in R (version 3.6.3). We resampled all 5x5 km squares to 1x1 km resolution using nearest-neighbour allocation, so that all grid cells within each 5x5 km square contain the same value. To include the influence of landscape context (i.e. bird density and landcover predictors) beyond the properties of the grid cell, we applied isotropic bivariate Gaussian smoothers to the grid layers, with bandwidths of 2.5, 5 and 10 km (Holland et al., 2004; de Knegt et al., 2010). The unsmoothed raster layers contain essentially no information on environmental context beyond the 5x5 km square, and thus only contain site-specific information. The smoothed raster layers contain information on spatial context beyond the 5x5 km square.

Model training and evaluation

We analysed the feature importance of wild bird densities and land cover data for the prediction of HPAI outbreak risk on poultry farms. Prior to the analyses, all features were rescaled and normalized to a range 0-1. Because of the large number of features compared to the limited number of cases, we performed a univariate conditional logistic regression analysis (using the survival package, (Therneau, 2020) to identify features with a negative association with the HPAI cases. Features with a strict negative association on all scales were removed from further analyses (n = 4). The final dataset for further analyses contained 54 bird species and five land cover classes (Table S2).

Subsequently, a random forest classifier (using the ranger package, (Wright and Ziegler, 2017)) was performed. This was chosen because it can deal with a very large number of input variables, generally performs very well in classifying data, and by constraining the hyperparameters it can be robust against overfitting (Breiman, 2001). We used the following hyperparameters: number of trees: 100,000; number of variables per tree: 3; minimum node size: 2; and, maximum tree depth: 3. We applied a leave-one-group-out cross validation approach by iteratively leaving one selected case farm and its matched controls out while fitting the random forest, and using

the fitted model to predict the left-out data. A random forest permutation variable importance approach to quantify feature importance values for all bird species and landscape variables was used (Altmann et al., 2010), which we summarized for each spatial scaling level in the model. Feature importance was standardized: importance/mean(importance), so that the average feature importance value is 1 (Table S2). To reduce the number of features for the final model, we performed another series of leave-one-group-out cross validation (LoGo) random forest, in which we started with the 3 most important features at their most important scale, adding the subsequent most important feature for every round of leave-one-group-out cross validation. This was used to draw a precision-recall curve, and determine the area under the precision-recall curve, i.e. average precision (Sofaer et al., 2019), of the LoGo random forest per number of most important features, with which we determined the optimal number of features for the final model (Figure S1). Average precision is a threshold independent metric that calculates the area under the precision-recall curve, which is defined by a trade-off between different aspects of performance (i.e. precision and recall) as the threshold to the model's predictions varies (Sofaer et al., 2019). Model performance was determined by calculating its average precision, as well as the sensitivity and F_1 scores of different decisions thresholds. A prediction of HPAI risk across all 5x5 km squares in the Netherlands was made by averaging the cross-validated predicted HPAI probability surfaces. Moreover, we computed the standard deviation in HPAI risk prediction (on a logit scale) across all squares in the Netherlands. Additionally, as a form of evaluation of model performance, we plotted the locations of confirmed HPAI infections in dead wild birds between 2014-2018. To minimize bias, we only included locations for which the specific bird species was known and only included bird species with a homogenous distribution across the Netherlands (n = 19 out of 68, Table S4).

RESULTS

A random forest model with 19 features resulted in the highest average precision compared to the number of features ratio (Figure S1), and the average precision of this model to classify HPAI outbreak risk on poultry farms was 88% (Figure 1, Table 2). The spatial distribution of mallard (*Anas platyrhynchos*) contributed most to the prediction of HPAI risk in this model (mean feature importance 5.27 ± 0.80 SD), followed by mute swans (*Cygnus olor*, mean feature importance 5.23 ± 0.72) and common shelduck (*Tadorna tadorna*, mean feature importance 4.29 ± 0.51, Figure 2, Table S2). In the list of bird species of which the spatial distribution contributed most to model the HPAI risk, 11 bird species belonged to the family of Anatidae, two to the family of Ardeidae, two to the family of Falconidae, and four to other families all considered waterbird species (Figure 2). No land cover variables were selected in the process of variable reduction for the final model (Figure 2). Table 2 summarizes the performance of the reduced model to predict HPAI outbreaks on poultry farms. The optimal F1 score of the values listed in Table 2, is at a predicted HPAI risk threshold of 0.5, but misclassification of cases is then highest (n = 3) with one misclassification of the controls (Figure 1, Table 2).

Table 2: Classification metrics for different classification thresholds of predicted HPAI risk (Figure 2). A precision-recall curve was generated to determine area under the precision-recall-curve known as average precision, which was 88%. Accuracy is the ratio of correctly predicted observation to the total observations, whereas the F_1 score is a weighted averaged of precision and recall and gives a measure of the incorrectly classified cases.

Classification threshold	Recall	Precision	F_1	Accuracy
0.5	0.81	0.93	0.87	0.97
0.3	0.88	0.58	0.70	0.92
0.2	1	0.59	0.74	0.93

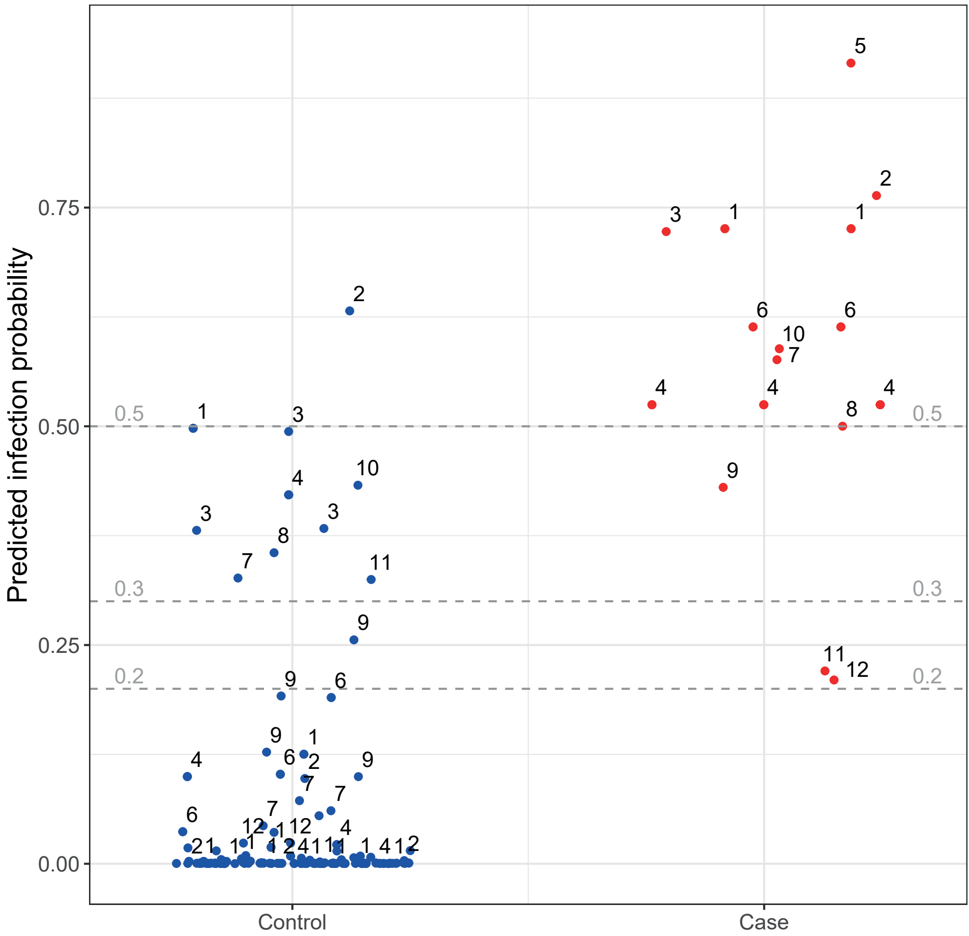

Figure 1: Results of final leave-one-group-out random forest (LoGo random forest), using the input of the 19 most important features at their most important scale. Each dot represents an individual highly pathogenic avian influenza case on a poultry farm (red) or control farm (blue). Labels indicate case ID of each farm. The predicted probability is given for each case and control farms within a set, after training of the LoGo random forest on the remaining cases and controls. The horizontal lines represent the different cut-off values for test performance analyses (Table 2).

In Figure 3A, the predicted HPAI risk across the Netherlands was mapped. This map shows generally a high risk in the north-western parts of the Netherlands and around the river Rhine, and lower risk in south-eastern areas. A negative correlation (-0.6) between the logit-scale of the standard deviation of the average predicted infection probability and the predicted infection probability was found, indicating that variation in areas with high

predicted infection probability was lower than in areas where infection probability was low (Figure 3B). Also, the predicted HPAI risk agrees well with the 19 locations where dead wild birds with HPAI were found, with an average mean infection probability of 0.58 ± 0.20 (SD) and 68% of locations with a predicted infection probability of 0.5 (optimal F_1 score) or higher (Table S4).

To include the influence of landscape context beyond the grid cell, we applied spatial smoothing to each variable at the raw (i.e. no spatial smoothing), 2.5, 5 and 10 km Scale. The distribution of three bird species (out of 19) had the strongest association with HPAI risk at the raw scale, including the Eurasian wigeon that is known to graze close to farms. Four species exhibited the strongest association at a 2.5 km smoothing scale, five species at 5 km, and seven species at 10 km scale of the Gaussian smoother (Figure 2), showing that the landscape contexts differs among species.

DISCUSSION

We showed that a model using wild bird species densities can accurately predict HPAI risk areas for poultry farms in the Netherlands. Seventeen waterbird and two raptor species were most strongly associated with the HPAI outbreak risks. The risk map of HPAI-infection probability across the Netherlands also correctly predicted locations where HPAI infected dead wild birds were reported in the same period.

The mallard and mute swan had the highest feature importance, and have both been found infected with HPAIVs in several studies (Beerens et al., 2017, 2018; Napp et al., 2018). Other species selected by the model, i.e. tufted duck (*Aythya fuligula*), Eurasian wigeon, peregrine falcon (*Falco peregrinus*), and great black-backed gulls (*Larus marinus*), were found to have high mortality rates during the H5N8 epidemic in 2016 (Kleyheeg et al., 2017), and wild birds of the family of Anatidae and of the order of Charadriiformes are often mentioned as reservoir species for HPAIVs (Caron et al., 2017). Our model also found other species to be associated with HPAI infection risk, suggesting that more bird species than those often diagnosed in passive or active surveillance activities are involved in HPAIV transmission. AIV

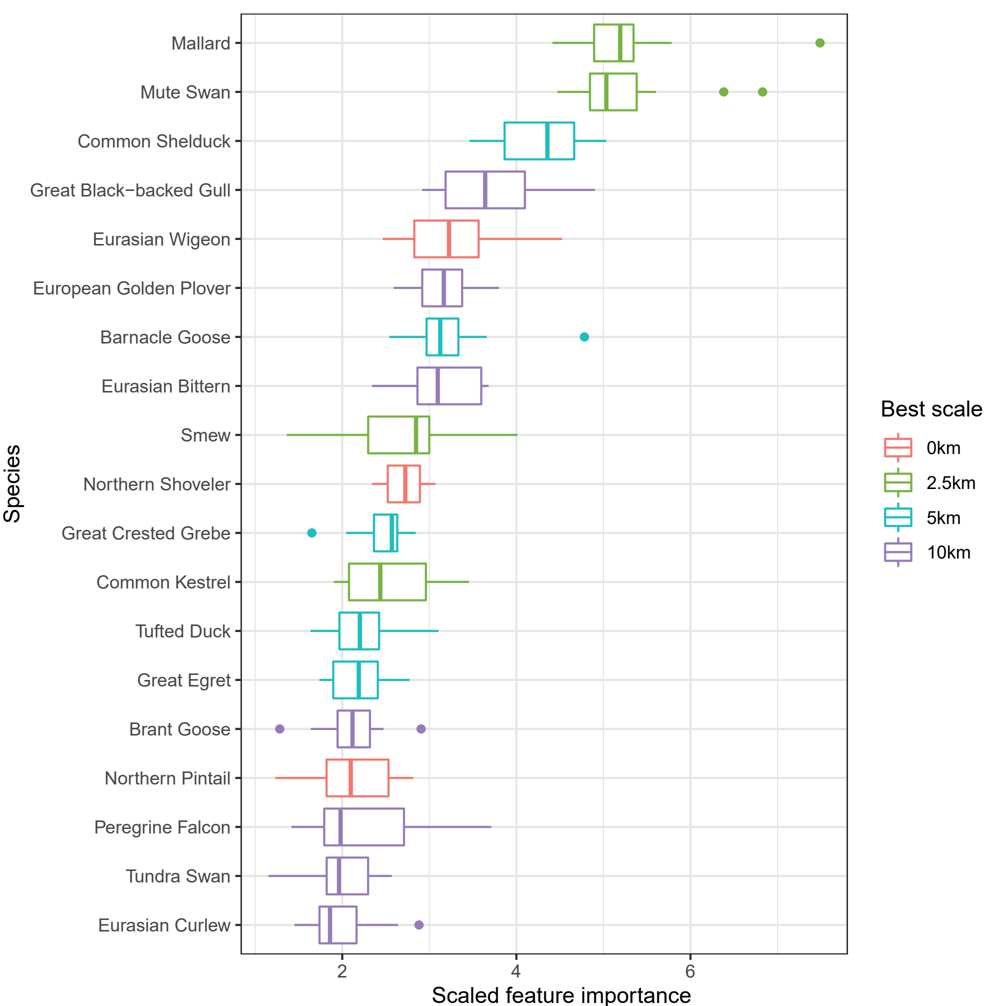

Figure 2: Scaled importance of 19 most important features that were used for the final leave-one-group-out random forest (LoGo random forest). Feature importance was standardized: importance/mean(importance), so that the average feature importance value is 1. The most important scale of each feature (highest median value, Table S3) was used in the final LoGo random forest. The boxplots indicate the variation in the feature importance across the 16 LoGo random forest runs. Colors of the boxplots indicate the spatial scale with the highest feature importance for each variable that was used for smoothing of the data.

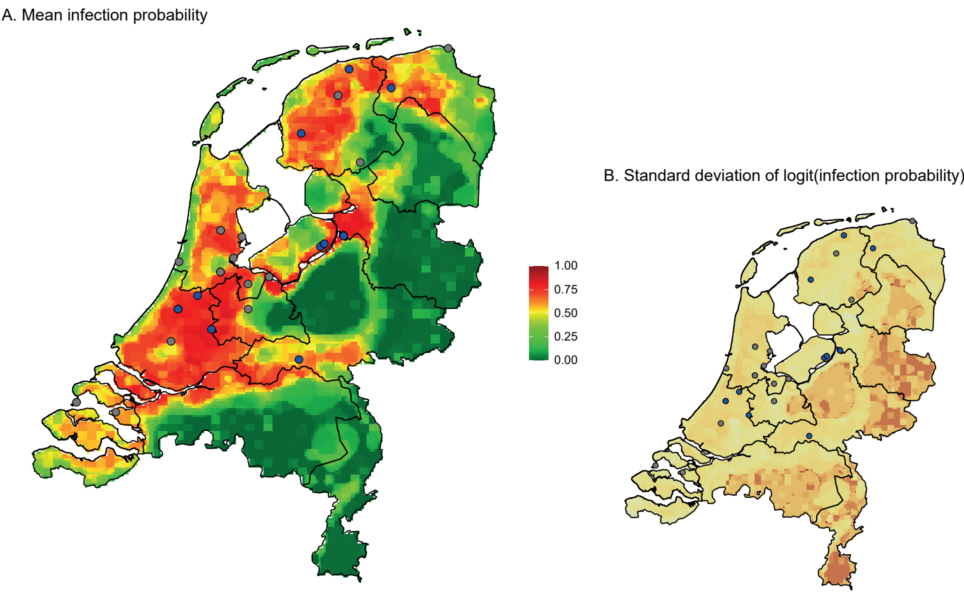

Figure 3: Mean infection probability of highly pathogenic avian influenza (HPAI) across all 1x1 km grid-cells in the Netherlands (A) using the final leave-one-group-out random forest model with 19 most important features. The prediction of HPAI risk ranges between 0 (low, dark green) to 1 (high, dark red). Standard deviation of the predicted HPAI probabilities on logit-scale across all 1x1 km grid-cells in the Netherlands (B). Locations of poultry farms with HPAI outbreaks (i.e. cases, blue) and selected locations with HPAIV infected dead wild birds between 2014-2018 (grey) are shown.

infections among wild bird species depends on a complex multispecies system, influenced by ecosystem properties, bird species diversity and community structure, the specific circulating HPAIV strain(s), and the clinical impact it has among the different hosts species (Caron et al., 2017; Huang et al., 2019). This study does not indicate how many of the identified wild birds were infected, or to what extent their presence contributed to disseminating virus in the farms' surroundings, nor does it allow the distinction between the role of migratory bird species (e.g. Eurasian wigeon and tufted duck), which likely play a role in long distance dispersal, and species that are less migratory (e.g. mute swan, and mallard), which could act as local amplifiers, or bridge species (Hill et al., 2012; Alarcon et al., 2018). Therefore, we have to be careful when drawing conclusions on the exact roles of specific wild bird species in the epidemiological processes at the wild bird/domestic

bird interface, or on that of other bird species not included in this study. Nevertheless, the identified bird species give important clues for target species for future studies with regard to infection probability.

In contrast to previous research (Si et al., 2013; Belkhiria et al., 2018), none of the land cover variables included in the initial model were selected in the final model, indicating that densities of wild bird species were better predictors of HPAI infection risk. However, previous studies did not include wild bird species distribution to analyse HPAIV introductions on poultry farms, but rather tried to explain variation in occurrence of HPAIV introductions only with environmental variables, like distance to waterways and vegetation index. Environmental variables can be considered a proxy for habitat selection of wild birds, and were in our study less suitable predictors than the densities of the actual bird species associated with HPAIV infection in poultry. This does not mean that land cover data could not be of great value. For example the presence of wetlands is vital as they are an important habitat for many waterfowl species (Belkhiria et al., 2018). In the current study, the original land cover classes were aggregated into five major classes, which decreased the resolution of land cover classification in the analyses, and may have reduced sensitivity of these variables for the prediction of infection probability. Therefore, especially in countries where detailed quantitative wild bird density data is not available, land cover data could still be a suitable proxy for predicting HPAI risk, as these data are likely more easily gathered across large areas compared to wild bird density distributions.

Our model accurately predicted the HPAI risk in 68% of the locations with HPAI infected dead wild birds between 2014-2018. However, during the 2016-outbreak, H5N8 was detected in live Eurasian wigeons and mallards (Poen et al., 2018), and in the current 2020 epidemic apparently healthy ducks and geese have also been reported to be infected in Germany, Italy, the Netherlands and Denmark (Adlhoch et al., 2020). These findings highlight that detection and reporting of dead wild birds found via passive surveillance is helpful, but not enough to predict HPAI outbreak risk, as reporting of dead wild birds can be biased by factors such as human presence,

and size and habitat of bird species. It is therefore not possible to know what other species are affected by these HPAIV strains if the species do not show clinical signs, including mortality (Alarcon et al., 2018; Adlhoch et al., 2020). Besides the spatial distribution of wild birds, seasonality and the arrival of migratory birds also plays a role in the prediction of HPAI outbreak risk (Hill et al., 2015; Alarcon et al., 2018; Gonzales et al., 2020; Velkers et al., 2020), e.g. Velkers et al. (2020) found that the timing of peak densities of Anatidae species observed around farms, coincided with the timing of outbreaks. We only used long-term averages of bird count data, collected at set moments each winter between 2012/2013 – 2014/2015. However, the observed spatiotemporal relationships between outbreaks on poultry farms and HPAIV wild bird detections represent complex dynamics. For future research, including spatiotemporal analyses on HPAIV infection probability to improve predictive power, and new HPAI cases to further train and validate the model is recommended.

We determined the best fitting spatial scale for each species separately. This is in line with ecological studies assessing the influence of environmental context, varied over different scales, on the analysis and prediction of habitat selection (de Knegt et al., 2011). This is important as local dispersal patterns and ecology of bird species differ. Diving ducks, e.g. tufted ducks, are mainly found on large open waterbodies, often at considerable distance from farms, and have relatively few movements over land between foraging and roosting sites. In contrast, Eurasian wigeons and mallards forage on grass- and agricultural lands, and are found more closely to farms (Kleyheeg et al., 2017; Belkhiria et al., 2018), which is in line with our results.

We realize that our dataset was limited with 16 confirmed HPAI cases on poultry farms. Although some farms had multiple outbreaks over the years, these were all new introductions, and thus independent of one another. Furthermore, we tuned the random forest analysis in a way to minimize the risk of overfitting and, used a cross-validation approach for the random forest, testing its robustness despite the limited dataset.

In conclusion, we show that spatial variation in HPAI outbreak risk

in the Netherlands was accurately predicted based on wild bird density data, rather than land cover variables. The spatial distributions of several waterbird species were important contributors to model the HPAI outbreak risk. New HPAI outbreaks will be used to validate and improve the risk map, but already in its current form, areas classified as high risk for HPAIV introduction on poultry farms should be considered as important targets for surveillance, preventive measures against HPAIV introduction, and may assist in decision making on locations for new poultry farms. The described modelling approach allows for inclusion of the best predictors based on the available data, which may include land cover variables in addition to bird data, depending on the local situation. Identification of high-risk areas for development of country or region-specific control programs, would be a proactive strategy to combat the global threat of these recurring HPAI outbreaks.

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CONTRIBUTION OF THE AUTHORS

WdB, AS, AE, JSt, FV and JSc initiated this project. AS, WdB, HdK, JSt, RS, JSc, and FV contributed to the design of the experiment. JSc and HdK did data processing and analysis. JSc performed manuscript writing. WdB, AS, FV, AE, HdK, JSt and RS contributed to the development of the manuscript by giving constructive feedback on the manuscript during its preparation. All authors gave approval of the manuscript for publication.

SUPPLEMENTARY INFORMATION

Supplement S1

Dutch bird count data collected by Sovon, the Dutch Center for Field Ornithology (Nijmegen, the Netherlands), and published in the bird atlas (Sovon, 2018), were used for the analyses. The bird atlas was compiled through a citizen science approach of structured bird counts across the whole of the Netherlands by (largely voluntary) observers in three winter seasons from December – February 2012/2013 – 2014/2015. We only used winter bird density data, as HPAI outbreaks only occurred between November and March. For organizing the field work and processing the data the whole of the Netherlands was divided in 5x5 km squares (1769 squares in total, including open water areas of lake IJsselmeer, the Wadden Sea and the large river deltas). Each square was assigned to an observer who performed bird counts in pre-defined spring/summer and winter months. Each observer was instructed to perform a minimum of three bird counts in the assigned square between December and February, visiting all main habitats within the square. As a final result after counts were finished, the observer made an estimate of the number of individuals per bird species present in the square in the winter according to the following classes: 1-3; 4-10; 11-25; 26-50; 51-100; 101-250; 251-500; 501-1000; >1000.

Sovon used the obtained data to construct maps with numbers per species per square based on the estimated numbers per square. An internal reviewing process checked the constructed maps for each bird species. Quality checks entailed if the pattern of distribution of a bird species over the Netherlands was accurate compared to what is known from the distribution of the species from other projects lead by Sovon.

References

Sovon, Dutch Center for Field Ornithology. Bird atlas of the Netherlands. 1st ed. Kosmos Publishers Utrecht/Antwerp; 2018. 1–640 p.

Table S1: Overview of all highly pathogenic avian influenza case farms with the selected control farms. Case Farm_IDs 1, 4 and 6 had multiple outbreaks over different years, which are indicated with Case_ID a, b and c. Poultry Type indicates the poultry type that was affected in the farm.

Case Farm_ID	Case_ID	Case/Control	Poultry Type	Year of outbreak	Month of outbreak
1	1a	Case	Layer	2014	11
1	1a	Control	Layer	2014	11
1	1a	Control	Layer	2014	11
1	1a	Control	Layer	2014	11
1	1a	Control	Layer	2014	11
1	1a	Control	Layer	2014	11
1	1a	Control	Layer	2014	11
1	1a	Control	Layer	2014	11
1	1a	Control	Layer	2014	11
1	1a	Control	Layer	2014	11
1	1a	Control	Layer	2014	11
2	2	Case	Layer	2014	11
2	2	Control	Layer	2014	11
2	2	Control	Layer	2014	11
2	2	Control	Layer	2014	11
2	2	Control	Layer	2014	11
2	2	Control	Layer	2014	11
2	2	Control	Layer	2014	11
2	2	Control	Layer	2014	11
2	2	Control	Layer	2014	11
2	2	Control	Layer	2014	11
2	2	Control	Layer	2014	11
3	3	Case	Layer	2014	11
3	3	Control	Layer	2014	11
3	3	Control	Layer	2014	11
3	3	Control	Layer	2014	11
3	3	Control	Layer	2014	11
3	3	Control	Layer	2014	11
3	3	Control	Layer	2014	11
3	3	Control	Layer	2014	11
3	3	Control	Layer	2014	11
3	3	Control	Layer	2014	11
3	3	Control	Layer	2014	11
4	4a	Case	Pekin duck	2014	11
4	4a	Control	Pekin duck	2014	11
4	4a	Control	Pekin duck	2014	11

Case Farm_ID	Case_ID	Case/Control	Poultry Type	Year of outbreak	Month of outbreak
4	4a	Control	Pekin duck	2014	11
4	4a	Control	Pekin duck	2014	11
4	4a	Control	Pekin duck	2014	11
4	4a	Control	Pekin duck	2014	11
4	4a	Control	Pekin duck	2014	11
5	5	Case	Broiler Breeder	2014	11
5	5	Control	Broiler Breeder	2014	11
5	5	Control	Broiler Breeder	2014	11
5	5	Control	Broiler Breeder	2014	11
5	5	Control	Broiler Breeder	2014	11
5	5	Control	Broiler Breeder	2014	11
5	5	Control	Broiler Breeder	2014	11
5	5	Control	Broiler Breeder	2014	11
5	5	Control	Broiler Breeder	2014	11
5	5	Control	Broiler Breeder	2014	11
5	5	Control	Broiler Breeder	2014	11
6	6a	Case	Pekin duck	2016	12
6	6a	Control	Pekin duck	2016	12
6	6a	Control	Pekin duck	2016	12
6	6a	Control	Pekin duck	2016	12
6	6a	Control	Pekin duck	2016	12
7	7	Case	Pekin duck	2016	11
7	7	Control	Pekin duck	2016	11
7	7	Control	Pekin duck	2016	11
7	7	Control	Pekin duck	2016	11
7	7	Control	Pekin duck	2016	11
7	7	Control	Pekin duck	2016	11
7	7	Control	Pekin duck	2016	11
7	7	Control	Pekin duck	2016	11
8	8	Case	Pekin duck	2016	11
8	8	Control	Pekin duck	2016	11
8	8	Control	Pekin duck	2016	11
8	8	Control	Pekin duck	2016	11
8	8	Control	Pekin duck	2016	11
8	8	Control	Pekin duck	2016	11
1	1b	Case	Layer	2016	12
1	1b	Control	Layer	2016	12
1	1b	Control	Layer	2016	12
1	1b	Control	Layer	2016	12

Case Farm_ID	Case_ID	Case/Control	Poultry Type	Year of outbreak	Month of outbreak
1	1b	Control	Layer	2016	12
1	1b	Control	Layer	2016	12
1	1b	Control	Layer	2016	12
1	1b	Control	Layer	2016	12
1	1b	Control	Layer	2016	12
1	1b	Control	Layer	2016	12
1	1b	Control	Layer	2016	12
9	9	Case	Layer	2016	12
9	9	Control	Layer	2016	12
9	9	Control	Layer	2016	12
9	9	Control	Layer	2016	12
9	9	Control	Layer	2016	12
9	9	Control	Layer	2016	12
9	9	Control	Layer	2016	12
9	9	Control	Layer	2016	12
9	9	Control	Layer	2016	12
9	9	Control	Layer	2016	12
9	9	Control	Layer	2016	12
4	4b	Case	Pekin duck	2016	12
4	4b	Control	Pekin duck	2016	12
4	4b	Control	Pekin duck	2016	12
4	4b	Control	Pekin duck	2016	12
4	4b	Control	Pekin duck	2016	12
4	4b	Control	Pekin duck	2016	12
10	10	Case	Layer	2016	12
10	10	Control	Layer	2016	12
10	10	Control	Layer	2016	12
10	10	Control	Layer	2016	12
10	10	Control	Layer	2016	12
10	10	Control	Layer	2016	12
10	10	Control	Layer	2016	12
10	10	Control	Layer	2016	12
10	10	Control	Layer	2016	12
10	10	Control	Layer	2016	12
10	10	Control	Layer	2016	12
11	11	Case	Broiler Breeder	2016	12
11	11	Control	Broiler Breeder	2016	12
11	11	Control	Broiler Breeder	2016	12
11	11	Control	Broiler Breeder	2016	12

Case Farm_ID	Case_ID	Case/Control	Poultry Type	Year of outbreak	Month of outbreak
11	11	Control	Broiler Breeder	2016	12
11	11	Control	Broiler Breeder	2016	12
11	11	Control	Broiler Breeder	2016	12
11	11	Control	Broiler Breeder	2016	12
11	11	Control	Broiler Breeder	2016	12
11	11	Control	Broiler Breeder	2016	12
11	11	Control	Broiler Breeder	2016	12
6	6b	Case	Pekin duck	2017	12
6	6b	Control	Pekin duck	2017	12
6	6b	Control	Pekin duck	2017	12
6	6b	Control	Pekin duck	2017	12
6	6b	Control	Pekin duck	2017	12
6	6b	Control	Pekin duck	2017	12
6	6b	Control	Pekin duck	2017	12
12	12	Case	Broiler Breeder	2018	2
12	12	Control	Broiler Breeder	2018	2
12	12	Control	Broiler Breeder	2018	2
12	12	Control	Broiler Breeder	2018	2
12	12	Control	Broiler Breeder	2018	2
12	12	Control	Broiler Breeder	2018	2
12	12	Control	Broiler Breeder	2018	2
12	12	Control	Broiler Breeder	2018	2
12	12	Control	Broiler Breeder	2018	2
12	12	Control	Broiler Breeder	2018	2
12	12	Control	Broiler Breeder	2018	2
4	4c	Case	Pekin duck	2018	3
4	4c	Control	Pekin duck	2018	3
4	4c	Control	Pekin duck	2018	3
4	4c	Control	Pekin duck	2018	3
4	4c	Control	Pekin duck	2018	3
4	4c	Control	Pekin duck	2018	3
4	4c	Control	Pekin duck	2018	3
4	4c	Control	Pekin duck	2018	3
4	4c	Control	Pekin duck	2018	3

Table S2: list of all bird species and land cover variables included in the random forest. Each variable was included at 4 different Gaussian smoother scales (0, 2.5, 5 and 10 km). Overall rank indicates the rank of that variable in the random forest of the full model. Median and mean prediction are the scaled feature importance across all 4 smoothing scales for that specific variable. Varscale_mean and median are the feature importance for each smoothing scale of a variable. Varscale_rank is the rank of importance for the different smoothing scales of each variable. The highest rank of the 19 most important features was chosen for the final model.

Variable (English)	Latin name bird species	Family bird species	Gaussian scale	Overall rank full model	Rank final model	Median prediction	Mean prediction	Standard deviation of prediction	Varscale_ mean	Varscale_ median	Varscale_ sd	Varscale_ rank
Common Shelduck	*Tadorna tadorna*	Anatidae	0km	1		3.21	2.69	1.51	0.69	0.66	0.27	4
Common Shelduck			2.5km	1		3.21	2.69	1.51	2.00	1.93	0.54	3
Common Shelduck			5km	1	3	3.21	2.69	1.51	4.29	4.36	0.51	1
Common Shelduck			10km	1		3.21	2.69	1.51	3.79	3.87	0.32	2
Mute Swan	*Cygnus olor*	Anatidae	0km	2		3.09	3.45	1.25	2.97	2.77	0.62	3
Mute Swan			2.5km	2	2	3.09	3.45	1.25	5.24	5.03	0.72	1
Mute Swan			5km	2		3.09	3.45	1.25	3.39	3.42	0.45	2
Mute Swan			10km	2		3.09	3.45	1.25	2.20	2.26	0.30	4
European Golden Plover	*Pluvialis apricaria*	Charadriidae	0km	3		2.89	2.75	0.73	1.82	1.69	0.56	4
European Golden Plover			2.5km	3		2.89	2.75	0.73	2.90	2.89	0.59	3
European Golden Plover			5km	3		2.89	2.75	0.73	3.13	3.10	0.42	2
European Golden Plover			10km	3	6	2.89	2.75	0.73	3.16	3.17	0.34	1
Mallard	*Anas platyrhynchos*	Anatidae	0km	4		2.74	3.10	1.47	2.10	2.03	0.41	3
Mallard			2.5km	4	1	2.74	3.10	1.47	5.27	5.19	0.80	1
Mallard			5km	4		2.74	3.10	1.47	3.24	3.15	0.48	2
Mallard			10km	4		2.74	3.10	1.47	1.80	1.78	0.31	4
Eurasian Wigeon	*Mareca penelope*	Anatidae	0km	5	5	2.63	2.74	0.63	3.26	3.23	0.57	1
Eurasian Wigeon			2.5km	5		2.63	2.74	0.63	3.11	3.13	0.45	2

Eurasian Wigeon			5km	5		2.63	2.74	0.63	2.17	2.20	0.15	4
Eurasian Wigeon			10km	5		2.63	2.74	0.63	2.42	2.40	0.46	3
Great Black-backed Gull	*Larus marinus*	Laridae	0km	6		2.45	2.25	1.48	0.17	0.18	0.09	4
Great Black-backed Gull			2.5km	6		2.45	2.25	1.48	1.78	1.72	0.34	3
Great Black-backed Gull			5km	6		2.45	2.25	1.48	3.37	3.20	0.59	2
Great Black-backed Gull			10km	6	4	2.45	2.25	1.48	3.68	3.64	0.58	1
Barnacle Goose	*Branta leucopsis*	Anatidae	0km	7		2.22	2.07	1.03	0.59	0.55	0.13	4
Barnacle Goose			2.5km	7		2.22	2.07	1.03	2.25	2.22	0.41	2
Barnacle Goose			5km	7	7	2.22	2.07	1.03	3.22	3.13	0.59	1
Barnacle Goose			10km	7		2.22	2.07	1.03	2.20	2.22	0.28	3
Common Kestrel	*Falco tinnunculus*	Falconidae	0km	8		1.90	1.72	0.87	0.53	0.59	0.21	4
Common Kestrel			2.5km	8	12	1.90	1.72	0.87	2.56	2.44	0.58	1
Common Kestrel			5km	8		1.90	1.72	0.87	2.20	2.17	0.37	2
Common Kestrel			10km	8		1.90	1.72	0.87	1.61	1.58	0.44	3
Northern Pintail	*Anas acuta*	Anatidae	0km	9	16	1.86	1.81	0.48	2.11	2.10	0.51	1
Northern Pintail			2.5km	9		1.86	1.81	0.48	1.75	1.77	0.42	3
Northern Pintail			5km	9		1.86	1.81	0.48	1.66	1.70	0.41	4
Northern Pintail			10km	9		1.86	1.81	0.48	1.71	1.88	0.49	2
Great Egret	*Ardea alba*	Ardeidae	0km	10		1.65	1.64	0.53	1.03	1.07	0.32	4
Great Egret			2.5km	10		1.65	1.64	0.53	1.87	1.92	0.33	2
Great Egret			5km	10	14	1.65	1.64	0.53	2.18	2.19	0.31	1
Great Egret			10km	10		1.65	1.64	0.53	1.51	1.42	0.29	3
Tundra Swan	*Cygnus columbianus*	Anatidae	0km	11		1.55	1.51	0.55	0.92	0.91	0.33	4
Tundra Swan			2.5km	11		1.55	1.51	0.55	1.62	1.73	0.58	2
Tundra Swan			5km	11		1.55	1.51	0.55	1.52	1.47	0.20	3

Tundra Swan			10km	11	18	1.55	1.51	0.55	1.99	1.96	0.40	1
Great Crested Grebe	*Podiceps cristatus*	Podicipedidae	0km	12		1.46	1.62	0.74	0.69	0.69	0.25	4
Great Crested Grebe			2.5km	12		1.46	1.62	0.74	1.96	1.98	0.45	2
Great Crested Grebe			5km	12	11	1.46	1.62	0.74	2.45	2.57	0.34	1
Great Crested Grebe			10km	12		1.46	1.62	0.74	1.37	1.38	0.23	3
Northern Shoveler	*Spatula clypeata*	Anatidae	0km	13	10	1.45	1.62	0.72	2.71	2.72	0.24	1
Northern Shoveler			2.5km	13		1.45	1.62	0.72	1.66	1.68	0.26	2
Northern Shoveler			5km	13		1.45	1.62	0.72	1.09	1.07	0.26	4
Northern Shoveler			10km	13		1.45	1.62	0.72	1.04	1.09	0.27	3
Eurasian Bittern	*Botaurus stellaris*	Ardeidae	0km	14		1.40	1.62	1.31	0.08	0.06	0.08	4
Eurasian Bittern			2.5km	14		1.40	1.62	1.31	0.76	0.76	0.27	3
Eurasian Bittern			5km	14		1.40	1.62	1.31	2.49	2.47	0.56	2
Eurasian Bittern			10km	14	8	1.40	1.62	1.31	3.15	3.10	0.46	1
Grey Heron	*Ardea cinerea*	Ardeidae	0km	15		1.31	1.16	0.54	0.35	0.30	0.15	4
Grey Heron			2.5km	15		1.31	1.16	0.54	1.58	1.52	0.38	1
Grey Heron			5km	15		1.31	1.16	0.54	1.36	1.39	0.21	2
Grey Heron			10km	15		1.31	1.16	0.54	1.34	1.38	0.24	3
Smew	*Mergellus albellus*	Anatidae	0km	16		1.31	1.53	0.86	0.98	1.05	0.28	3
Smew			2.5km	16	9	1.31	1.53	0.86	2.74	2.85	0.68	1
Smew			5km	16		1.31	1.53	0.86	1.56	1.54	0.29	2
Smew			10km	16		1.31	1.53	0.86	0.84	0.79	0.19	4
Brant Goose	*Branta Bernicla*	Anatidae	0km	17		1.28	1.16	0.88	0.00	0.00	0.00	4
Brant Goose			2.5km	17		1.28	1.16	0.88	0.83	0.73	0.31	3
Brant Goose			5km	17		1.28	1.16	0.88	1.71	1.71	0.40	2
Brant Goose			10km	17	15	1.28	1.16	0.88	2.11	2.12	0.42	1
Eurasian Curlew	*Numenius arquata*	Scolopacidae	0km	18		1.23	1.31	0.65	0.60	0.61	0.18	4
Eurasian Curlew			2.5km	18		1.23	1.31	0.65	0.91	0.92	0.13	3

Eurasian Curlew			5km	18		1.23	1.31	0.65	1.73	1.63	0.36	2
Eurasian Curlew			10km	18	19	1.23	1.31	0.65	2.00	1.86	0.42	1
Great Cormorant	*Phalacrocorax carbo*	Phalacrocoracidae	0km	19		1.11	1.08	0.35	0.82	0.78	0.24	4
Great Cormorant			2.5km	19		1.11	1.08	0.35	1.39	1.51	0.27	1
Great Cormorant			5km	19		1.11	1.08	0.35	0.85	0.91	0.28	3
Great Cormorant			10km	19		1.11	1.08	0.35	1.29	1.30	0.15	2
Tufted Duck	*Aythya fuligula*	Anatidae	0km	20		1.10	1.20	0.81	0.24	0.25	0.07	4
Tufted Duck			2.5km	20		1.10	1.20	0.81	0.77	0.76	0.20	3
Tufted Duck			5km	20	13	1.10	1.20	0.81	2.24	2.20	0.38	1
Tufted Duck			10km	20		1.10	1.20	0.81	1.56	1.54	0.24	2
Common Goldeneye	*Bucephala clangula*	Anatidae	0km	21		1.08	0.99	0.42	0.41	0.39	0.11	4
Common Goldeneye			2.5km	21		1.08	0.99	0.42	1.11	1.12	0.28	3
Common Goldeneye			5km	21		1.08	0.99	0.42	1.28	1.36	0.30	1
Common Goldeneye			10km	21		1.08	0.99	0.42	1.14	1.16	0.27	2
Peregrine Falcon	*Falco peregrinus*	Falconidae	0km	22		1.02	1.10	0.88	0.05	0.06	0.03	4
Peregrine Falcon			2.5km	22		1.02	1.10	0.88	1.06	1.07	0.23	2
Peregrine Falcon			5km	22		1.02	1.10	0.88	1.02	1.02	0.22	3
Peregrine Falcon			10km	22	17	1.02	1.10	0.88	2.26	1.98	0.72	1
Freshwater systems			0km	23		0.92	0.99	0.27	1.11	1.16	0.32	1
Freshwater systems			2.5km	23		0.92	0.99	0.27	1.05	0.94	0.31	3
Freshwater systems			5km	23		0.92	0.99	0.27	0.77	0.76	0.13	4
Freshwater systems			10km	23		0.92	0.99	0.27	1.02	1.05	0.18	2
Common Buzzard	*Buteo buteo*	Accipitridae	0km	24		0.91	0.85	0.36	0.48	0.47	0.23	4
Common Buzzard			2.5km	24		0.91	0.85	0.36	0.99	1.02	0.35	1
Common Buzzard			5km	24		0.91	0.85	0.36	0.90	0.90	0.31	3
Common Buzzard			10km	24		0.91	0.85	0.36	1.02	0.97	0.28	2
European Herring Gull	*Larus argentatus*	Laridae	0km	25		0.90	1.03	0.65	0.29	0.27	0.11	4

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European Herring Gull			2.5km	25	0.90	1.03	0.65	0.77	0.83	0.18	3
European Herring Gull			5km	25	0.90	1.03	0.65	1.58	1.72	0.57	1
European Herring Gull			10km	25	0.90	1.03	0.65	1.48	1.58	0.44	2
Lesser Black-backed Gull	*Larus fuscus*	Laridae	0km	26	0.88	0.82	0.26	0.56	0.55	0.19	4
Lesser Black-backed Gull			2.5km	26	0.88	0.82	0.26	0.99	0.96	0.24	1
Lesser Black-backed Gull			5km	26	0.88	0.82	0.26	0.86	0.91	0.23	3
Lesser Black-backed Gull			10km	26	0.88	0.82	0.26	0.88	0.92	0.20	2
Common Pochard	*Aythya ferina*	Anatidae	0km	27	0.85	0.86	0.36	0.48	0.52	0.19	4
Common Pochard			2.5km	27	0.85	0.86	0.36	0.83	0.82	0.28	3
Common Pochard			5km	27	0.85	0.86	0.36	0.90	0.90	0.27	2
Common Pochard			10km	27	0.85	0.86	0.36	1.24	1.28	0.24	1
Water Rail	*Rallus aquaticus*	Rallidae	0km	28	0.80	0.76	0.46	0.20	0.19	0.13	4
Water Rail			2.5km	28	0.80	0.76	0.46	0.65	0.65	0.22	3
Water Rail			5km	28	0.80	0.76	0.46	1.01	1.00	0.36	2
Water Rail			10km	28	0.80	0.76	0.46	1.20	1.20	0.23	1
Eurasian Coot	*Fulica atra*	Rallidae	0km	29	0.77	0.81	0.35	0.45	0.47	0.14	4
Eurasian Coot			2.5km	29	0.77	0.81	0.35	0.74	0.70	0.30	3
Eurasian Coot			5km	29	0.77	0.81	0.35	0.91	0.90	0.22	2
Eurasian Coot			10km	29	0.77	0.81	0.35	1.16	1.05	0.26	1
Pied Avocet	*Recurvirostra avosetta*	Recurvirostridae	0km	30	0.76	0.67	0.46	0.00	0.00	0.01	4

Pied Avocet			2.5km	30	0.76	0.67	0.46	1.13	1.12	0.30	1
Pied Avocet			5km	30	0.76	0.67	0.46	0.73	0.76	0.14	3
Pied Avocet			10km	30	0.76	0.67	0.46	0.83	0.82	0.17	2
Dunlin	*Calidris alpina*	Scolopacidae	0km	31	0.75	0.69	0.43	0.09	0.08	0.06	4
Dunlin			2.5km	31	0.75	0.69	0.43	0.81	0.84	0.25	2
Dunlin			5km	31	0.75	0.69	0.43	0.75	0.76	0.18	3
Dunlin			10km	31	0.75	0.69	0.43	1.10	1.12	0.32	1
Eurasian Oystercatcher	*Haematopus ostralegus*	Haematopodidae	0km	32	0.69	0.85	0.61	0.22	0.16	0.14	4
Eurasian Oystercatcher			2.5km	32	0.69	0.85	0.61	0.44	0.44	0.17	3
Eurasian Oystercatcher			5km	32	0.69	0.85	0.61	1.51	1.55	0.32	1
Eurasian Oystercatcher			10km	32	0.69	0.85	0.61	1.22	1.17	0.45	2
Mew Gull	*Larus canus*	Laridae	0km	33	0.62	0.70	0.34	0.39	0.34	0.19	4
Mew Gull			2.5km	33	0.62	0.70	0.34	0.64	0.54	0.25	3
Mew Gull			5km	33	0.62	0.70	0.34	0.96	0.94	0.34	1
Mew Gull			10km	33	0.62	0.70	0.34	0.79	0.75	0.29	2
Greater White-fronted Goose	*Anser Albifrons*	Anatidae	0km	34	0.58	0.53	0.20	0.26	0.23	0.10	4
Greater White-fronted Goose			2.5km	34	0.58	0.53	0.20	0.62	0.63	0.12	2
Greater White-fronted Goose			5km	34	0.58	0.53	0.20	0.70	0.71	0.16	1
Greater White-fronted Goose			10km	34	0.58	0.53	0.20	0.55	0.60	0.11	3
Agriculture			0km	35	0.54	0.73	0.47	1.09	1.05	0.28	2
Agriculture			2.5km	35	0.54	0.73	0.47	1.21	1.17	0.23	1
Agriculture			5km	35	0.54	0.73	0.47	0.41	0.40	0.10	3
Agriculture			10km	35	0.54	0.73	0.47	0.22	0.22	0.09	4

Common Moorhen	*Gallinula chloropus*	Rallidae	0km	36	0.53	0.53	0.22	0.29	0.30	0.09	4
Common Moorhen			2.5km	36	0.53	0.53	0.22	0.60	0.59	0.09	2
Common Moorhen			5km	36	0.53	0.53	0.22	0.55	0.53	0.17	3
Common Moorhen			10km	36	0.53	0.53	0.22	0.69	0.68	0.24	1
Common Merganser	*Mergus merganser*	Anatidae	0km	37	0.50	0.49	0.20	0.26	0.30	0.13	4
Common Merganser			2.5km	37	0.50	0.49	0.20	0.64	0.64	0.21	1
Common Merganser			5km	37	0.50	0.49	0.20	0.59	0.58	0.10	2
Common Merganser			10km	37	0.50	0.49	0.20	0.46	0.45	0.12	3
Pink-footed Goose	*Anser brachyrhynchus*	Anatidae	0km	38	0.50	0.47	0.29	0.04	0.04	0.05	4
Pink-footed Goose			2.5km	38	0.50	0.47	0.29	0.65	0.64	0.22	1
Pink-footed Goose			5km	38	0.50	0.47	0.29	0.66	0.64	0.18	2
Pink-footed Goose			10km	38	0.50	0.47	0.29	0.52	0.49	0.10	3
Gadwall	*Mareca strepera*	Anatidae	0km	39	0.48	0.55	0.29	0.25	0.23	0.09	4
Gadwall			2.5km	39	0.48	0.55	0.29	0.39	0.43	0.09	3
Gadwall			5km	39	0.48	0.55	0.29	0.68	0.63	0.24	2
Gadwall			10km	39	0.48	0.55	0.29	0.87	0.86	0.17	1
Common Redshank	*Tringa totanus*	Scolopacidae	0km	40	0.46	0.45	0.31	0.01	0.01	0.03	4
Common Redshank			2.5km	40	0.46	0.45	0.31	0.61	0.54	0.21	2
Common Redshank			5km	40	0.46	0.45	0.31	0.43	0.44	0.11	3
Common Redshank			10km	40	0.46	0.45	0.31	0.74	0.75	0.14	1
Whooper Swan	*Cygnus cygnus*	Anatidae	0km	41	0.40	0.44	0.31	0.10	0.09	0.04	4
Whooper Swan			2.5km	41	0.40	0.44	0.31	0.37	0.39	0.14	3
Whooper Swan			5km	41	0.40	0.44	0.31	0.41	0.43	0.14	2
Whooper Swan			10km	41	0.40	0.44	0.31	0.87	0.84	0.20	1
Eurasian Teal	*Anas crecca*	Anatidae	0km	42	0.38	0.52	0.36	0.22	0.24	0.09	4
Eurasian Teal			2.5km	42	0.38	0.52	0.36	0.24	0.24	0.10	3
Eurasian Teal			5km	42	0.38	0.52	0.36	0.58	0.61	0.19	2

Eurasian Teal			10km	42	0.38	0.52	0.36	1.04	1.03	0.20	1
Greater Scaup	*Aythya marila*	Anatidae	0km	43	0.37	0.35	0.21	0.08	0.07	0.05	4
Greater Scaup			2.5km	43	0.37	0.35	0.21	0.36	0.36	0.08	3
Greater Scaup			5km	43	0.37	0.35	0.21	0.39	0.42	0.11	2
Greater Scaup			10km	43	0.37	0.35	0.21	0.59	0.56	0.12	1
Common Snipe	*Gallinago gallinago*	Scolopacidae	0km	44	0.37	0.52	0.40	0.22	0.21	0.13	4
Common Snipe			2.5km	44	0.37	0.52	0.40	0.35	0.35	0.09	3
Common Snipe			5km	44	0.37	0.52	0.40	0.36	0.36	0.09	2
Common Snipe			10km	44	0.37	0.52	0.40	1.13	1.08	0.28	1
Greylag Goose	*Anser anser*	Anatidae	0km	45	0.36	0.41	0.21	0.20	0.16	0.10	4
Greylag Goose			2.5km	45	0.36	0.41	0.21	0.28	0.27	0.05	3
Greylag Goose			5km	45	0.36	0.41	0.21	0.55	0.57	0.15	2
Greylag Goose			10km	45	0.36	0.41	0.21	0.61	0.64	0.16	1
Carrion Crow	*Corvus corone*	Corvidae	0km	46	0.33	0.30	0.15	0.07	0.08	0.03	4
Carrion Crow			2.5km	46	0.33	0.30	0.15	0.44	0.44	0.08	1
Carrion Crow			5km	46	0.33	0.30	0.15	0.37	0.37	0.07	2
Carrion Crow			10km	46	0.33	0.30	0.15	0.31	0.31	0.07	3
Ruff	*Calidris pugnax*	Scolopacidae	0km	47	0.31	0.34	0.15	0.44	0.43	0.22	1
Ruff			2.5km	47	0.31	0.34	0.15	0.23	0.21	0.07	4
Ruff			5km	47	0.31	0.34	0.15	0.31	0.29	0.06	3
Ruff			10km	47	0.31	0.34	0.15	0.38	0.37	0.08	2
Greater Canada Goose	*Branta canadensis canadensis*	Anatidae	0km	48	0.31	0.28	0.14	0.11	0.11	0.05	4
Greater Canada Goose			2.5km	48	0.31	0.28	0.14	0.30	0.31	0.10	3
Greater Canada Goose			5km	48	0.31	0.28	0.14	0.32	0.32	0.13	2

Greater Canada Goose			10km	48	0.31	0.28	0.14	0.39	0.39	0.09	1
Little Grebe	*Tachybaptus ruficollis*	Podicipedidae	0km	49	0.30	0.36	0.22	0.14	0.14	0.08	4
Little Grebe			2.5km	49	0.30	0.36	0.22	0.51	0.52	0.15	2
Little Grebe			5km	49	0.30	0.36	0.22	0.53	0.55	0.21	1
Little Grebe			10km	49	0.30	0.36	0.22	0.24	0.27	0.06	3
swamps and peat			0km	50	0.28	0.30	0.11	0.33	0.33	0.11	2
swamps and peat			2.5km	50	0.28	0.30	0.11	0.20	0.21	0.08	4
swamps and peat			5km	50	0.28	0.30	0.11	0.28	0.28	0.03	3
swamps and peat			10km	50	0.28	0.30	0.11	0.39	0.36	0.11	1
Grasslands			0km	51	0.27	0.29	0.09	0.35	0.32	0.13	1
Grasslands			2.5km	51	0.27	0.29	0.09	0.29	0.30	0.07	2
Grasslands			5km	51	0.27	0.29	0.09	0.25	0.27	0.07	3
Grasslands			10km	51	0.27	0.29	0.09	0.26	0.26	0.07	4
Egyptian Goose	*Alopochen aegyptiaca*	Anatidae	0km	52	0.27	0.31	0.16	0.28	0.27	0.11	2
Egyptian Goose			2.5km	52	0.27	0.31	0.16	0.24	0.23	0.09	4
Egyptian Goose			5km	52	0.27	0.31	0.16	0.23	0.24	0.08	3
Egyptian Goose			10km	52	0.27	0.31	0.16	0.50	0.49	0.17	1
Tundra Bean Goose	*Anser serrirostris*	Anatidae	0km	53	0.24	0.25	0.16	0.07	0.08	0.06	4
Tundra Bean Goose			2.5km	53	0.24	0.25	0.16	0.39	0.40	0.05	2
Tundra Bean Goose			5km	53	0.24	0.25	0.16	0.38	0.40	0.11	1
Tundra Bean Goose			10km	53	0.24	0.25	0.16	0.16	0.16	0.05	3
Green Sandpiper	*Tringa ochropus*	Scolopacidae	0km	54	0.24	0.25	0.08	0.19	0.18	0.05	4
Green Sandpiper			2.5km	54	0.24	0.25	0.08	0.30	0.33	0.09	1
Green Sandpiper			5km	54	0.24	0.25	0.08	0.23	0.23	0.06	3
Green Sandpiper			10km	54	0.24	0.25	0.08	0.27	0.25	0.07	2
Common Eider	*Somateria mollissima*	Anatidae	2.5km	55	0.21	0.29	0.27	0.03	0.03	0.02	3

Common Eider			5km	55	0.21	0.29	0.27	0.25	0.21	0.11	2
Common Eider			10km	55	0.21	0.29	0.27	0.59	0.55	0.22	1
White Stork	*Ciconia ciconia*	Ciconiidae	0km	55	0.21	0.21	0.11	0.07	0.08	0.04	4
White Stork			2.5km	55	0.21	0.21	0.11	0.22	0.23	0.08	3
White Stork			5km	55	0.21	0.21	0.11	0.30	0.32	0.06	1
White Stork			10km	55	0.21	0.21	0.11	0.26	0.29	0.10	2
Black-headed Gull	*Chroicocephalus ridibundus*	Laridae	0km	56	0.19	0.20	0.08	0.17	0.19	0.07	3
Black-headed Gull			2.5km	56	0.19	0.20	0.08	0.22	0.20	0.09	2
Black-headed Gull			5km	56	0.19	0.20	0.08	0.24	0.24	0.07	1
Black-headed Gull			10km	56	0.19	0.20	0.08	0.16	0.16	0.05	4
Saltwater systems			0km	57	0.14	0.17	0.19	-0.01	-0.01	0.01	4
Saltwater systems			2.5km	57	0.14	0.17	0.19	0.04	0.03	0.05	3
Saltwater systems			5km	57	0.14	0.17	0.19	0.32	0.30	0.14	2
Saltwater systems			10km	57	0.14	0.17	0.19	0.35	0.32	0.15	1
Jack Snipe	*Lymnocryptes minimus*	Scolopacidae	0km	58	0.08	0.12	0.11	0.06	0.06	0.05	3
Jack Snipe			2.5km	58	0.08	0.12	0.11	0.11	0.10	0.07	2
Jack Snipe			5km	58	0.08	0.12	0.11	0.05	0.05	0.03	4
Jack Snipe			10km	58	0.08	0.12	0.11	0.27	0.27	0.09	1

Table S3: Several land cover classes were aggregated to five major classes which were used in the random forest

Class	Aggregated Class used in random forest
Agricultural grass	Agriculture
Maze	Agriculture
Potatoes	Agriculture
Beets	Agriculture
Grains	Agriculture
Freshwater	Fresh water systems
Saltwater	Salt water systems
Grass in primary built-up areas	Grasslands
Grass in secondary built-up areas	Grasslands
Salt marshes	Salt water systems
Peat moor	Swamps and peats area
Other marsh vegetation	Swamps and peats area
Reed vegetation	Swamps and peats area
Nature grasslands	Grasslands

Table S4: List of locations of highly pathogenic avian influenza virus (HPAIV) infected dead wild birds. The predicted risk is the infection probability as predicted by the final random forest model for the location the bird was found

Year found dead	Bird species	HPAIV classification	KM-BLOK
2018	Mallard	H5N6	265234
2018	Western marsh-harrier	H5N6	265234
2016	Buzzard	H5N8	64244
2017	Mallard	H5N8	195512
2017	Mallard	H5N8	195512
2017	Mallard	H5N8	254552
2017	Mallard	H5N8	254552
2017	Mallard	H5N8	254552
2016	Mallard	H5N8	372743
2016	Buzzard	H5N8	253624
2017	Peregrine falcon	H5N8	311822
2018	Buzzard	H5N6	435152
2017	Buzzard	H5N8	314812
2016	Peregrine falcon	H5N8	424445
2016	Peregrine falcon	H5N8	424445
2016	Eurasian Magpie	H5N8	195754
2016	White-tailed eagle	H5N8	162531

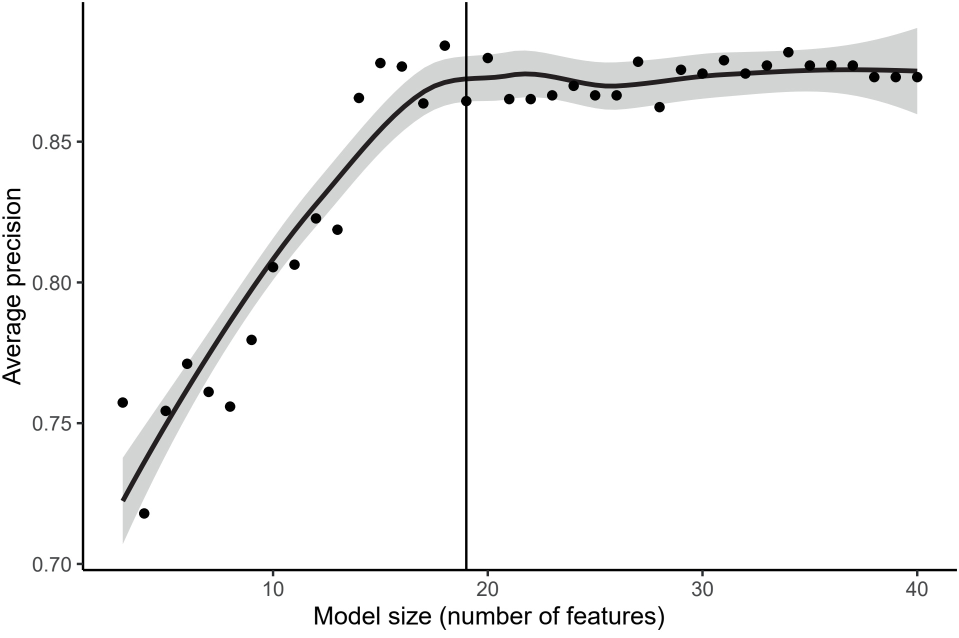
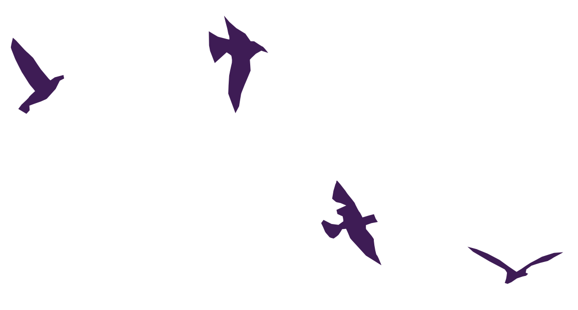


Figure S1: Average increase in precision of runs of the leave-one-group-out random forest (LoGo random forest) while adding the next most important feature for each run, as based on Table S2. On the x-axis, model size indicates the number of features included in the LoGo random forest. After inclusion of 19 features, the curve flattens and average precision does not increase any more with increasing the model size with the next most important feature. The black line tracks the calculated average precision estimates, and the shaded areas represents the 95% confidence interval

6

Highly pathogenic avian influenza subtype H5Nx clade 2.3.4.4 outbreaks in Dutch poultry farms, 2014–2018: Clinical signs and mortality



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ABSTRACT

In recent years, different subtypes of highly pathogenic avian influenza (HPAI) viruses caused outbreaks in several poultry types worldwide. Early detection of HPAI virus infection is crucial to reduce virus spread. Previously, the use of a mortality ratio threshold to expedite notification of suspicion in layer farms was proposed. The purpose of this study was to describe the clinical signs reported in the early stages of HPAI H5N8 and H5N6 outbreaks on chicken and Pekin duck farms between 2014 and 2018 in the Netherlands and compare them with the onset of an increased mortality ratio (MR). Data on daily mortality and clinical signs from nine egg-producing chicken farms and seven Pekin duck farms infected with HPAI H5N8 (2014 and 2016) and H5N6 (2017–2018) in the Netherlands were analysed. In 12 out of 15 outbreaks for which a MR was available, MR increase preceded or coincided with the first observation of clinical signs by the farmer. In one chicken and two Pekin duck outbreaks, clinical signs were observed prior to MR increase. On all farms, veterinarians observed clinical signs of general disease. Nervous or locomotor signs were reported in all Pekin duck outbreaks, but only in two chicken outbreaks. Other clinical signs were observed less frequently in both chickens and Pekin ducks. Compared to veterinarians, farmers observed and reported clinical signs, especially respiratory and gastrointestinal signs, less frequently. This case series suggests that a MR with a set threshold could be an objective parameter to detect HPAI infection on chicken and Pekin duck farms at an early stage. Observation of clinical signs may provide additional indication for farmers and veterinarians for notifying a clinical suspicion of HPAI infection. Further assessment and validation of a MR threshold in Pekin ducks are important as it could serve as an important tool in HPAI surveillance programs.

Keywords: H5N6 subtype, H5N8 subtype, influenza A virus, mortality, poultry, signs and symptoms

INTRODUCTION

In recent years, different subtypes of highly pathogenic avian influenza A (HPAI) viruses have caused outbreaks in different poultry types worldwide (Lee et al., 2017a; Napp et al., 2018).

Clearly, early detection of HPAI virus infection on poultry farms is essential to reduce risks for virus spread and minimize the socio-economic impact of the disease (Elbers et al., 2004; Backer et al., 2015), which is also increasingly reflected in legislation and contingency plans worldwide. European Union legislation on the control of HPAI (EU, 2005a, 2005b) stipulates that early detection systems, aimed at a rapid reporting of any sign of avian influenza in poultry and other captive birds by owners or keepers to the competent veterinary authority, need to be in place. For both LPAI and HPAI outbreaks, sudden changes in mortality have shown to be an indicator of infection (Elbers et al., 2007; Malladi et al., 2011; Gonzales and Elbers, 2018), as well as clinical signs (Elbers et al., 2004, 2005; Velkers et al., 2006).

These indicators have been used to formulate criteria in European Union legislation for reporting suspicion of a notifiable disease such as avian influenza in poultry, with even more detailed criteria implemented in national regulations in the Netherlands (Box 1). However, the current reporting thresholds may not be sensitive enough for timely detection of HPAI virus infections (Gonzales and Elbers, 2018). Published reports on analyses of mortality data from previous outbreaks, that is HPAI H7N7 in 2003 (Stegeman et al., 2004; Marian E.H. Bos et al., 2007) and HPAI H5N8 in 2014 and 2016 (Velkers et al., 2015) have shown that (a) it takes several days after the start of increased mortality due to HPAI virus infections to reach the official reporting threshold of 0.5% mortality for two consecutive days; and (b) many flocks have already been depopulated well before reaching these thresholds. To improve sensitivity of detection of LPAI and HPAI virus infections and at the same time maintain a high level of specificity, Gonzales and Elbers (2018) developed new reporting thresholds based on increased mortality and drops in egg production for layer farms, and evaluated the performance of those indicators with HPAI H7N7 outbreak

Box 1 European legislation and Dutch regulations on reporting criteria for avian influenza detection

European Commission Decision 2005/734/EC (EU, 2005a):
Article 2 stipulates that Member States shall introduce early detection systems, aimed at a rapid reporting of any sign of avian influenza in poultry and other captive birds by the owners or keepers to the competent veterinary authority.
Annex II: criteria to be considered when applying the measure set out in Article 2: drop in feed and water intake higher than 20%; drop in egg production higher than 5% for more than two days; mortality rate higher than 3% in a week; and any clinical sign or post-mortem lesion suggesting avian influenza.
Dutch Ministerial Regulation TRCJZ/2005/1411 concerning the prevention, control and monitoring of infectious animal diseases, zoonoses and transmissible spongiform encephalopathies (TSEs), Article 84 (Dutch State Journal, 2005):
Poultry keepers have to report increased mortality in layers, reproduction birds or broilers (older than 10 days) to the authorities in case of 0.5% mortality or more per flock per day for two consecutive days; in turkeys in case of 1% mortality or more per day for two consecutive days; and in AI susceptible birds in case of 3% or more mortality per week.
Poultry keepers of AI susceptible birds need to consult their veterinarian in case of a clinical problem; reduction in feed intake or water intake of 5% or more per day for two consecutive days; in layers and breeders a reduction in egg production of 5% or more per day for two consecutive days.
Approved veterinary programme of the Netherlands under EU Regulation 652/2014 (EC, 2019; Elbers et al., 2010):
Additionally, to ensure timely detection and minimize spread of infections with low pathogenic avian influenza (LPAI) viruses, that can mutate to HPAI viruses, an intensive monitoring program that includes all commercial poultry holdings in The Netherlands is in place. Because LPAI virus infections can be asymptomatic or might generate only mild symptoms, veterinarians in the Netherlands can submit cloacal or pharyngeal swabs to exclude LPAI virus infection as a possible cause for clinical problems.

data from 110 infected layer flocks in the Netherlands in 2003. The mortality ratio (MR), with a reporting threshold of 2.9 times higher mortality than the average weekly mortality of the previous week for that particular flock, had a 95.3% sensitivity to signal HPAI virus infection in laying hens and would have resulted in 2 days earlier detection compared with the current Dutch national thresholds for HPAI and in 7 days earlier detection for LPAI virus infection (Gonzales and Elbers, 2018).

For early detection of HPAI virus infections, the suggested MR ratio threshold of 2.9 may also be applicable to other poultry types. Ssematimba et al. (2019) recently explored efficacy of mortality-based triggers for HPAI virus detection in game birds, but for commercial ducks and turkeys, which

are also commonly affected during HPAI outbreaks, mortality thresholds have not yet been evaluated. Furthermore, as clinical signs have proven to be indicators of HPAI virus infections, taking both MR and clinical signs into account may potentially further enhance early detection in different poultry types.

Therefore, the aim of this study was to describe the clinical signs reported in the early stages of HPAI H5N8 and H5N6 outbreaks on chicken and Pekin duck farms between 2014 and 2018 in the Netherlands and compare them with the onset of an increased MR. For this purpose, we collected data on mortality, production characteristics and clinical signs from 16 HPAI (H5N8 and H5N6) outbreaks on poultry farms between 2014 and 2018 in the Netherlands. We calculated the MR and daily mortality for each outbreak and provide an extensive inventory of the species-specific clinical signs and how these developed over time in the days before official notification, as observed by poultry farmers and veterinarians.

MATERIALS AND METHODS

Data collection

A case series study was performed on a total of 16 poultry farms that were diagnosed with HPAI infection caused by viruses of subtypes H5N8 or H5N6 in the Netherlands between 2014 and 2018, which included six farms with laying hens, three farms with broiler breeders and seven farms with Pekin ducks (Table 1). The only other HPAI H5N8 outbreak in this period (OIE, 2017) not included in the analysis, was a wild water bird trading farm, that also housed domestic poultry in 2016. The day of notification (Table 1; Figure 1) refers to the day when the farmer or the veterinary practitioner reported a suspicion of avian influenza to the Netherlands Food and Consumer Product Safety Authority (NVWA). Only for outbreak D-1, samples were submitted to the national reference laboratory by the veterinary practitioner in the Dutch national diagnostic framework of excluding LPAI (as described in Box 1; EC, 2019). In this outbreak, we considered the day of the positive result of these swabs as day of notification. In all outbreaks, a team consisting of a (state)

veterinarian of the NVWA, a poultry veterinarian from GD Animal Health, and in most outbreaks the veterinary practitioner, visited the farms within 9 hr after notification for clinical inspection and official sample collection (referred to as veterinary inspection visit [VIV]). Inquiries on the history of the clinical situation observed by the farmer and clinical signs observed by the veterinarians during this inspection were recorded in a standardized form (see Section 2.3). Twenty cloacal and pharyngeal swabs were collected per flock. Swabs were tested at the national reference laboratory by PCR for antigen detection (Beerens et al., 2018). According to the protocol of HPAI virus-positive farms, NVWA performed an epidemiological investigation to trace dangerous contacts prior to culling. This included a standardized interview with the farmer and collection of charts with at least daily records of mortality and production data, for example feed and water intake, and egg production. All birds on the HPAI virus-positive farms were culled within 1–2 days after the day of notification (Table 1).

Additionally, an in-depth epidemiological investigation was performed by specialized poultry veterinarians of the Faculty of Veterinary Medicine of Utrecht University. This investigation was performed for all farms between 9 days to 3 months after culling and was aimed to facilitate retrospective identification of the most likely moment and route of HPAI virus introduction and/or spread (referred to as Detailed Epidemiological Investigation [DEI]). For all farms, all available data collected by NVWA and laboratory results were evaluated, additional in-depth interviews with farmers and farm employees, veterinarians from NVWA, GD Animal Health and the farms' veterinary practitioner were conducted retrospectively, and detailed production records were gathered. The farmers and veterinarians were inquired in detail about the course of infection and observed clinical and post-mortem signs in the 2 weeks prior to notification up to and including the day of the VIV. These data were used for further data analyses as described below.

Table 1: Highly pathogenic avian influenza (HPAI) virus-infected commercial chicken and duck farms in the Netherlands between 2014 and 2018 included in the study: notification and culling dates, flock data and HPAI virus subtype

Outbreak no.[a]	Date of notification	Date of culling	Poultry type	Flock size	Affected houses / total houses	Flock age at notification	HPAI virus type[b]
L-1	14-Nov-14	16-Nov-14	Laying hens	124,000	1/6	55 wk	H5N8
L-2	19-Nov-14	21-Nov-14	Laying hens	41,400	1/3	67 wk	H5N8
BB-1	20-Nov-14	21-Nov-14	Broiler breeders	11,000	1/2	61 wk	H5N8
D-1[e]	21-Nov-14[c]	22-Nov-14	Pekin ducks	14,500	1/2	18 d	H5N8
L-3[f]	29-Nov-14	30-Nov-14	Laying hens	28,000	1/1	22 wk	H5N8
D-2	25-Nov-16	26-Nov-16	Pekin ducks	10,000	1/1	40 d	H5N8
D-3	30-Nov-16	1-Dec-16	Pekin ducks	8,500	1/1	24 d	H5N8
D-4[g]	1-Dec-16	2-Dec-16	Pekin ducks	15,400	2/2	15 & 43 d[d]	H5N8
L-4	12-Dec-16	14-Dec-16	Laying hens	63,000	1/3	38 wk	H5N8
D-5[e]	16-Dec-16	17-Dec-16	Pekin ducks	14,000	1/2	23 d	H5N8
L-5	17-Dec-16	19-Dec-16	Laying hens	28,500	1/2	25 wk	H5N8
BB-2	19-Dec-16	20-Dec-16	Broiler breeders	48,000	1/4	30 wk	H5N8
L-6[f]	24-Dec-16	25-Dec-16	Laying hens	28,000	1/1	52 wk	H5N8
D-6[g]	7-Dec-17	8-Dec-17	Pekin ducks	16,000	1/2	29 d	H5N6
BB-3	24-Feb-18	26-Feb-18	Broiler breeders	39,100	1/3	31 wk	H5N6
D-7[e]	12-Mrt-18	13-Mrt-18	Pekin ducks	29,700	1/2	32 d	H5N6

[a]*Outbreaks on Laying hen (L), Broiler Breeder (BB) and Duck (D) farms.*
[b]*Diagnosis of HPAI, tested positive on real-time PCR on the matrix gene, H5-PCR and sequencing of the haemagglutinin and neuraminidase (Beerens et al., 2018).*
[c]*Samples were submitted to the national reference laboratory by the veterinary practitioner in the framework of the Dutch early-warning system, we considered the day of the positive result of these samples as day of notification.*
[d]*Two flocks infected with HPAI virus present on the farm, one flock age 15 days the other age 43 days.*
[e]*D-1, D-5 and D-7 are outbreaks of HPAI on the same duck farm.*
[f]*L-3 and L-6 are outbreaks of HPAI on the same laying hen farm.*
[g]*D-4 and D-6 are outbreaks of HPAI on the same duck farm.*

Mortality and production parameters

Mortality ratio (MR) and egg production ratio (EPR) were calculated as described by Gonzales and Elbers (2018) for each of the flocks, using available flock records of at least 5 days to approximately 1 month before notification. The threshold of 2.9 for MR, as applied for laying hens by Gonzales and Elbers (2018), was used and the first day the MR exceeded the threshold was

considered as an increase in MR and used for further analyses. We were not able to calculate the MR for one Pekin duck farm (D-2) due to incomplete mortality data in the weeks prior to the outbreak. The current applied daily mortality (DM) threshold of 0.5% per flock (see Box 1) was also used for comparisons. In layer farms, an EPR of below 0.94 was considered as presence of reproduction tract signs. The use of this threshold alone, and in combination with the MR, was validated as a way to detect LPAI and HPAI outbreaks at an early stage by Gonzales and Elbers (2018). Data on daily growth were not recorded in any of the affected farms. In farms where records of water and feed intake were available, a decrease in feed or water intake of 5% compared with the previous day was classified under general clinical signs as described below.

Clinical signs

The standardized form used to record clinical signs observed during the VIV included a yes or no checklist with questions on feed and water intake, sudden death, ruffled feathers, diarrhoea, egg quality, oedema and cyanosis, nervous signs, abnormal conjunctivae, lacrimation, respiratory distress and decreased activity. Furthermore, the veterinarians recorded findings on mortality, production and feed and water intake based on the flock records if available at time of VIV. At the DEI, poultry veterinarians of GD Animal Health and the farms' veterinary practitioners were questioned in more detail on the clinical signs on day of notification. In two outbreaks, the veterinary practitioner had visited the farm prior to notification, that is for outbreak D-1 2 days and for BB-3 1 day prior to notification. The observed clinical signs did not differ from the clinical signs observed at day of the VIV (data not shown). The farmers were queried at the DEI on the clinical signs observed in the period between 14 days prior to and the day of culling, but only the data until day of notification were used for the analyses. Also, the flock records were checked for notes on clinical signs.

A list of clinical signs, categorized in different categories, was used to compile all the data from the veterinarians from VIV and DEI, and only from de DEI for the farmers separately. The observed clinical signs were

categorized in six categories, that is as clinical signs attributed to nervous and locomotor system; mucosal membranes and skin; respiratory tract; gastrointestinal tract; and reproduction tract (Tables S1–S4) or as general clinical signs. The latter category included signs of general illness, which could not be related to a specific organ system or were associated with signs of systemic disease, for example depression, reduced feed or water intake, ruffled feathers or hunched posture, cold or warm extremities and sudden death (Tables S1–S4). Signs of the nervous and locomotor system were categorized together as these were difficult to distinguish based on the information from the farmers. Mucosal membranes and skin signs included discolorations or oedema, most likely because of the endothelial damage caused by the virus, for example cyanosis, oedema and haemorrhages, including those in the conjunctivae. Excessive lacrimation and conjunctivitis without haemorrhages were categorized under (upper) respiratory signs. Decreased egg production (EPR < 0.94) and abnormal eggs were classified as signs of the reproduction tract. These data were used to report the frequency of detection of clinical signs for each of the six categories in Pekin duck and chicken farms (layers and broiler breeders) and for veterinarians and farmers separately.

RESULTS

Outbreaks

Five, eight and three farms were infected in the autumn-winter period of 2014, 2016 and 2017–2018, respectively. No outbreaks occurred in 2015. In 2014 and 2016 six farms with laying hens, two broiler breeder farms and five Pekin duck farms were infected with HPAI virus H5N8. In the winter of 2017–2018, two Pekin duck farms and a broiler breeder farm tested positive for HPAI virus H5N6. Some farms were affected repeatedly. This was the case for Pekin duck farms D-1, D-5 and D-7, for D-4 and D-6 and for laying hen flocks L3 and L6. On 13 farms, more than one poultry house was present, but only in one duck farm (D-4) two houses tested HPAI virus positive. The age of infected Pekin ducks varied between 15 and 43 days and chicken flocks were between 22 and 67 weeks of age at notification.

Clinical signs

A detailed list of the observed clinical signs by farmers and veterinarians in the chicken and duck flocks based on VIV and DEI is provided in Tables S1–S4.

Chicken farms

Figure 1 summarizes the clinical signs that were observed by farmers in their flocks in the 5 day period prior to notification to the authorities, and occurrences where the current official DM threshold for reporting (>0.5%) or the MR threshold (>2.9) were exceeded. In both parameters, the first day the parameter exceeded its threshold was used in the further analyses. For the chicken farms, the first signs observed by the farmers were those of general disease in outbreak L-3 at 3 days prior to notification. On the day of notification, the farmers of the chicken farms (n = 9 outbreaks) observed general clinical signs in all nine outbreaks, clinical signs of the reproduction tract in six outbreaks, clinical signs of mucosal membranes and skin in three outbreaks, clinical signs of the gastrointestinal tract in three outbreaks, and clinical signs of the respiratory tract in two outbreaks. MR exceeded the threshold in all nine outbreaks, but only in six outbreaks the DM exceeded 0.5% per day on day of notification.

The frequency of observed clinical signs on the chicken farms (n = 9 outbreaks), as reported by the farmers (at day of notification) or veterinarians (during the VIV) for the six different categories is summarized in the left part of Figure 2. Similar to the farmers, the veterinarians reported general clinical signs in all nine chicken outbreaks. The frequency of the clinical signs reported by the veterinarians was higher for signs of the gastrointestinal tract (seven outbreaks), mucosal membranes and skin (five outbreaks), and respiratory tract (five outbreaks), but lower for reproduction tract (four outbreaks) compared with the farmers. None of the farmers reported nervous or locomotor signs, whereas veterinarians reported this in two outbreaks.

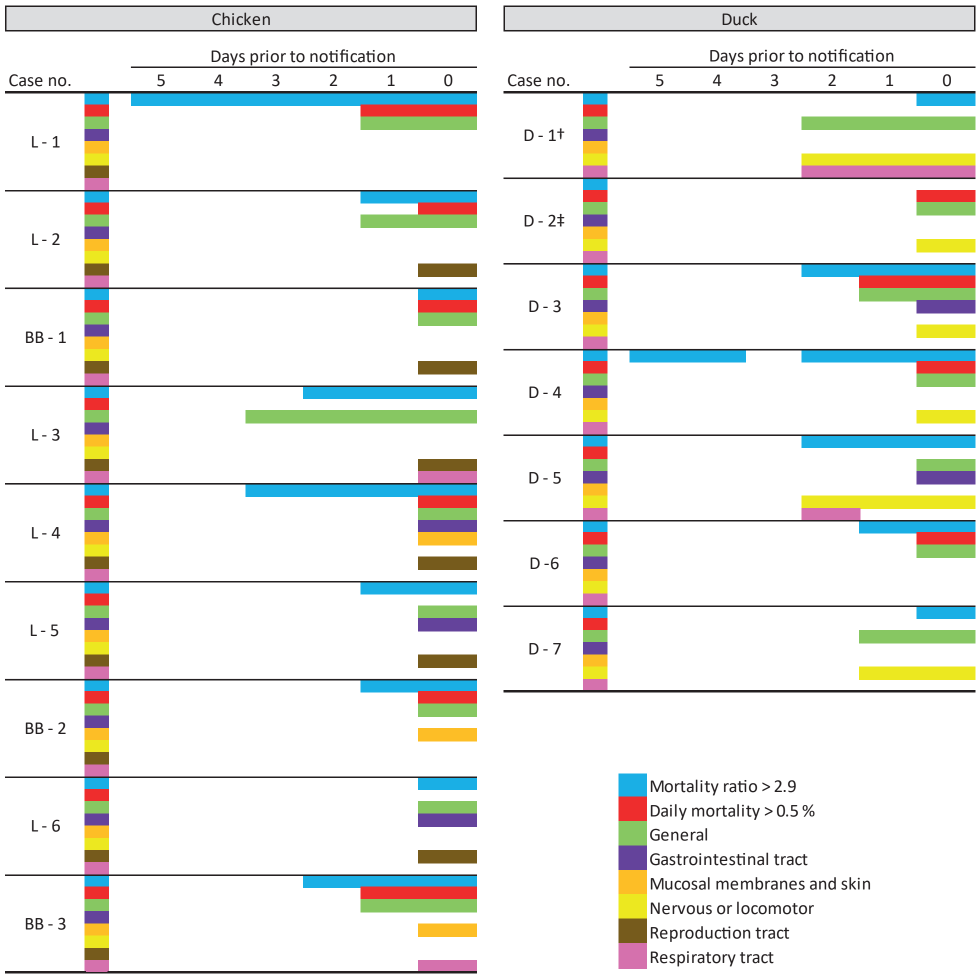

Figure 1: Clinical signs observed by the farmers, categorized by organ system, for the highly pathogenic avian influenza virus-infected chicken (left) and duck farms (right) and exceedance of daily mortality (>0.5%) and mortality ratio (MR) thresholds in the 5 day period prior to notification. †Day of notification for D-1 was the day a positive result was found in the early warning swabs sent in by the veterinary practitioner. ‡Not enough mortality data were available to calculate the mortality ratio

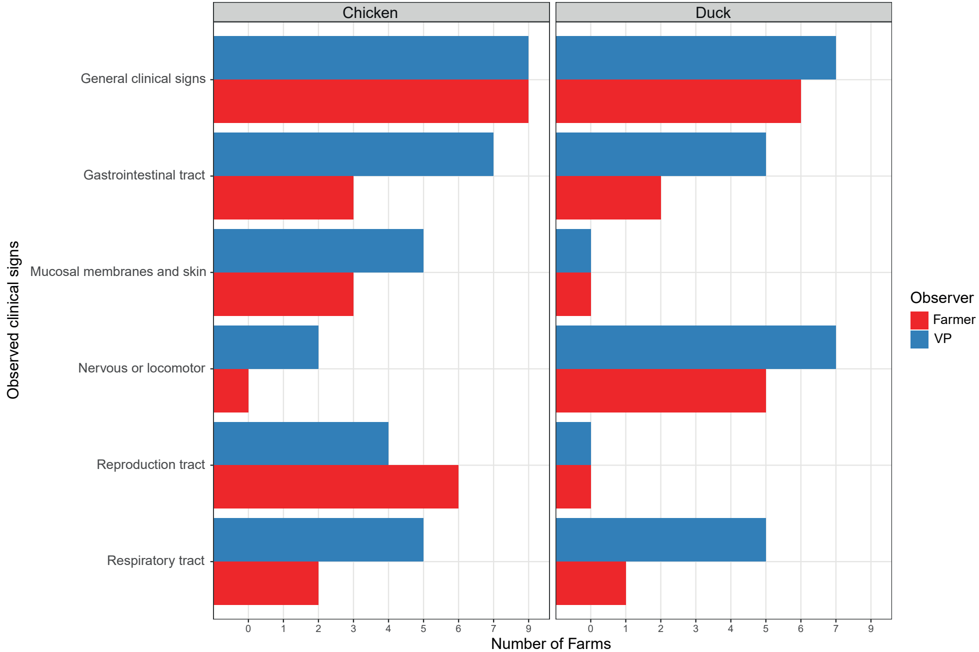

Figure 2: Overview of the frequency of detection of clinical signs, as categorized by organ system, observed on day of notification by farmers (in red) and veterinarians (in blue) on the day of veterinary inspection on highly pathogenic avian influenza virus-infected chicken (n = 9, left) and duck farms (n = 7, right)

Pekin duck farms

In the Pekin duck farms (n = 7 outbreaks), the first clinical signs were observed 2 days prior to the notification in two outbreaks (D-1 and D-5), which included general clinical signs and signs of the nervous or locomotor system and respiratory tract (Figure 1). For outbreak D-5, temporary sneezing was only observed at day two before notification. A day prior to notification farmers observed clinical signs of the nervous or locomotor system in three, and of the respiratory tract in one of the outbreaks. On the day of notification, general clinical signs were observed in all seven outbreaks, signs of the nervous or locomotor system in six, gastrointestinal signs in two and respiratory tract signs in one of the outbreaks. The DM exceeded the 0.5% threshold in only four outbreaks whereas the MR exceeded the threshold of 2.9 in six outbreaks at day of notification. For D–2, the DM was only available from 1 day prior to notification and therefore the MR could not be calculated.

The frequency of observed clinical signs on the seven duck farms, as reported by the farmers (at day of notification) or veterinarians (during the VIV) for the six different categories are summarized in the right part of Figure 2. Overall, the frequency of clinical signs reported by the veterinarians was higher compared with the frequency of the clinical signs reported by the farmer. Similar to the farmers, the most prominent signs reported by the veterinarians were general clinical signs. Clinical signs of the nervous or locomotor system were observed in all seven Pekin duck outbreaks, and clinical signs of the respiratory tract and the gastrointestinal tract were observed in five outbreaks. In contrast with the veterinarians, farmers only reported respiratory signs in one outbreak. Unlike the clinical signs observed on chicken farms, no signs of the membranes and skin were observed in the duck flocks by farmers nor veterinarians.

Mortality

Chicken farms

For chicken flock L-1, the MR exceeded the threshold 5 days prior to the day of notification, whereas in all others outbreaks the MR exceeded the threshold three or fewer days prior to notification (Figure 1). On the day of notification, the MR of all chicken farms exceeded the threshold. The DM exceeded the 0.5% threshold in two farms 1 day prior to notification and in six farms at day of notification. The MR exceeded 2.9 for only a single day on eight occasions on six different farms in the 30 days period prior to notification (Figure 3).

In five out of nine outbreaks, the MR exceeded the proposed threshold prior to observing of clinical signs by the farmer, in three out of nine outbreaks the increase of the MR and first observation of clinical sign coincided, and in one outbreak the clinical signs were observed prior to an increased MR (Figure 1).

Pekin duck farms

The MR exceeded the threshold the first time 5 days prior to day of notification in one house of one Pekin duck farm (D-4.1; Figure 1). At the day of notification, the MR exceeded the threshold in all six outbreaks for which a MR was available. The DM exceeded 0.5% in four of seven outbreaks on day of notification and only in one outbreak (D-3) mortality exceeded 0.5% 1 day prior to notification (Figure 1). The MR exceeded 2.9 for only a single day on seven occasions on four different farms in the 30 days period prior to the notification (Figure 3). On six occasions the MR exceeded the threshold for a single day and was <2.9 the following day. On one occasion, the MR exceeded the threshold on two consecutive days in one house of a farm (D-4.1). This house also had the most occasions (five out of seven) in which the MR temporarily exceeded the threshold.

The MR exceeded the threshold in three out of six outbreaks prior to observation of clinical signs. In one outbreak, the increase of MR coincided with the first observation of clinical signs, and in two out of six outbreaks the clinical signs were observed prior to an increase in MR (Figure 1).

DISCUSSION

The purpose of this case series was to describe the observed clinical signs in HPAI H5N8 and H5N6 outbreaks on chicken and Pekin duck farms between 2014 and 2018 in the Netherlands and compare this with the onset of an increased MR threshold (Gonzales and Elbers, 2018). We describe that in 12 out of 15 outbreaks for which a MR was available on chicken and Pekin duck farms, the MR increase preceded or coincided with the first observation of clinical signs by the farmer. In one chicken and two Pekin duck outbreaks, clinical signs were observed prior to a MR increase. Additionally, in most cases the first clinical signs were seen within a day or two after the onset of an increased MR. Although these observations conveyed the idea that MR could be an earlier indicator of HPAI infection, when MR is less affected, for instance for less virulent AI virus strains, the observation of clinical signs in combination with MR may provide additional indication for farmers and veterinarians and prompt them to notifying the disease.

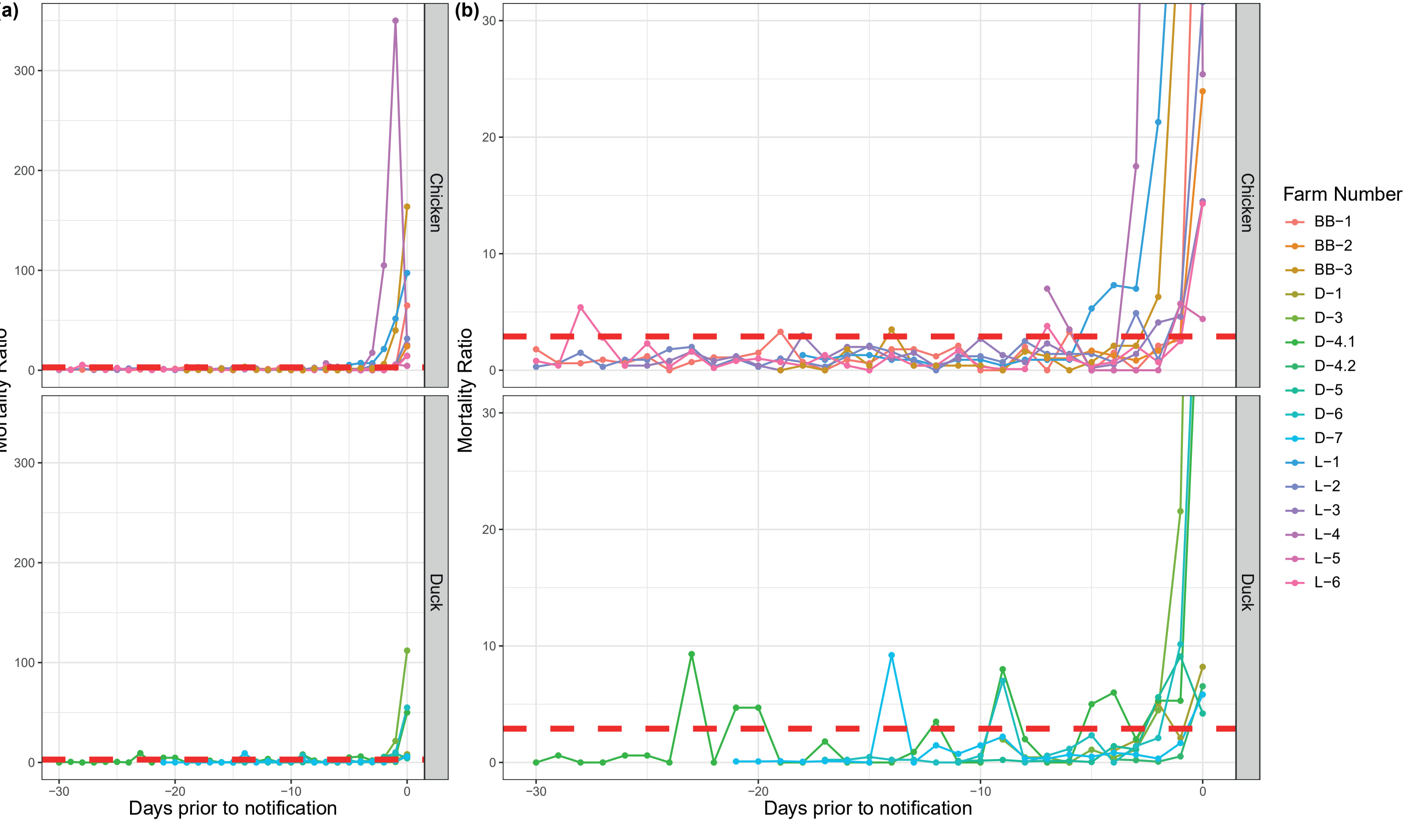

Figure 3: (a) Calculated mortality ratio’s in the 30 day period prior to highly pathogenic avian influenza (HPAI) notification for the nine chicken farms (top), of which three broiler breeder (BB) and six layer (L) farms, and six Pekin duck farms (D; bottom). The mortality ratio (MR) threshold of 2.9 is shown in red. In one outbreak on a duck farm, 2 houses were affected: D-4.1 and D-4.2. (b) A more detailed plot of the calculated mortality ratio’s in the 30 day period prior to HPAI notification in chicken and Pekin duck outbreaks. Cut-off on the Y-axis was set to 30 to better visualize the behaviour of the MR in days prior to notification. The MR threshold of 2.9 is shown in red

It should be noted that we looked at the first day the MR exceeded the threshold and compared that with the first observation of clinical signs according to the interviews with the farmers, because we were interested in the timing of detection of clinical signs in relation to an increase of the MR. Gonzales and Elbers (2018), however, proposed that the MR should be implemented in practice to notify authorities only after the MR exceeds the threshold for two consecutive days, to reduce false-positive signals (i.e. increase specificity). By using that logic, an increased MR still preceded or coincided with the first observation of clinical signs in eight out of 15 outbreaks (five outbreaks on chicken farms, three outbreaks on Pekin duck farms).

To our knowledge, this is the first report to apply this MR threshold in Pekin duck outbreaks. Our results show that the MR fluctuated more in Pekin duck farms in comparison with the layer farms and exceeded the threshold more often in the 30 day period prior to the HPAI virus infection. However, in six of the seven occasions that the MR exceeded the threshold in Pekin duck farms, the MR only exceeded the set threshold for 1 day, which would not lead to a notification to the authorities when the MR is applied as suggested by Gonzales and Elbers (2018). Furthermore, the MR exceeded the threshold in all outbreaks on Pekin duck and chicken farms, whereas the DM only exceeded 0.5% in four out of seven outbreaks on the Pekin duck farms and in six out of nine outbreaks on chicken farms. Moreover, in eight out of nine outbreaks in chicken and Pekin duck farms where the DM did exceed 0.5%, the MR had already exceeded its set thresholds 1–4 days prior. In pheasants, however, it was found that exceeding a set absolute threshold on two consecutive days resulted in the best trade-off between false-alarm rate and early detection compared with a 7 day moving average or exceeding a set absolute threshold for 1 day (Ssematimba et al., 2019). Due to the limited data set, we were not able to evaluate these trade-offs appropriately, but the results obtained from these H5Nx outbreaks in the Netherlands suggest that the MR could be a more sensitive parameter to monitor for HPAI virus infection in Pekin ducks compared with the current DM used in Dutch legislation for notification to the authorities. As the

choice of an effective mortality threshold requires evaluation of the trade-off between lowering the threshold to enhance early detection of infected flocks and the corresponding increase in false alarm rates in uninfected flocks, more research is needed. To assess and validate the currently used MR, and determine the best set threshold for an optimal sensitivity and specificity for Pekin ducks, and where possible also for other poultry species, flock data from outbreaks with preferably different HPAI virus strains should be analysed.

In chickens, veterinarians reported general clinical signs in all nine outbreaks, signs of the gastrointestinal tract in seven outbreaks, mucosal membrane and skin in five and respiratory tract also in five outbreaks at the day of notification. The clinical sings were not notably different over the years, although the outbreaks in 2014 and 2016 were caused by subtype H5N8 and in 2017–2018 by H5N6. These findings are in line with earlier reports about H5Nx infections in chickens (Sun et al., 2016; Pohlmann et al., 2017). Sun et al. (2016) found that naturally infected H5Nx chickens developed systemic disease, congestion and haemorrhage of the comb, wattles and feet, subcutaneous haemorrhages and oedema around the hock and shanks, which are similar to the clinical signs that were reported in the mucosal membrane and skin (Table S1). Early in the flock infection process, however, the farmers in our study mainly observed clinical signs that could only be considered as general clinical signs, which are not specific for HPAI virus infection (Elbers et al., 2007; Swayne et al., 2013) suggesting that in early stages of the infection process it is difficult to distinguish HPAI virus infection from other diseases that lead to systemic disease. Clearly, when the farmer or veterinarian suspects HPAI infection, immediate notification is needed. However, in cases with rather mild clinical signs or limited increased mortality not specific for HPAI, the submission of cloacal or pharyngeal swabs to exclude AI infection is recommended to facilitate detection of circulating AI virus at an early stage. This is already implemented in the Netherlands, as mentioned in Box 1, and has shown to be effective in detecting LPAI outbreaks, and incidentally, as described in this study for duck farm D-1, also for detection of HPAI outbreaks at an early stage (Elbers et al., 2010).

In Pekin ducks, veterinarians reported general clinical signs and nervous or locomotor signs most often and in all outbreaks. This was followed by respiratory and gastrointestinal signs, which were both reported in five out of seven outbreaks. The high incidence of nervous and locomotor signs, also observed by six of the Pekin duck farmers, is in contrast with the incidence in chickens, where nervous and locomotor signs were only reported in two outbreaks by veterinarians. Although the outbreaks in 2014 and 2016 were caused by different subtypes of H5Nx, the clinical signs were not notably different over the years in Pekin ducks. The observation of neurological signs (mainly head tremors, torticollis and ataxia) in our study in Pekin ducks is in line with findings reported in an outbreak of H5N8 among fattening Pekin ducks in Hungary in 2015 (Bányai et al., 2016) where affected ducks showed neurologic signs, including torticollis. These findings are further supported with the results of studies where Pekin ducks were infected experimentally with different H5Nx subtypes of clade 2.3.4.4 (Sun et al., 2016). However, in other experimental inoculated domestic ducks with H5N8 viruses of the same clade (2.3.4.4), a wide range of pathobiological outcomes, from no clinical signs to some neurological signs to severe disease, were reported (Kang et al., 2015; Shivaprasad et al., 2016; Pantin-Jackwood et al., 2017). Although previous cases have shown that clinical manifestation and mortality in Anseriformes species highly depends on the phenotypic characteristics of the HPAI virus (EFSA Panel on Animal Health and Welfare (EFSA AHAW Panel), 2017), the current case series emphasizes that Pekin duck farmers and veterinarians should be aware that observation of neurological signs in a flock could be an indication of HPAI virus infection and might require further diagnostic follow-up.

Compared to the veterinarians, farmers observed and reported less specific clinical signs, especially regarding respiratory and gastrointestinal signs in both chicken and ducks. This difference may be due to the specialized training and experience of the veterinarians in poultry veterinary medicine to observe signs of disease, and veterinarians may be better equipped with a repertoire of specific words to indicate their observations and relate that to a specific organ system. The discrepancy in observation of clinical signs

between farmers and veterinarians is, however, smaller than we anticipated, suggesting that the farmers were aware of signs to look for. This shows that training and awareness of the farmer in detecting clinical signs is an important tool in detecting HPAI virus infection at an early stage.

The willingness of the farmer and practitioners to report a suspicion of a notifiable disease to the authorities may be different for the very first suspicion compared with suspicions after the first confirmed HPAI outbreak (Elbers et al., 2010). To prevent the spread of HPAI viruses to other farms, it is crucial to notify a suspicion as early as possible to be able to adequately diagnose and quickly depopulate the farms. The first outbreak of a HPAI (H5Nx; outbreak no. L-1) in 2014 had increased mortality (>2.9) for 5 days prior to notification. In the outbreaks after 2014, the mortality ratios exceeded the threshold 0–3 days prior to notification, which suggest that farmers were more alert and reported a suspicion of notifiable disease more rapidly. Additionally, two Pekin duck farms and one chicken farm had multiple outbreaks of HPAI in 2014, 2016–2017 on their farms, making the farmers even more aware of the risk of a new outbreak. Due to the fast reporting of HPAI suspicion of farmers and veterinarians to the authorities, the spread of HPAI viruses to other poultry facilities was minimized.

To conclude, the current study gives an indication that the use of an objective MR with a set threshold could be a reliable parameter to detect HPAI virus infection on chicken and Pekin duck farms at an early stage and may perform even better when complemented with detection of clinical signs in poultry farms, provided farmers are well trained to notice them. These results underline the need to validate the MR in Pekin ducks and other poultry species, and it should encourage farmers, veterinarians and veterinary institutes in other countries to monitor and register mortality on farms more rigorously, because a poultry-specific MR could serve as an important indicator in HPAI poultry surveillance programs.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Most of the data that support the findings of this study are available in the article or supplementary materials. Additional data are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

SUPPORTING INFORMATION

Additional supporting information may be found online at https://onlinelibrary.wiley.com/doi/full/10.1111/tbed.13597

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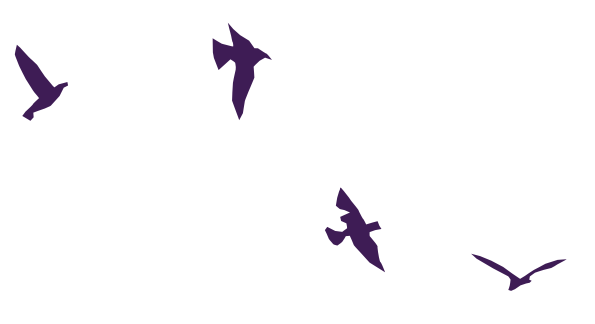
General discussion



The main aim of the research described in this thesis is to evaluate tools that can be used to predict and detect highly pathogenic avian influenza (HPAI) outbreaks on poultry farms in order to reduce risks of HPAIV introductions and prevent further spread to other farms via appropriate control measures. In this chapter the major findings of this research are summarized and discussed, and perspectives for future studies are provided. Furthermore, additional analyses using data from ten new HPAI outbreaks that occurred in the Netherlands in the autumn-winter months of 2020-2021 are presented.

Part 1 – The microbiome as a proxy for contact with wild birds

Microbiota dynamics

The main conclusion of chapters 2-4 is that the microbiome is not a suitable proxy for contact between wild birds and poultry, at least not with the techniques that were applied during our experimental and field studies. We found no detectable effect of a fecal transplant with wild bird feces to chickens (Chapter 2) and also found little to no differences between indoor- and outdoor-housed chickens in a cross-sectional study of poultry flocks with and without access to an outdoor range (Chapter 3). Even over a period of 16 weeks, no major shifts in gut microbial community were detected when flocks were given access to an outdoor range (Chapter 4). We did find high temporal variability of the cloacal microbiota within chickens over time (Chapter 2) and between chickens within the same flock (Chapters 2, 3 and 4). This indicates that, like in humans, the adult chickens' gut microbiome is a dynamic system on the one hand, but it is also stable (Fassarella et al., 2021). As we did not detect any effect on the microbiota community after a single oral inoculation of adult chickens with wild duck feces (Chapter 2), nor found differences over time in the community composition of layer flocks exposed to an outdoor environment (Chapter 4), the microbiota communities of the laying hens in our studies were likely not only stable, but also resistant to the applied perturbations.

A well-developed intestinal microbiota community is highly beneficial for the host. It forms a complex ecosystem which is characterized

by a capacity for self-regeneration after an external perturbation, i.e. it shows resilience (Lozupone et al., 2012; Lahti et al., 2014; Sommer et al., 2017), and protects the host by creating a gastrointestinal resistant environments which helps prevent external microbiota from colonizing, i.e. it is resistant (Lawley and Walker, 2013; Han et al., 2017). As an animal host ages, its influence on microbial selection in the development of the intestinal microbiota increases (Björk et al., 2019). Previous studies have shown that layers above the age of 25 weeks (Ngunjiri et al., 2019) or 28 weeks (Videnska et al., 2014) reach an adult microbial equilibrium. The age of the chickens in our studies varied between 24 – 49 weeks, and the effect of the exposure to an outdoor environment was therefore likely dampened by the adults' host homeostatic responses. In contrast, the developing microbiota community in young chickens is easily modified. Studies in broiler chickens showed that microbial treatment supplied after hatch did result in changes in the development of bacterial taxa (Ballou et al., 2016; Schokker et al., 2017), and that, besides age and feed, the living environment predominantly impacted the microbiota composition development (Kers et al., 2019b; Schokker et al., 2021). We also still measured an effect of the environmental conditions of the rearing farm on the chickens' microbiota in both the cross-sectional (Chapter 3) and longitudinal field studies (Chapter 4). This indicates that the microbiome in young layers is more plastic compared to the community in adult chickens, and this is an important aspect to consider in studies of the microbiota composition.

In humans, fecal transfer is often preceded by antibiotic treatment or bowel lavage (Schmidt et al., 2018). These treatments cause a short term decrease in microbial diversity, and enable the donor microbiota to colonize (Voigt et al., 2015; Schmidt et al., 2018). In Chapter 2, where layers were inoculated with duck feces, the chickens were not exposed to such perturbations prior to the experiment. As a consequence, the healthy adults layers microbiome was most likely quite resistant to change, making it difficult for duck microbiota to outcompete the chicken's microbiota and successfully colonize the gut of the chicken. Oral inoculation with duck feces did not result in an increase in richness, which is a measure for the total

number of different observed species in a sample. Previous research has shown that colonization after fecal microbiota transfer is more successful for genera which were already present in the recipient before fecal transplant, and that rare genera are less likely to colonize (Li et al., 2016; Schmidt et al., 2018), indicating that it is difficult for the unique duck microbiota to colonize the new environment of the layers' gut. Hence, a detectable shift in the gut microbiota of the adult chicken upon exposure to duck feces could not be measured, making the gut microbiota composition as measured with 16S rRNA gene amplicon sequencing unsuitable as a proxy for contact with wild birds.

It is more likely that chickens that visit the outdoor range pick up on soil microbiota, including possible fecal microbiota from wild birds. However, the microbiota they pick up from the environment are likely unique to each location and the abundance of these microbiota fluctuate over time, not resulting in one consistent signal which is unique for having access to an outdoor range. In the cross-sectional study in Chapter 3 it was found that only outdoor-housed layers harboured microbiota of *Dietzia maris*, a taxon which is related to soil (Rainey et al., 1995). However, this was a rare taxon, and we did not detect *Dietzia maris* in the fecal samples of outdoor layers in the longitudinal study of Chapter 4. Furthermore, we found that most fluctuations in the composition of the microbiota over time within each poultry house were unique for each flock, and were mainly driven by changes in the abundance of rare, rather than dominant taxa (Chapter 4). Indeed in baboons, it was found that variation in gut microbiota between populations of baboons from 14 distinct geographical sites was best explained by the baboons' environments, especially the soil's geologic history and exchangeable sodium (Grieneisen et al., 2019). Local soil effects were 15 times stronger than those of genetic distance between host populations, perhaps because soil predicts which foods are present, or because baboons are terrestrial animals and consume soil microbiota while foraging (Grieneisen et al., 2019). Furthermore, in zebra finches, exposure to distinct experimental microbial environments (i.e. high and low diversity environments) led to differences in the composition, richness and dynamics

of the cloacal microbiota (van Veelen et al., 2020). Given the latter studies, one would expect that the environment of the outdoor range, which reflects an exposure to a more diverse environment rather than just the environment of the poultry house, would have had an impact on the cloacal microbiota composition in the laying hens studied. However, in contrast to wild animals, which have a continuous exposure to their direct living environments, laying hens in the Dutch poultry industry spent most of their time in the poultry house, and the time spent in the outdoor range is limited. In general, the doors to the outdoor range in the Dutch table egg industry only open between 10-11 am until sunset. Furthermore, only a limited number, between 3-15 %, of chickens of the flock actually use the range (Bestman and Wagenaar, 2003; Hegelund et al., 2005), and individual hens use the range differently of which not all enter the outdoor range daily (Gebhardt-Henrich et al., 2014). Therefore, it makes sense that the direct environment of the poultry house, which also includes feed and litter material in which the chickens live, explains most of the variation in cloacal microbiota between chickens of different flocks which was found in Chapters 3 and 4.

Perspectives for future studies

In the studies presented in this thesis, changes in the microbiota that could be used as an indicator for contact between poultry and wild birds could not be identified. As in any research field, the power to detect associations in microbiome research strongly depends on effect size, heterogeneity of the background noise, and sample size in a study (Hasin et al., 2017). In the study designs of Chapters 3 and 4 we randomly selected chickens during the sampling and did not specifically select chickens that used the outdoor range. This choice was made from an epidemiological perspective, as we wanted to gain insight into the dynamics of the microbiota community at the level of the population of the entire flock housed in one poultry house, rather than study the changes in the microbiota community of individual chickens. However, to specifically study the effect of the outdoor range one could consider an animal experiment in which chickens can be followed up individually and environmental conditions are more easily controlled.

Furthermore, it is advisable to sample more chickens of the same flock to improve the power of the study, and get a better insight into the dynamics and variation of the microbiota community at flock level. To study these microbiota dynamics on flock level, taking boot socks samples, which is a method to sample the microbiota composition of the feces of the chickens on the litter, could be considered. This is, like cloacal swabs, a minimally invasive technique, and has been shown to be a good representative of the cecal microbiota community composition and shows less variation between samples compared to cloacal swabs (Kers et al., 2019a).

In Chapters 2-4 16S rRNA gene amplicon sequencing on the V3-V4 hypervariable regions of the 16S rRNA gene was used. The ~1500 bp 16S rRNA gene comprises nine variable regions interspersed throughout the highly conserved 16S sequence. This technique is sensitive enough to accurately discriminate between different genera and give an approximation of 16S diversity, but does not sequence deep enough to capture sufficient sequence variation to make the distinction between different species, let alone strains (Yarza et al., 2014; Johnson et al., 2019). Consequently, this technique might not have been sensitive enough to pick up on small shifts in the microbiota after exposure to an outdoor environment. To accurately determine the fate of donor microbiota after fecal microbiota transfer, it has been proposed that one should track the microbiota at the resolution of strains rather than at the level of genera or species as is done with 16S rRNA gene amplicon sequencing (Li et al., 2016; Schmidt et al., 2018). In the future, sequencing the entire 16S rRNA may be advisable, as it was shown that when a full-length sequence with all variable regions was used, it was possible to classify nearly all sequences as the correct species (Johnson et al., 2019).

Furthermore, instead of looking for specific microbiota indicators or strains in the chicken gut, a combination with other omics analyses (i.e. using the transcriptome, epigenome, metabolome or proteome), that can facilitate a more holistic understanding of biological mechanisms, may reveal associations between exposure to wild bird feces/pathogens from an outdoor range, the response of the host and disease susceptibility, and may help pinpoint relevant biomarkers (Hasin et al., 2017; Koh and Hwang, 2019).

Instead of sampling chickens for contact with wild birds, another approach to identify areas or farms at higher risk for AIV introductions could be to look for bio-indicators for the presence of wild bird feces or AIVs in the immediate environment of the farm. Environmental sampling of feces, feathers, water or ice, has shown to be a promising strategy to pick up on AIV in the environment and might be used in AIV surveillance, particularly in the early detection of HPAI subtypes (Himsworth et al., 2019; Coombe et al., 2020; Hood et al., 2021). Also, microbial source tracking (MST) methodologies and environmental DNA sampling techniques that are becoming increasingly available, and allow for high-throughput analyses, could be considered as well to assess the presence of specific wild bird (feces) in the vicinity of farms to determine risks for exposure of poultry to these species (Harwood et al., 2014; Ohad et al., 2016; Ushio et al., 2018).

Part 2 – Identification of high risk areas and identification of early onset

Exposure to avian influenza viruses

Although the range use of the chickens does not have a measurable effect on the microbiome composition of adult layers on flock level, chickens that use the range can still pick up AIVs, which poses a risk for AIV incursions for outdoor housed poultry flocks. Unlike the commensal microbiota in the duck inoculum of the fecal transplant in Chapter 2 (which was checked for absence of specific pathogens, i.e. AIV and salmonella), pathogenic microbes have aggressive tools for invasion of the host (Beutler, 2016; Li et al., 2019). As stocking densities of chickens are generally high in commercial poultry farms, only one or a few chickens in a susceptible flock have to become infected with AIVs harboured in the environment, for direct transmissions between the chickens in the flock to induce a major outbreak in the flock (Bouma et al., 2009; Rohani et al., 2009). Indeed, the risk of introduction of avian influenza for outdoor-layer farms is six-times higher than for indoor-layer farms in the Netherlands (Bouwstra et al., 2017). As wild waterfowl excrete large amounts of virus in their feces (Webster et al., 1978; França et al.,

2012), and AIVs can persist and remain infective in a water rich environment for several days (Stallknecht et al., 1990; Brown et al., 2007; Beerens et al., 2020), the viral load around poultry farms may build-up due to repeated visits of AIV infected wild birds. This scenario especially occurs during autumn-winter months when conditions for viral survival are optimal due to increased rainfall and lower temperatures (Beerens et al., 2020), which also explains why the risk of low pathogenic avian influenza (LPAI) and HPAI incursions were four times higher in the autumn-winter months than in the summer months in the Netherlands (Gonzales et al., 2020), and global HPAIV spread among poultry starts to increase in October and peaks in February (Awada et al., 2018).

Prediction of HPAI outbreaks in poultry farms

As described in the introduction, HPAI epidemics now show a recurrent pattern, with great economic and social consequences during each epidemic. This is a major concern for the poultry industry worldwide, and underlines the need for a better prediction of HPAI outbreak risk. In Chapter 5 we showed that wild bird density data of 19 bird species could accurately predict HPAI outbreaks on poultry farms in the Netherlands that occurred between 2014-2018 with an average precision of 88%. In the most recent autumn-winter period of 2020-2021 a new HPAI epidemic ravaged across Europe and caused massive wild bird mortality and HPAI outbreaks in poultry farms. Many countries, bird species and poultry types were affected (Adlhoch et al., 2020). In the Netherlands, ten new HPAI outbreaks occurred on poultry farms of different poultry types, since the analyses of Chapter 5 was made (Table 1). In contrast to previous years, this year's outbreaks also occurred in broiler farms (n = 2, Table 1) and one turkey farm. Other poultry types that were affected were laying hens (n = 4), broiler breeders (n = 2) and Pekin ducks (n = 1). In Figure 1A the locations of these ten cases are plotted in the predicted HPAI risk map as modelled in Chapter 5. In Table 1 these new cases are characterized and the predicted outbreak risk using the model of Chapter 5 is shown. Surprisingly, case IDs 16, 20 and 21 have very low predicted probabilities, and in the risk map one can see that especially cases

20 and 21 are outliers and occur in very dark green areas. There are also no areas with higher predicted probabilities close to these cases (Figure 1A). The situation is different for Case 16, though, which is located in the vicinity of the river IJssel, which also has a higher predicted probability.

To optimize the predictive power of the model in Chapter 5, we used the locations of these ten new outbreaks and added them to the dataset of Chapter 5, and ran the model again. In short, we first selected four controls from the Netherlands Food and Consumer Product Safety Authority (NVWA) database for each new HPAI outbreak according to the same poultry type and within a 100 km radius of the case farm. Then, we ran a leave-one-group-out random forest on the full model with all 19 bird species that were identified in Chapter 5 at their optimal scale, i.e. raw scale, 2.5 km, 5 km, and 10 km, to include the influence of landscape context. Additionally, Sovon, the Dutch Center for Field Ornithology (Nijmegen, the Netherlands), provided us with counts of waterbirds within a 2 km radius of each infected farm. These waterbird counts were carried out within 7-31 days after a HPAI positive result on nine out of ten infected farms (Table 1). It is important to emphasise that these data were based on a single count. As such a snapshot can be influenced by many factors, such as time of the day, weather conditions, incidental disturbances (for instance due to activities of people, vehicles and other wild animals) etc., these bird counts provide only an impression of the situation around the time of the HPAI infection on each farm (R. Slaterus, pers. comm). Furthermore, the incursion of the virus is likely to have occurred some time before detection and diagnosis of the farm, suggesting that the situation may have been different at time of virus introduction.

Inclusion of the 2020-2021 outbreaks to the model results in an average precision of 70% (Table 2), which is lower than the average precision in the initial model of 88% in Chapter 5. It is shown that in the new model, the HPAI prediction in areas around the big rivers are reduced, as well as the predictions in the province of Zeeland and other coastal areas (Figure 1A-1B). Furthermore, there was some change in the order of feature importance

Table 1: Overview of all highly pathogenic avian influenza (HPAI) outbreaks on poultry farms in the Netherlands between 2014-2020. For each case ID the poultry type is indicated and in which year(s) the farm had a confirmed HPAI outbreak. For the cases in the autumn-winter of 2020, the predicted probability as modelled by the model used in Chapter 5, are given and the days between a confirmed HPAI infection and the waterbird count which was carried out by Sovon.

Case ID	Poultry type	Predicted probability	2014 H5N8	2016 H5N8	2017 H5N6	2020 H5N8	Days between confirmed HPAI and bird count
1	Layer		x	x			
2	Layer	0.785	x			x	9
3	Layer		x				
4	Pekin duck		x	x	x		
5	Broiler Breeder		x				
6	Pekin duck			x	x		
7	Pekin duck			x			
8	Pekin duck			x			
9	Layer			x			
10	Layer			x			
11	Broiler Breeder			x			
12	Pekin duck				x		
13	Broiler Breeder	0.611				x	31
14	Layer	0.598				x	24
15	Layer	0.698				x	19
16	Pekin duck	0.059				x	13
17	Broiler	0.657				x	11
18	Broiler	0.571				x	7
19	Broiler Breeder	0.751				x	7
20	Turkey	0.002				x	8
21	Layer	0.000				x	

in the 19 bird species that were identified in Chapter 5. The mute swan (*Cygnus olor*) however, remains the species of which the distribution across the Netherlands contributes most to the prediction of the model (Figure 2). Of the birds with the highest feature importance, the mallard (n = 9), Eurasian wigeon (*Mareca penelope*, n = 8) and mute swan (n = 7) were observed around nine, eight, and seven of the infected poultry farms (Figure 3). Northern shoveler (*Spatula clypeata*, n = 2), Tufted duck (*Aythya fuligula*, n = 4) and Eurasian curlew (*Numenius arquata*, n = 5) were also observed within the 2 km radius of several poultry farms, but around less farms. Furthermore, several

gull and heron species were also observed often within the 2 km radius of the infected farms. Although the distribution of the latter birds in the Netherlands might not contribute a lot in the prediction of HPAI risk across the Netherlands (Figure 2), they may still play a role in the dissemination of the virus in the environment around the farm. AIV infections among wild bird species depend on a complex multispecies system, influenced by ecosystem properties, bird species diversity and community structure, the specific circulating HPAIV strain(s), and the clinical impact it has among the different hosts species (Caron et al., 2017; Huang et al., 2019; Verhagen et al., 2021). Bridge hosts can be an important 'missing link' that bring AIV from the habitat of the infected waterfowl (the maintenance hosts) closer to the poultry farm, and are likely to be common peri-domestic bird species that are widely distributed over the Netherlands (Caron et al., 2015). Therefore, we have to be careful when drawing conclusions on the exact roles of specific wild bird species in the epidemiological processes at the wild bird/domestic bird interface, or on that of other bird species not included in this study.

In the new model, case ID 16, 20 and 21 still have low predicted probabilities and lie in dark green areas (Figure 1B). However, especially around case ID 20, a lot of waterbirds were observed by Sovon during the count shortly (8 days) after the outbreak. In the direct vicinity of this farm, a wetland area was created in the past two years, aimed at attracting waterbirds. This might explain why high number of waterbirds were reported by Sovon. For case ID 21, Sovon did not perform a bird count. However, four days before the notification of the HPAI infection in case ID 21, a 9-day frost period had passed the Netherlands, freezing a lot of waters and ponds. A small river flows close to this farm (at approximately 2 km), and although the river does not usually attract a lot of waterbirds, during the frost period ice holes in this river might have attracted more waterbird than usual because other water sources were frozen (Reperant et al., 2010). Although the latter could not be confirmed as we did not have bird count data of that location at the time of the frost period, the local situations at both farms underline the limitations of the model which was used to make the risk map. In the model, we used the spatial distribution of wild birds of Sovon

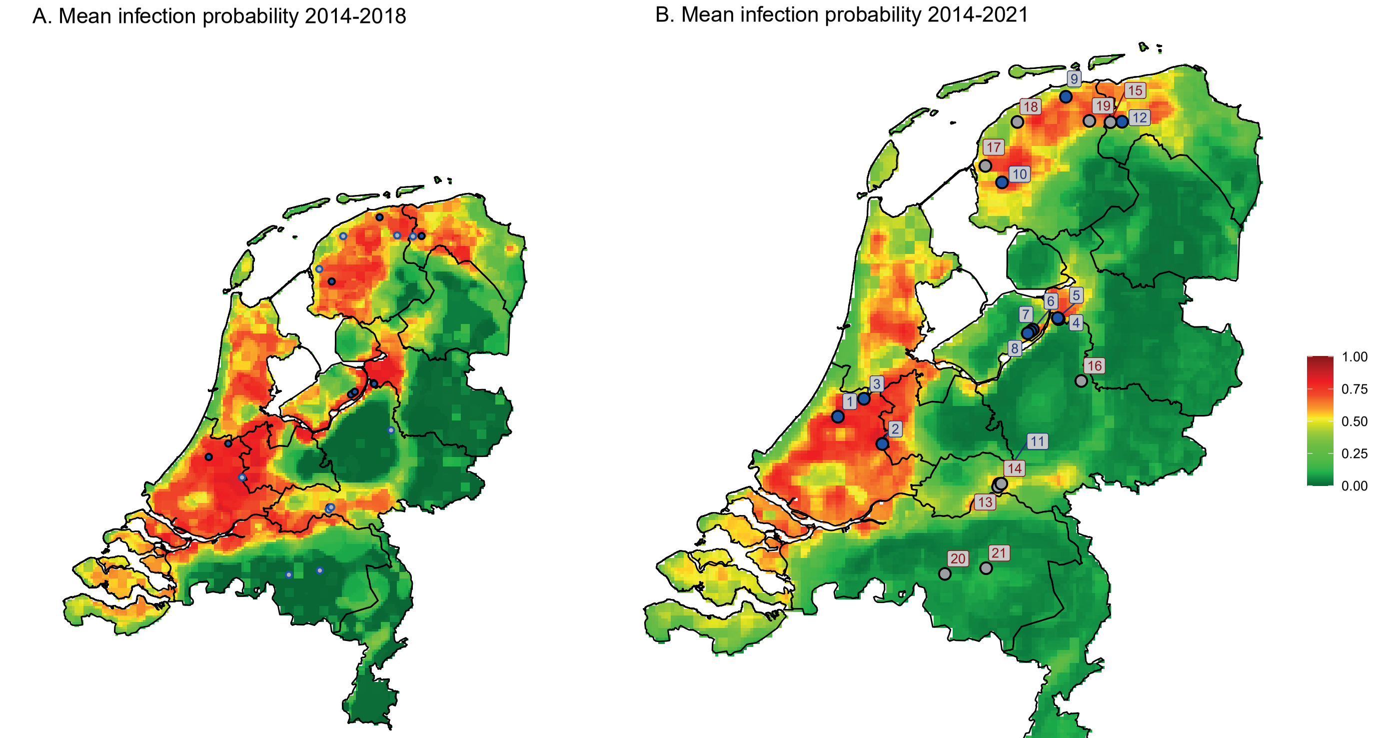


Figure 1: Predicted HPAI outbreak risk across the Netherlands. A. the predicted HPAI risk using 2014-2018 HPAI outbreaks. Blue dots indicate infected HPAI farms between 2014-2018 on which the primary model was built. Light blue dots indicate new HPAI outbreaks on poultry farms in the autumn-winter period of 2020-2021. The latter were not used in the modelling of the predicted risk. B. The predicted HPAI risk using all HPAI outbreaks between 2014-2021. Blue dots are HPAI infected farms between 2014-2018. Grey dots are HPAI infected farms in 2020-2021. Labels indicate case IDs of the farm.

which was a long-term average of bird count data per 1 by 1 km square, collected at the set moments each winter between 2012/2013 – 2014/2015. Although the average bird counts over several years and months gives a robust estimate of the number of birds counted per square, more recent temporal changes in wild bird densities due to either nature development or for instance, weather influences, are not accounted for. Despite these limitations, we show with the inclusion of the ten new outbreak cases in the Netherlands that the model gives a more specific identification of the most important high risk areas. Although we also demonstrate that outbreaks can still occur in low risk areas, high risk areas should be considered as important targets for surveillance and preventive measures against HPAIV introduction, and may assist in decision making on locations for new poultry farms. To account for the local fluctuations over time that can influence HPAI outbreak risk, like changes in bird densities, circulating AIV virus strains and weather conditions, it is advised to include temporal data, for example data concerning specific flyway migration patterns and surveillance data on AIV infected wild birds and poultry farms, in the model (Astill et al., 2018). Ideally, the model should be able to incorporate these data real-time in order for rapid and accurate identification of areas that are at risk.

Table 3: Classification metrics optimal classification thresholds of predicted HPAI risk (Figure 1). A precision-recall curve was generated to determine area under the precision-recall-curve known as average precision, which was 70%. Accuracy is the ratio of correctly predicted observation to the total observations, whereas the F_1 score is a weighted averaged of precision and recall and gives a measure of the incorrectly classified cases.

Classification threshold	**Recall**	**Precision**	**F_1**	**Accuracy**
0.42	0.89	0.74	0.81	0.91

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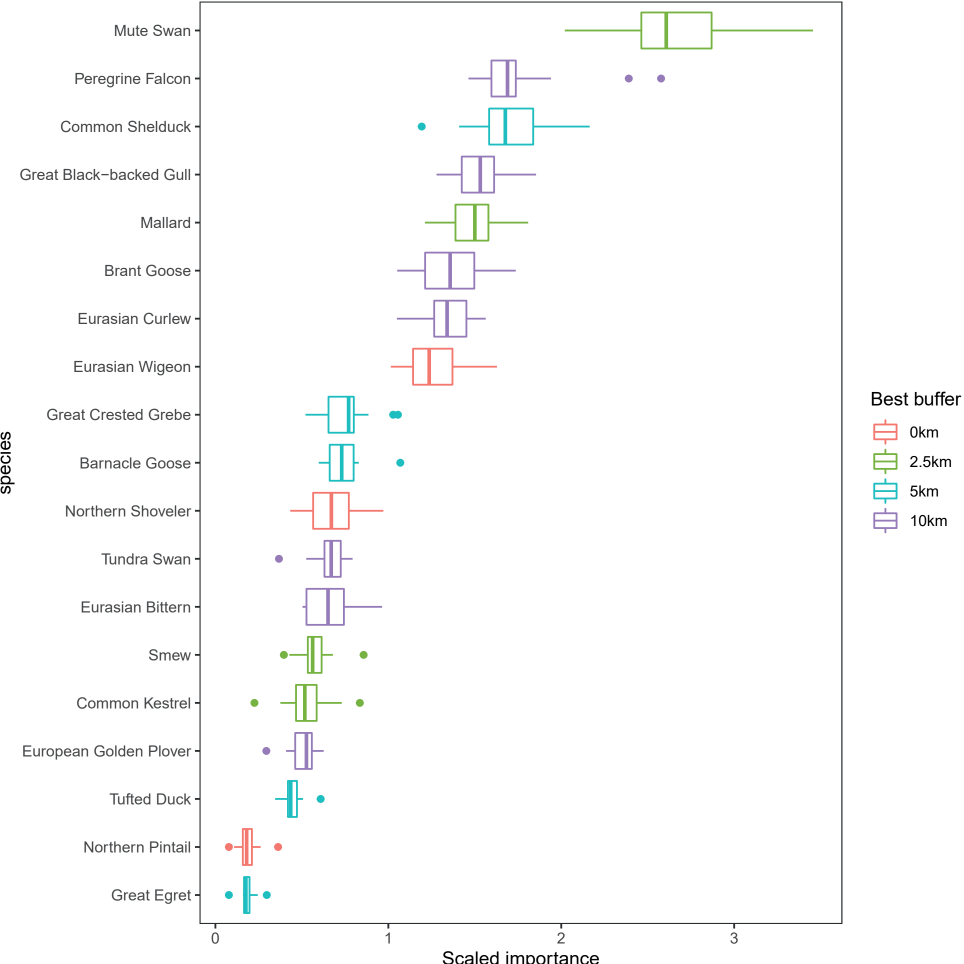


Figure 2: Scaled importance of 19 most important features that were used for the final leave-one-group-out random forest (LoGo random forest). Feature importance was standardized: importance/mean(importance), so that the average feature importance value is 1. The most important scale of each feature was used in the final LoGo random forest. The boxplots indicate the variation in the feature importance across the 21 LoGo random forest runs. Colours of the boxplots indicate the spatial scale with the highest feature importance for each variable that was used for smoothing of the data.

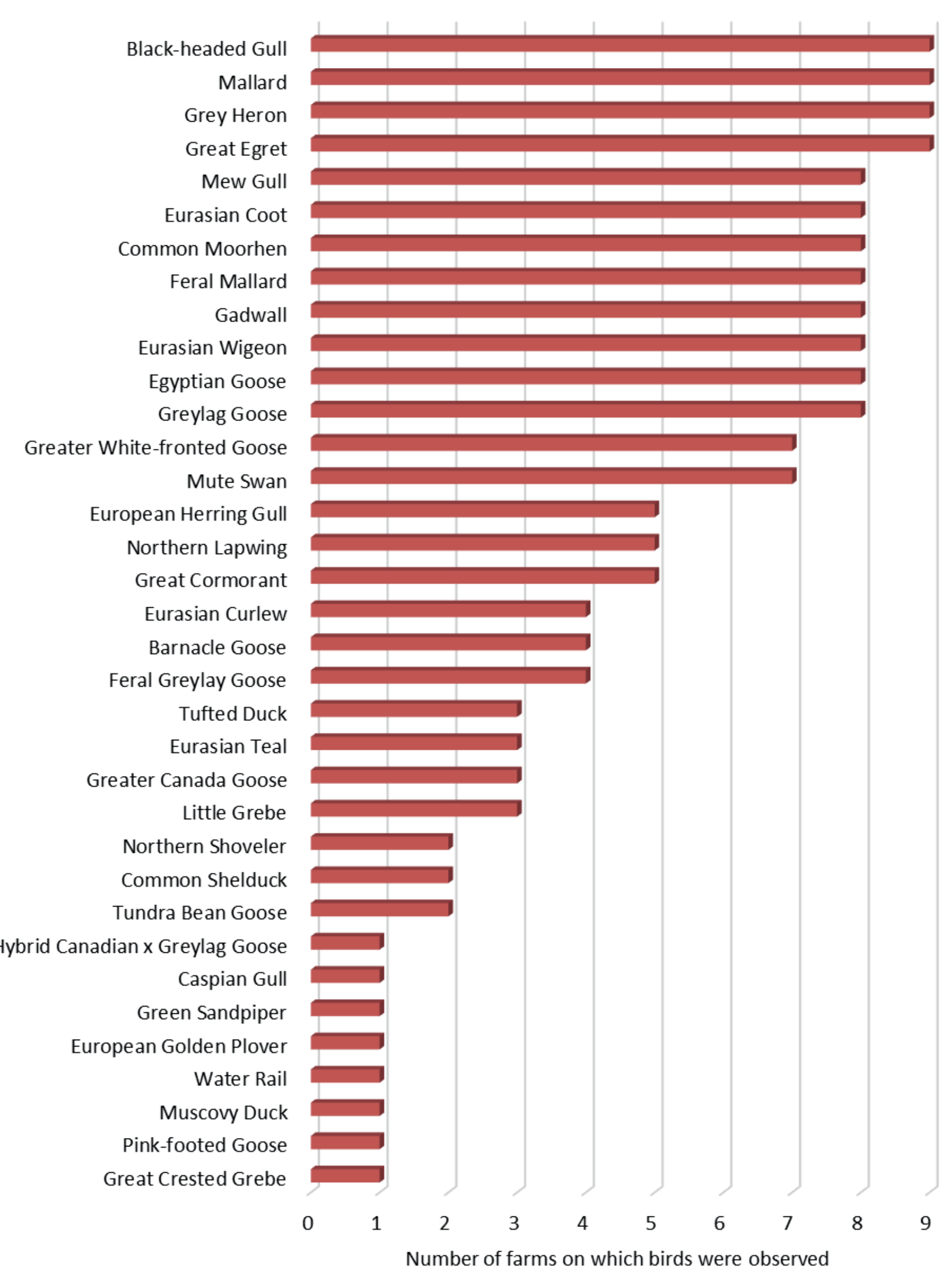


Figure 3: Frequency count of how often specific waterbird species were observed within a 2 km radius around an infected poultry farm in the autumn-winter period of 2020-2021. Waterbird counts were executed by Sovon on nine out of ten infected poultry farms within 7-31 days after confirmed HPAI infection on the farm.

Using clinical signs and mortality to detect outbreaks
Beerens et al. (2020) compared the pathogenicity of the three recent HPAI viruses involved in outbreaks in the Netherlands, i.e. H5N8-2014, H5N8-2016 and H5N6 2017, for chickens, Pekin ducks and Eurasian wigeons. They showed that all virus types had high intravenous pathogenicity indexes (IVPI values, ranges from 0, no pathogenicity, to 3.0, highest pathogenicity) for chickens, ranging between 2.7 – 2.8. Furthermore, all chickens died within two to three days after infection, and only showed some listlessness and ruffled feathers (Beerens et al., 2020). This is in line with the findings in Chapter 6, where we found that the mortality ratio's (MR) in most affected chicken farms had already increased prior to the observation of general clinical signs by the farmer, indicating that the chickens died a sudden death, without showing a lot of symptoms prior to death. This is also corroborated by data of the ten new outbreak cases in 2020-2021. Farmers mostly reported general clinical signs (including sudden death) prior to notification, and the MR increased either before or simultaneously with the onset of clinical sign (Figure 4). Moreover, the MR showed a very steep increase on the day of notification, reaching values above 30 (Figure 5).

In contrast, Beerens et al. (2020) reported more variation in virulence of different HPAI viruses strains for Pekin ducks and a wider range in IVPI, ranging between 0 (no ducks died) and 1.23. There was also more variation in the clinical signs observed compared to the infected chickens, and reported signs were listlessness, lethargy, ruffled feathers, sneezing, loss of appetite, nasal and ocular discharge, watery eyes, conjunctivitis and neurological signs (Beerens et al., 2020). This suggests that there was a difference in pathogenicity for Pekin ducks between the different virus types circulating every year. The broader scale of clinical signs in Pekin ducks is in line with our findings in Chapter 6, in which particularly the presence of neurological signs was more pronounced, and is corroborated with the broad range of clinical signs reported in the HPAI outbreak on a Pekin duck farm in 2020 (Figure 4). Furthermore, unlike in chicken farms, the mortality ratio in the Pekin duck farms was only increased in three out of seven affected farms between 2014-2018 prior to the first observation of clinical

symptoms, whereas in the other four farms, clinical signs were observed 1-2 days before or on the same day as the increase in mortality ratio (Chapter 6). In the outbreak of 2020, it is clear that the MR in the affected Pekin duck farm passed the reporting threshold of 2.9 several days prior to notification, although the increase in MR seems to be less steep compared to the MR in layers. Therefore, as the virulence of HPAIVs in Pekin duck variates more between strains (Beerens et al., 2020; Vergne et al., 2020) and the MR might be less affected, the observation of clinical signs may provide an additional indication for farmers and veterinarians in combination with MR and prompt them to notifying the disease.

In the HPAI epidemic in the autumn-winter period of 2020-2021, the first cases of HPAI H5N8 on two broiler farms and a turkey farm were reported in the Netherlands (Table 1). The clinical signs reported in the days prior to notification in the broiler farms vary more compared to clinical signs reported in layers, and are more similar to those reported in Pekin ducks (Figure 4). Remarkably, there was also a more insidious course of infection, where the MR passed the threshold already several days prior to infection, but the death rate did not increase as steeply as reported in laying hen farms (Figure 5). For the turkey farm the clinical signs were more in line with previous reports of HPAI infection in turkey flocks (Burcham et al., 2017), and were similar to the clinical signs reported in layer farms (Figure 4). The farmer also reported sudden death in the animals, although this cannot be shown in the graph with the MR (Figure 5), likely because the farmer reported the outbreak at a very early stage due to sudden increase in deaths.

The above data confirm the finding in Chapter 6 that the MR threshold is a sensitive threshold to detect HPAI outbreaks at an early stage of infection of poultry farms. As this approach has mostly been developed based on data from layers, future outbreak data in other poultry species should be incorporated for further validation. For Pekin duck and broiler farms (given the limited data), it is shown that it remains vital to consistently monitor mortality, but also monitor clinical signs carefully, as the course of infection seems to be less severe compared to affected layer flocks and a combination of MR and clinical symptoms is needed to make an early diagnosis.

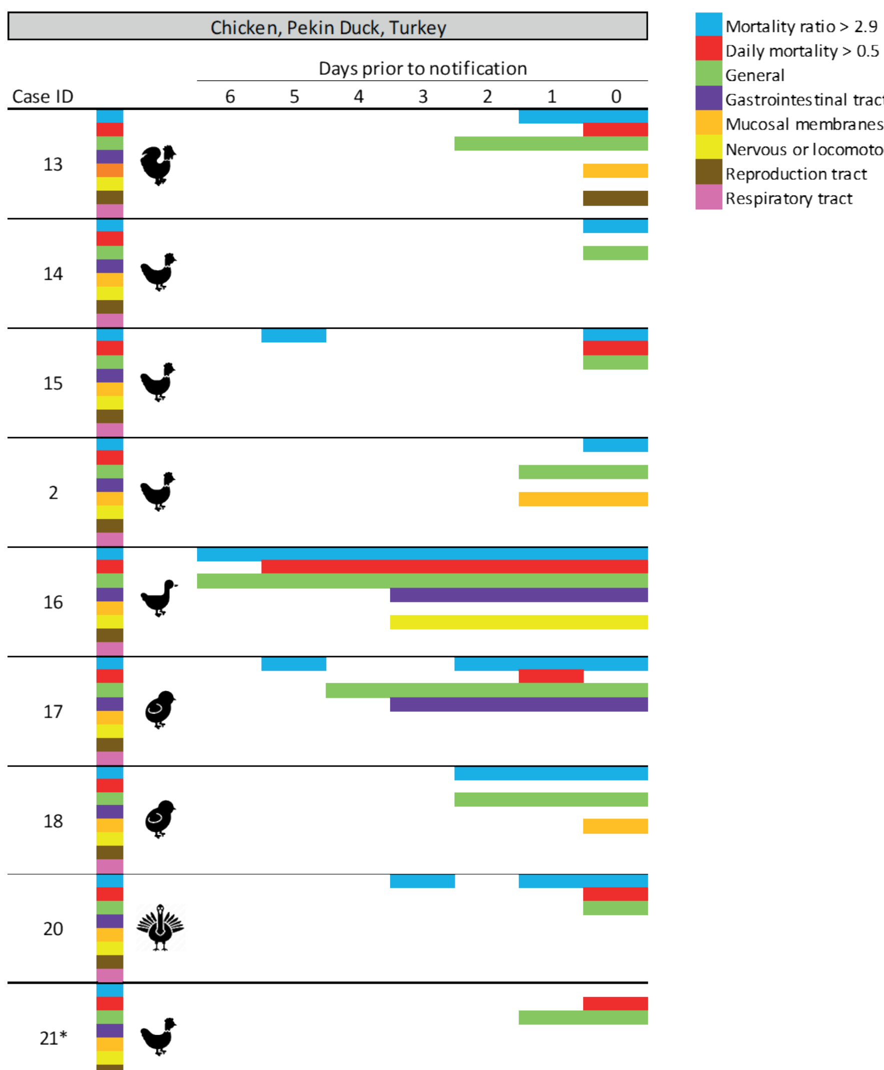
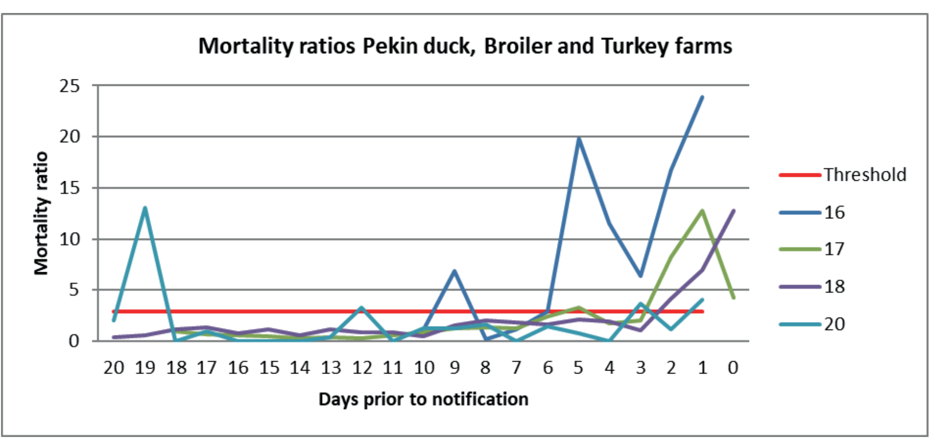


Figure 4: Clinical signs observed by the farmer in 10 new HPAI cases in the autumn-winter period of 2020-2021 an exceedance of daily mortality (>0.5%) and mortality ratio (MR) thresholds in the 5 day period prior to notification. Clinical signs are categorized by organ system. The symbol behind each case ID depicts the poultry type affected.



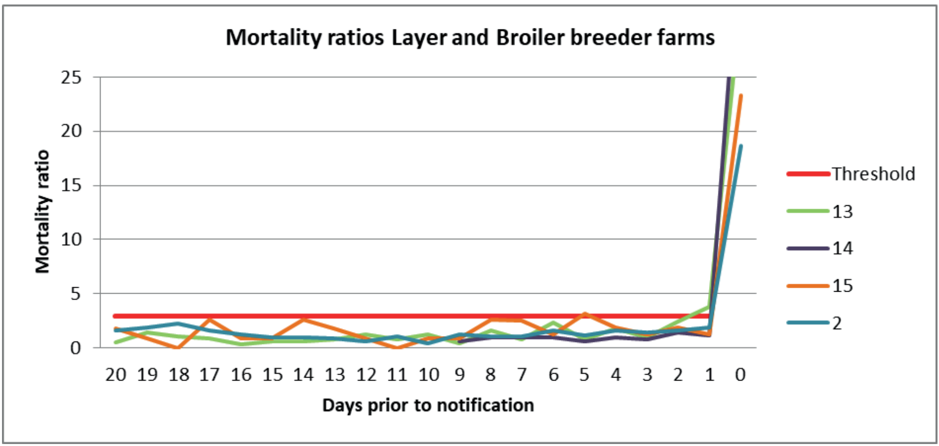


Figure 5: Calculated mortality ratio's in the 20 day period prior to highly pathogenic avian influenza (HPAI) notification in 2020-2021 for the four chicken farms (top), of which three layer and one broiler breeder farms, and four Pekin duck, broiler or turkey farms (bottom), shown per case-ID as mentioned in Table 1. The mortality ratio (MR) threshold of 2.9 is shown in red. Cut-off on the Y-axis was set to 25 to better visualize the behaviour of the MR in days prior to notification.

CONCLUSIONS

Risk assessment and risk management strategies to inform decisions on preventive and control measures are important for control of AIV infections in poultry around the globe. For this purpose, tools for prediction and early detection are needed. In this thesis, several strategies were evaluated that could be used for this purpose.

The first strategy was the use of the gut microbiota of chickens as

a proxy for contact with wild birds. Although it was shown that the gut microbiota are an unsuitable proxy to identify farms with increased risk of exposure to AIVs, we did show that the adult microbiota community in chickens is a highly complex ecosystem, which is rather stable over time and resistant to external perturbations. This is important knowledge, as it gives insight into the dynamics of the microbiota dynamics in healthy layers and future studies should use this knowledge when looking for ways to modulate the microbiota of chickens in favour of health and production.

The second strategy was the use of wild bird density data to spatially model HPAI outbreak risk across the Netherlands on poultry farms. It was shown that wild bird density data of HPAI high risk bird species could successfully be used for this purpose. Like the gut microbiota community, AIV infection among wild birds represent a complex multispecies system where we cannot designate one species as the most important contributor. However, we did identify several species of which the dispersal across the Netherlands robustly contributed most to the predictive model, indicating that these species might be important targets for future surveillance. Notably, the generated risk map gives insight into high risk areas for primary introductions, and these geographical areas should also be considered as important targets for surveillance and preventive measures against HPAIV introduction. Including the additional HPAI outbreaks of 2020-2021 resulted in a more robust prediction of geographical areas which have a high HPAI outbreak risk, and future outbreak data should be used to further improve and validate the model. Nevertheless, the risk map in its current form may already be utilized to inform decision making processes for policymakers, for instance regarding locations for new poultry farms, as well as nature development aimed at attracting more waterfowl in poultry dense areas which could unintentionally increase HPAI infection risks.

Thirdly, the mortality ratio, in combination with clinical signs, was shown to be a sensitive tool to detect HPAI infection on poultry farms at an early stage. As this approach has mostly been developed based on data from layers, and needs further validation for more poultry species, it is already an important tool for poultry farmers and veterinarians to notify suspected

HPAI infection to the authorities at an early stage. This will facilitate quick implementation of appropriate measures, e.g. culling and transport restraints, to prevent further spread of HPAIVs to other farms and reduce zoonotic risks.

As the HPAI outbreaks in the Netherlands from 2014 onwards are mostly considered primary introductions in poultry farms, future research should be aimed at further improvements of the prediction of HPAI risks for poultry farms. Therefore, it remains worthwhile to investigate other approaches, like multi-omics or environmental sampling, which could help identify bio-indicators for exposure of poultry to wild birds or pathogens in order to improve surveillance strategies and risk assessment. Also, to achieve a more accurate identification of areas at risk of HPAI outbreaks, temporal data on e.g. AIV surveillance in wild birds, flyway migration patterns and detections of AIV outbreaks on poultry farms should be included to the current model in real-time to further improve HPAI prediction based on current events.

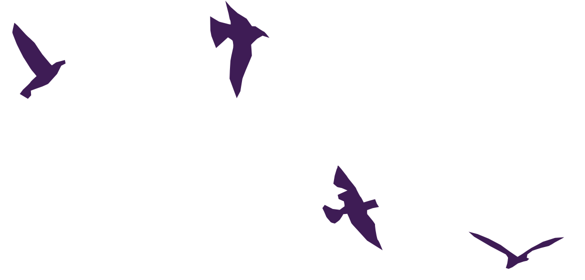
Bibliography

Summary

Samenvatting

About the author

Dankwoord



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SUMMARY

Outbreaks of avian influenza are a major concern for the poultry industry, as well as for society. It is therefore important to prevent outbreaks as much as possible, and to detect outbreaks at an early stage when they do occur to prevent between-farm spread. Aquatic birds, especially wild waterfowl, are the natural reservoir of avian influenza viruses (AIV). Infected birds can excrete high amounts of AIVs in their feces and AIVs can survive in the environment for a long time under favourable conditions. Other species can become infected with AIVs via oral ingestion of infected wild bird feces directly or indirectly via an infected environment. Several studies have indicated that for laying hens with access to an outdoor range, the risk of AIV introduction is higher compared to indoor housed poultry, but outbreaks in both indoor and outdoor housed poultry occur. The main aim of the research described in this thesis was to evaluate tools that can be used to predict and detect avian influenza outbreaks on poultry farms in order to reduce risks of AIV introductions and prevent further spread to other farms via appropriate control measures.

The first part of the thesis explored if exposure of layers to an outdoor environment results in detectable changes in the gut microbiota community, and if these changes might be used as a proxy for contact of layers with wild birds. In Chapter 2, as a proof of principle, laying hens were inoculated with wild duck feces to determine if bacteria in the feces of wild ducks could be transmitted to laying hens. Only limited changes in the bacterial community in the feces of the laying hens were found after the inoculation, indicating that the bacterial community of adult laying hens is rather stable. As the layers in the experiments of Chapter 2 were only inoculated once with wild duck feces and repeated exposure to microbiota from the environment may have different effects, a cross-sectional field study was performed in Chapter 3. The aim of this study was to investigate the differences between the bacterial community in the feces of laying hens with and without access to an outdoor range. This cross-sectional field study showed that exposure to an outdoor environment was responsible for a relatively small proportion of the variation in the bacterial community of the laying hens. To study the effect of access to an outdoor range over time, a longitudinal field study was performed in Chapter 4 in which two layer flocks got access to the outdoor

range for the first time and two layer flocks remained inside. All flocks were followed-up over a period of 16 weeks. Results showed that the bacterial community in these adult layers was rather stable over time, even after a sudden environmental change. Furthermore, the dynamics in the bacterial community over time were unique to each layer flock and a strong influence of poultry house on the bacterial community composition of these layers was found. The results of the first part of this thesis illustrate that the community of gut bacteria in healthy, adult laying hens reaches a stable equilibrium, which is relatively resistant to external perturbations. Although the bacterial community cannot be used as a proxy for contact with (feces of) wild birds, this research gives valuable insights into the healthy dynamics of gut bacteria in the adult layer.

The second part of the thesis focused on the identification of highly pathogenic avian influenza (HPAI) high risk areas using wild bird density data and the timely diagnosis of HPAI outbreaks on poultry farms. In Chapter 5, density data of AIV high risk bird species and land cover variables were used to spatially model the HPAI outbreak risk across the Netherlands using data of HPAI outbreaks on poultry farms that occurred between 2014-2018. The HPAI outbreak risk in the Netherlands was accurately predicted using a random forest model. It was shown that the densities of several waterbird species were important contributors to model the HPAI outbreak risk. The risk map that was generated with the model, gave an accurate prediction of the previous HPAI outbreaks. The identification of high risk HPAI areas is an important tool to develop country or region-specific control programs for HPAI. These type of models and risk maps can help the fight against these recurrent outbreaks worldwide. In addition to identification of high risk areas for HPAI outbreaks in poultry farms, the early detection of a possible outbreak on a poultry farms is essential to prevent spread to other farms. In Chapter 6, mortality data and clinical signs of HPAI infected poultry farms between 2014-2018 were analysed for the early diagnosis of HPAI outbreaks. This study showed that the use of an objective mortality ratio with a set threshold could be a reliable parameter to detect HPAI virus infection at an early stage on chicken and Pekin duck farms. The use of a mortality ratio may

perform even better when complemented with detection of clinical signs in poultry farms, provided farmers are well trained to notice them. Further validation of the mortality ratio in Pekin ducks and other poultry species is needed, but these results should already encourage farmers, veterinarians and veterinary institutes in other countries to monitor and register mortality on farms more rigorously, because a poultry-specific mortality ratio could serve as an important indicator in HPAI poultry surveillance programs.

In Chapter 7, the main results of the thesis were discussed, additional data of HPAI outbreaks which occurred in 2020-2021 were included in previous analyses and recommendations for further research are given. Although investigating the bacterial community of layers did not result in a proxy for contact with wild birds, it remains worthwhile to investigate other approaches, like multi-omics or environmental sampling, which could help identify bio-indicators for exposure of poultry to wild birds or pathogens in order to improve surveillance strategies and risk assessment. Inclusion of the 2020-2021 outbreak data to the random forest model of Chapter 5, resulted in a more robust prediction of geographical areas which have a high HPAI outbreak risk. To achieve an even more accurate identification of areas at risk of HPAI outbreaks based on current events, real-time temporal data on e.g. AIV surveillance in wild birds, flyway migration patterns and detections of AIV outbreaks on poultry farms should be included to the current model. Nevertheless, the risk map in its current form may already support prioritization of areas for increased surveillance and biosecurity, and may be used to formulate recommendations for the establishment of new poultry farms to reduce the risk of HPAI outbreaks. Moreover, the evaluation of data of the 2020-2021 HPAI outbreaks underline that the use of a mortality ratio is a sensitive tool for the early detection and notification of suspected HPAI outbreaks on different types of poultry farms. In summary, the results of this thesis give leads for future research into the contact between layers and wild birds, and has resulted in an important first step towards mapping the risk of HPAI outbreaks across the Netherlands. Furthermore, this thesis provides useful criteria for the early detection of HPAI outbreaks, and together with the developed risk map provides important tools for the fight against avian flu globally.

SAMENVATTING

Uitbraken van vogelgriep zijn een groot risico voor de pluimvee industrie en de samenleving. Het is daarom belangrijk om vogelgriepuitbraken te voorkomen, en wanneer deze toch optreden, ze zo spoedig mogelijk op te sporen zodat verdere tussenbedrijf transmissie voorkomen kan worden. Wilde watervogels zijn een natuurlijk reservoir voor vogelgriepvirussen. Geïnfecteerde watervogels scheidden grote hoeveelheden virus uit in de mest en deze virussen kunnen, onder gunstige omstandigheden, lang overleven in de omgeving. Op deze manier kunnen vervolgens andere dieren direct besmet raken via orale opname van geïnfecteerde wilde vogelmest of indirect via opname van virusmateriaal uit een geïnfecteerde omgeving. Verschillende studies hebben aangetoond dat de kans om besmet te raken met vogelgriep groter is voor leghennenbedrijven met uitloop dan voor leghennenbedrijven zonder uitloop, maar vogelgriepuitbraken komen voor bij pluimveebedrijven met en zonder uitloop. Het doel van het onderzoek, zoals gepresenteerd in dit proefschrift, was om verschillende methoden te evalueren die ingezet kunnen worden voor het voorspellen en opsporen van vogelgriepuitbraken op pluimveebedrijven. Hierdoor kan het risico op uitbraken gereduceerd worden en verdere verspreiding voorkomen worden door het tijdig nemen van passende maatregelen.

In het eerste deel van dit proefschrift is onderzocht of leghennen die toegang hebben tot een uitloop een andere samenstelling van darmbacteriën hebben dan leghennen die geen uitloop hebben, en of de verschillen gebruikt kunnen worden als een indicator voor contact van leghennen met wilde vogels. In Hoofdstuk 2 is bij leghennen daarom, handmatig via de bek, mest van wilde eenden toegediend om vast te stellen of bacteriën uit de mest van de wilde eenden overgedragen kunnen worden op de leghennen. Er werden slechts beperkte effecten gemeten op de samenstelling van darmbacteriën van de leghennen, wat erop wijst dat de samenstelling van bacteriën in de darm van de volwassen kippen relatief stabiel is en ongevoelig is voor verstoringen van buitenaf. Echter, de leghennen in dit experiment werden slechts eenmalig blootgesteld aan wilde vogelmest, terwijl herhaaldelijke blootstelling mogelijk wel een effect zou kunnen hebben. Daarom is in Hoofdstuk 3 een cross-sectionele veldstudie uitgevoerd waarbij is onderzocht

of de samenstelling van darmbacteriën verschilt tussen kippen die toegang hebben tot een uitloop en kippen die binnen blijven. Uit deze veldstudie is gebleken dat slechts een klein deel van de variatie in darmbacteriën verklaard kon worden door het hebben van toegang tot de uitloop. De meeste variatie werd bepaald door de plek waar de kippen werden gehuisvest, namelijk de pluimveestal. Om het effect van blootstelling aan een uitloop over de tijd te bestuderen, werd een longitudinale studie uitgevoerd in Hoofdstuk 4. In deze studie zijn leghennen van twee pluimveestallen die voor het eerst toegang kregen tot een uitloop en hennen van twee pluimveestallen waar de kippen binnen bleven, vervolgd over een periode van 16 weken. Resultaten lieten zien dat ook hier de samenstelling van het darmbacteriën van de volwassen kippen relatief stabiel was over de tijd, zelfs na een plotselinge blootstelling aan een nieuwe omgeving (de uitloop). Daarnaast bleek dat de dynamiek in de samenstelling van de darmbacteriën over de tijd uniek was per pluimveestal. In lijn met de uitkomsten van de eerdere veldstudie verklaarde de pluimveestal het meest van de variatie over de tijd. Samenvattend laten de resultaten uit deze drie hoofdstukken zien dat de bacteriepopulatie van volwassen, gezonde leghennen relatief stabiel is en dat deze populatie goed in staat is om het evenwicht in samenstelling vast te houden en daarmee resistent is tegen verstoringen van buitenaf. Hoewel we de samenstelling van darmbacteriën niet kunnen gebruiken als maat voor de blootstelling van kippen aan (mest van) wilde vogels, heeft dit onderzoek wel waardevolle inzichten gegeven over de gezonde samenstelling van darmbacteriën in volwassen kippen.

Het tweede deel van dit proefschrift richtte zich op het gebruik van gegevens van wilde vogeldichtheden voor het identificeren van gebieden met verhoogd risico op uitbraken met hoog-pathogene vogelgriepvirussen (HPAI) en het tijdig opsporen van deze vogelgriepuitbraken op pluimveebedrijven. In Hoofdstuk 5 is een case-control studie uitgevoerd met locaties van HPAI uitbraken tussen 2014-2018. Hierbij is gekeken of gegevens over dichtheden van wilde vogels die beschouwd worden als ‘risicosoorten’ voor vogelgriep en kenmerken van landbedekking gebruikt kunnen worden om het risico op HPAI uitbraken over geheel Nederland in kaart te brengen.

De dichtheden van verschillende watervogelsoorten bleken belangrijk in het voorspellen van het risico op HPAI uitbraken. De risicokaart die werd gemaakt met behulp van het model gaf een nauwkeurige voorspelling van de locaties van eerdere HPAI uitbraken. Het identificeren van hoog-risicogebieden is een belangrijk hulpmiddel voor het ontwikkelen van land- en regiospecifieke maatregelen. Dit soort modellen en risicokaarten kunnen helpen bij de bestrijding van de steeds terugkerende vogelgriepuitbraken over de hele wereld. Daarnaast is het tijdig vaststellen van een mogelijke vogelgriepuitbraak op pluimveebedrijven van groot belang om verdere verspreiding naar andere bedrijven te voorkomen. In Hoofdstuk 6 zijn daarom gegevens van sterfte en van waargenomen klinische verschijnselen van besmette HPAI pluimveebedrijven tussen 2014-2018 geanalyseerd. Hieruit is gebleken dat het gebruik van een objectieve sterfteratio met een vaste drempelwaarde een betrouwbaar criterium kan zijn voor het tijdig opsporen van HPAI uitbraken op zowel kippen- als vleeseendenbedrijven. Het gebruik van de sterfteratio is mogelijk nog beter wanneer deze gecombineerd wordt met het waarnemen van klinische verschijnselen die passen bij HPAI uitbraken, gegeven dat de veehouders goed geïnformeerd zijn over de symptomen om deze goed en tijdig te kunnen waarnemen. Verder onderzoek naar het gebruik van de sterfteratio, bijvoorbeeld voor het aanpassen van de meldcriteria bij verdenking van HPAI uitbraken, is met name noodzakelijk in vleeseenden en andere pluimveesoorten. De resultaten tot nu toe geven evenwel al duidelijk aan dat registratie en monitoring van sterfte op pluimveebedrijven een belangrijke methode is voor veehouders, dierenartsen en veterinaire instituten om HPAI uitbraken tijdig te kunnen opsporen.

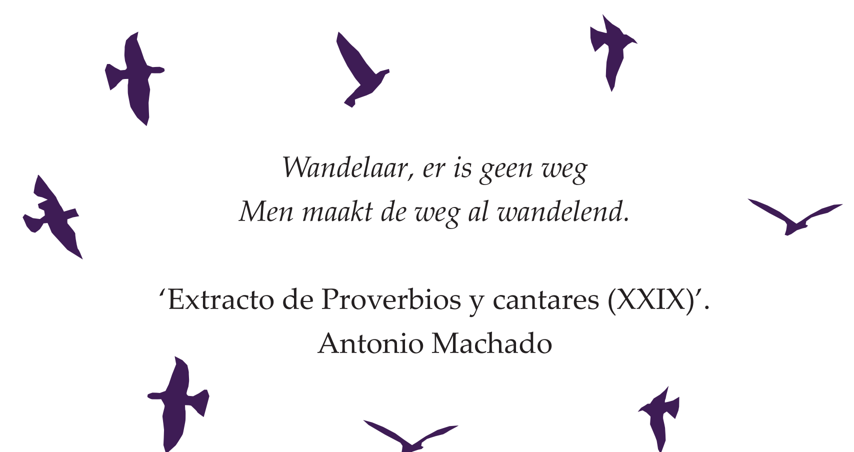
In hoofdstuk 7 worden de belangrijkste resultaten van het proefschrift bediscussieerd en aanvullende gegevens van HPAI uitbraken uit 2020-2021 toegevoegd aan eerdere analyses en worden suggesties gedaan voor verder onderzoek. Hoewel het onderzoek naar de samenstelling van darmbacteriën van leghennen niet heeft geleid tot een indicator voor het contact met wilde vogels, heeft dit onderzoek wel belangrijke inzichten gegeven voor de pluimvee-industrie. Daarnaast blijft het de moeite waard

om andere aanpakken te onderzoeken, zoals bijvoorbeeld multi-omics technieken en het onderzoeken van omgevingsmonsters met nieuwe moleculaire technieken, die kunnen bijdragen aan het identificeren van biomarkers voor de blootstelling van pluimvee aan wilde vogels en mogelijke ziekteverwekkers. De aanvullende analyses van het random forest model van Hoofdstuk 5 met de gegevens van HPAI uitbraken in 2020-2021 laten een meer robuuste voorspelling van geografische gebieden met een verhoogd risico op vogelgriepuitbraken in Nederland zien. Om de voorspelling nog nauwkeuriger te maken en aan te passen aan specifieke omstandigheden in de tijd, zou het huidige model uitgebreid kunnen worden met real-time temporele data, zoals data van vogelgriep surveillance in wilde vogels, migratiepatronen van wilde watervogels en data van vogelgriepuitbraken op pluimveebedrijven. Desalniettemin kan de huidige risicokaart al helpen bij het stellen van prioriteiten voor gebieden voor monitoring en bioveiligheidsmaatregelen en bij het formuleren van aanbevelingen voor de vestiging van nieuwe pluimveebedrijven om het risico op HPAI uitbraken te verminderen. Daarnaast heeft de evaluatie van de gegevens van de HPAI uitbraken van 2020-2021 onderstreept dat het gebruik van een sterfteratio een gevoelig meldcriterium kan zijn voor de vroege detectie van vogelgriepuitbraken op verschillende typen pluimveebedrijven. Samenvattend geven de resultaten van dit proefschrift aanknopingspunten voor verder onderzoek naar het contact tussen wilde vogels en pluimvee en levert het een belangrijke eerste stap voor het in kaart brengen van het hoog-pathogene vogelgrieprisico over Nederland. Bovendien biedt dit proefschrift bruikbare criteria voor de vroege detectie van HPAI uitbraken, en biedt het samen met de ontwikkelde risicokaart belangrijke methoden voor de wereldwijde bestrijding van vogelgriep.

ABOUT THE AUTHOR

Janneke Schreuder was born on the 3rd January 1986 in Nijmegen, the Netherlands. After attending high school at NSG Nijmegen in 2003, she took a gap year in which she traveled and volunteered in wildlife centers in South Africa for 3 months. In 2004, she started studying Biology at Radboud University Nijmegen and followed several courses in medical biology as well as ecology. In 2006, she got a spot to study Veterinary Medicine at the Utrecht University. During her studies she followed the equine-track, competed in the annual "Peerdenpieten"-race in 2009, followed the courses of the Descartes College, and did an excellent track research in 2010-2011 about Tuberculosis in Lions and Rhinoceroses, for which she lived in South Africa for 9 months. In 2013, she completed Veterinary Medicine and started working as an equine veterinarian in several practices in Germany. In the summer of 2015 she returned to Utrecht and started working as a junior lecturer in Anatomy and Physiology at the Faculty of Veterinary Medicine. In 2016, Janneke started her PhD on the "Fight Flu" project at the Faculty of Veterinary Medicine, department of Population Health Sciences at Utrecht University. The main results of this research are described in this thesis. In 2019, she obtained the postgraduate master degree in Epidemiology, with a specialisation in Veterinary Epidemiology, at Utrecht University. Currently, Janneke is working as a researcher Animal Health and Welfare at Wageningen Livestock Research, Wageningen, the Netherlands.

DANKWOORD



Wandelaar, er is geen weg
Men maakt de weg al wandelend.

'Extracto de Proverbios y cantares (XXIX)'.
Antonio Machado

Deze zin uit een gedicht van Antonio Machado is voor mij representatief voor het verloop van mijn PhD. Al gaandeweg heb ik mijn weg gevonden binnen het project Fight Flu en kreeg mijn proefschrift steeds meer vorm. Tijdens deze reis, hebben velen mij ondersteund en geholpen om het uiteindelijk succesvol af te ronden, waar ik erg dankbaar voor ben.

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Fred, mijn copromotor, bedankt voor je enthousiasme tijdens de vele

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