Optimising poultry flock health

Advances in understanding parasite infections of poultry: focus on protozoa and the red mite

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Introduction

A wide range of parasites can infect poultry, including multiple protozoans, cestodes, nematodes, trematodes and arthropods. As the global chicken population undergoes dramatic expansion, production systems are increasingly moving towards drug-free and/or extensive systems in much of Europe and North America, as well as greater intensification in many tropical regions, posing a series of new challenges to pathogen control. Parasites such as *Ascaridia galli*, *Capillaria obsignata* and *Heterakis gallinarum*, *Davainea proglottina* and *Raillietina cesticullus*, and a range of mites and other ectoparasites are returning to significance. Others, such as the *Eimeria* species, remain consistently challenging. Changes in legislation and husbandry systems are driving increased problems with *Histomonas meleagridis*, while genetic resistance to existing control measures is exacerbating difficulties with parasites such as *Dermanyssus gallinae*. Increased parasite occurrence affects the performance and welfare of poultry production. Here, we focus on three of the most widespread and economically relevant parasites, outlining current understanding and introducing recent advances with implications for detection, control and prevention.

Eimeria - coccidiosis

Target populations, Incidence and economic relevance

All livestock and poultry can be infected by multiple *Eimeria* species (Taylor et al., 2007). Most *Eimeria* are strictly limited to a single host-species, although examples such as *Eimeria innocua* can replicate successfully in domestic turkeys, grey partridge and bobwhite quail (Vrba and Pakandl, 2015). *Eimeria* that infect chickens are considered to be most economically important, primarily due to the large number of chickens that are produced every year and their rapid population turnover. More than 72 billion chickens were produced in 2019 (FAOSTAT, 2021), and production cycles lasting just five to seven weeks are common for broilers. Seven *Eimeria* species are widely recognised to infect chickens, all of which have been detected on every continent where chickens are farmed (Clark et al., 2016). *Eimeria acervulina*, *E. maxima* and *E. tenella* usually are most common (Haug et al., 2008, Clark et al., 2016, Hauck et al., 2019, Kumar et al., 2014), although highly pathogenic species such as *E. necatrix* can pose significant risks when an outbreak occurs (Sawale et al., 2018). Globally, between 2% and 80% of chicken flocks require therapeutic intervention to control coccidiosis, with between 1.5% and 7.5% of individuals expected to die during an outbreak if an appropriate intervention is available (Blake et al., 2020a). Despite the significance of losses due to mortality, the cost attributed to coccidiosis is

primarily associated with morbidity, were reduced weight gain is the biggest single loss (Blake et al., 2020a). The global cost incurred by *Eimeria* in chickens has recently been estimated to have exceeded UK£ 10.4 billion in 2016, equivalent to EU€ 11.9 billion or US$ 14.2 billion at the time of writing (February, 2021).

Host-pathogen interactions

Clinical signs, pathology and welfare

The seven *Eimeria* species that infect chickens can all cause disease with distinct, albeit overlapping pathognomonic characteristics. Each species follows an oral-faecal life cycle involving three phases of replication: asexual (schizogony, also known as merogony) and sexual (gametogony) within the host, followed by sporulation (sporogony; Figure 1) in the environment (Shirley et al., 2005). All seven species replicate within epithelial cells of the chicken intestine, although the precise site of infection varies from the duodenum (e.g. *E. acervulina*) to the caeca (*E. tenella*) and lower intestine (e.g. *E. brunetti*). *Eimeria brunetti*, *E. necatrix* and *E. tenella* are most pathogenic, causing a haemorrhagic form of coccidiosis in the mid (*E. necatrix*) or lower gastrointestinal tract (Reid et al., 2014). Pathology is most closely related to the asexual phase of replication (schizogony), when the large and relatively invasive schizonts rupture resulting in deep erosions and haemorrhage in the intestinal wall. *Eimeria acervulina*, *E. maxima*, *E. mitis* and *E. praecox* tend to be less pathogenic, causing a malabsorptive form of coccidiosis associated with replication of the sexual lifecycle stages during gametogony and subsequent oocyst development, although disease can still be severe in the event of a high-level challenge. *Eimeria maxima* is most pathogenic of the malabsorptive species, in part due to it large size. *Eimeria praecox* has been considered by some to be non-pathogenic, although evidence of pathogenic strains circulating in chicken populations has dispelled this view (Williams et al., 2009).

The clinical signs of coccidiosis, including both the location of gross pathology and the appearance of lesions, have been used to develop several lesion scoring systems to identify the infecting *Eimeria* species and describe the severity of an infection (e.g. (Johnson and Reid, 1970)). Less specific signs of infection include a hunched posture, ruffled feathers, lethargy, reduced body weight gain (BWG), and increased food conversion ratio (FCR). Water consumption can also be used as a non-specific indicator of ill health.

Host immune responses

A protective anti-*Eimeria* immune response is primarily reliant on T lymphocytes. Treatment of chickens with cyclosporin A to prevent T-lymphocyte proliferation confirmed their necessity for control of secondary infection (Lillehoj, 1987), while transfer of cell mediated immunity (CMI) to *E. maxima* has also been possible (Rose and Hesketh, 1982). In studies with mice, chosen due to the availability of a more comprehensive immunological toolbox, $CD4^+$ T cells appear to be most important in controlling primary *Eimeria* infection, supplemented by a smaller role for $CD8^+$ T cells (Rose et al., 1992). $CD8^+$ T cells appear to play a more significant role in secondary infections in the same study. However, several studies in chickens have suggested notable differences (Trout and Lillehoj, 1996, Cornelissen et al., 2009). A more recent study identified higher proportions of cytotoxic $CD8^+$ cells following primary infection (Wattrang et al., 2016). $CD8^+$ intraepithelial lymphocytes (IELs) are also increased following secondary *E. acervulina* infection, while genetic resistance to infection has been linked to increased $CD8^+$ IEL proportions (Lillehoj, 1994). It has been suggested that $CD8^+$ cells may function by killing infected epithelial cells (Lillehoj and Trout, 1994).

Studies of cytokines produced by T cells have indicated a key role for interferon-gamma (IFN-γ) in the immune response to primary, but possibly not secondary *Eimeria* infection. For example, blocking endogenous IFN-γ using a monoclonal antibody during *E. vermiformis* infection in mice increased susceptibility to primary but not secondary infection (Rose et al., 1989). In chickens, *E. maxima* infection has been shown to upregulate both Th1 and Th2 cytokine transcription in primary, but not secondary infection (Hong et al., 2006). The contribution from other cytokines has also been assessed. For example, increased tumour necrosis factor alpha (TNF-α) is induced by primary but not secondary *E. tenella* infection (Zhang et al., 1995), although it has been suggested that this may increase pathology (Byrnes et al., 1993).

A limited role has been suggested for B lymphocytes in the natural immune response to *Eimeria* infection. Surgical bursectomy removes the ability to generate an antibody response in chickens, but does not significantly reduce immune protection against secondary infection (Long and Pierce, 1963). However, it has been shown that antibodies can inhibit *Eimeria* replication under controlled circumstances, providing passive and maternal immunity against challenge and suggesting an alternative immune mechanism that is not usually induced during eimerian infection (Wallach, 2010).

Considerable variation has been described in the outcome of *Eimeria* infection by individual chickens. Distinct susceptible and resistant profiles have been described in terms of performance and pathology for *E. maxima* (Boulton et al., 2018b, Hamzic et al., 2015) and *E. tenella* (Boulton et al., 2018a). Interestingly a third resistance profile, considered to be tolerant of infection as defined by good performance despite significant pathology, has been reported in commercial broiler chickens (Boulton et al., 2018a).

Impact on enteric dysbiosis

Beyond the direct consequences of coccidosis, *Eimeria* can also induce enteric dysbiosis. *Eimeria* infection has been linked to poor litter quality, indirectly contributing to footpad dermatitis as well as reducing overall welfare and technical performance (Abd El-Wahab et al., 2012, de Jong et al., 2014). Microbiome sequencing enteric microbial populations has revealed notable differences in beta but not alpha diversity (i.e. variation in the levels, not presence or absence, of distinct bacterial populations), with significant variation for genera such as *Bacteroides* and *Lactobacillus* in association with *E. tenella* lesion score (Macdonald et al., 2017). Well known interactions with specific bacteria include *Clostridium perfringens*, combining to cause necrotic enteritis (NE) (Van Immerseel et al., 2016). Less well known interactions include increased colonisation and faecal shedding of bacterial zoonoses such as *Salmonella* Typhimurium and *Campylobacter jejuni* (Macdonald et al., 2019, Arakawa et al., 1981).

Eimeria **population dynamics**

All seven *Eimeria* species widely recognised to infect chickens have a global occurrence (Clark et al., 2016), but very little is known of their population structure or genetic diversity. It is clear from studies of antigenic diversity, using escape from strain-specific protective immunity as a phenotype, that genetic variation exists for several species including *E. acervulina* (Joyner, 1969), *E. mitis* (McDonald et al., 1985), *E. maxima* (Smith et al., 2002) and *E. tenella* (Awad et al., 2013). However, very few genetics-led studies have been undertaken. One of the most detailed studies focused on *E. tenella*,

assessing variation at the apical membrane antigen 1 locus (AMA1, an anticoccidial vaccine candidate (Pastor-Fernández et al., 2020)) and a genome-wide panel of single nucleotide polymorphisms (SNPs) (Blake et al., 2015). Comparison of SNP profiles revealed notable variation between countries and regions, supporting the suggestion that variables such as climate or husbandry system shape *Eimeria* population dynamics (Blake et al., 2015, Pegg et al., 2016).

The majority of genetics-led studies for *Eimeria* have focused on single loci within the nuclear or mitochondrial genomes (e.g. the ribosomal repeat unit including internal spacer sequences, and cytochrome C oxidase subunit I; (Blake et al., 2020b)). One such study described unexpected variation between Internal Transcribed Spacer (ITS)-2 sequences, suggesting the presence of diverse strains or cryptic species (Cantacessi et al., 2008). Recent studies including measures of oocyst morphology, pathology, genome sequencing and genetics suggest that all three, previously termed Operational Taxonomic Units (OTUs) X, Y and Z, are indeed new *Eimeria* species (Blake et al., 2021, Morgan and Godwin, 2017). The new species, tentatively been named *E. lata*, *E. nagambie* and *E. zaria*, have already been detected in parts of Africa, Asia, Australasia, North and South America, indicating a new challenge for control of coccidiosis (Clark et al., 2016, Hauck et al., 2019).

Current methods of detection for *Eimeria*

A range of techniques and tools are available for the detection and species-specific identification of *Eimeria* infection. Routine monitoring commonly relies on microscopy to detect oocysts in faecal or litter samples (Kumar et al., 2014). Flotation using saturated saline or sucrose solutions can be used to increase sensitivity. Detection and enumeration of total eimerian oocysts is relatively straightforward, but species-specific differentiation is much more challenging and can be highly subjective (Haug et al., 2008). For example, *E. necatrix* and *E. praecox,* species defined by extremes of pathogenicity, are very difficult to differentiate by variables such as oocyst morphology alone (Long et al., 1976). Attempts to automate species identification using microscopy by systems such as COCCIMORPH offer promise (Castañón et al., 2007), although uptake has been limited. Practically, post-mortem assessment of gross pathology (lesion scoring) remains an important technique for detection and species identification. The lesion scoring system published by Johnson and Reid for five of the seven recognised species (excluding *E. mitis* and *E. praecox*) is most widely cited (Johnson and Reid, 1970). Neither of these latter species routinely result in intestinal lesions during infection.

Advances in molecular biology have improved diagnosis of many veterinary pathogens, but have proven challenging for *Eimeria*. Accessing genomic DNA for use as template has often been limiting, requiring a laboratory for effective and reproducible extraction. Genus and species specific detection using polymerase chain reaction (PCR) was established nearly 30 years ago (Stucki et al., 1993) with a variety of multiplex and nested options developed to improve throughput and sensitivity (Fernandez et al., 2003, Lew et al., 2003, Schwarz et al., 2009), but none have been widely adopted by industry (Figure 2). The appearance of quantitative PCR assays specific for all recognised *Eimeria* species has had a greater impact (Vrba et al., 2010), with several companies offering qPCR as a diagnostic service. A panel of loop-mediated isothermal amplification (LAMP) assays have been published for the species-specific detection of *Eimeria* that infect chickens (Barkway et al., 2011), although accessing genomic DNA as template remains a challenge for routine application under field conditions.

Current methods of control for *Eimeria*

Control of *Eimeria* relies upon on good husbandry, including consideration of stocking density, ventilation rate and choice of substrate. lower stocking densities can reduce environmental contamination with oocysts. Dry, high quality litter can reduce oocyst sporulation, limiting infectivity (Figure 1). However, husbandry alone is insufficient to prevent coccidiosis. Anticoccidial drugs have long dominated control of coccidiosis, including a range of synthetic or chemical anticoccidials and ionophores, which are products of fermentation (Chapman, 1997). Ionophores have been especially successful since their use permits a low level of parasite replication, even in naïve field populations, supporting induction of a complementary protective immune response (Chapman, 1999). Importantly, ionophores are classified as antibiotics in some regions such as the USA. While ionophores are not used in human medicine and have limited direct relevance to human health, the appearance of "no antibiotics, ever" markets for chicken products has indirectly increased demand for alternatives (Blake et al., 2020a). Increasing reports of drug resistance and consumer concerns around drug use in livestock production have reinvigorated attempts to develop cost-effective, scalable anticoccidial vaccines. Where anticoccidial drugs remain in use, it is common to rotate between different anticoccidial drugs within and between flocks to limit and respond to selection for resistance.

The first anticoccidial vaccine was marketed in 1952 (Williams, 2002). Based upon live, unmodified *E. tenella* oocysts, the vaccine was quickly followed by other live anticoccidial vaccines including a range of different *Eimeria* species. Controlled infection using these parasite formulations induces a natural immune response and protection against subsequent challenge. Such "wild-type", or non-attenuated vaccines are highly effective and relatively cheap to produce, but risk compromising flock performance and occurrence of clinical disease if managed incorrectly (Shirley et al., 2005). The risk associated with live anticoccidial vaccines was recognised and addressed by development of a second generation of live vaccines using attenuated parasite lines. With few exceptions, attenuation has been achieved by selection of stable precocious lines from populations of virulent parasites. Attenuation results in shorter lifecycles and reduced replication, accompanied by lower pathogenicity whilst retaining immunogenicity (Shirley et al., 2005). Attenuated anticoccidial vaccines have become popular in the layer and breeder chicken sectors, but their relative cost and inherently limited production capacity has hindered application in the much larger broiler sector. However, demand for antibiotic free poultry products is now prompting a significant shift in anticoccidial control, with ~40% of broilers sold in the USA now vaccinated using a non-attenuated product (Blake et al., 2020a). A major selling point for live anticoccidial vaccines has been the use of drug-sensitive parasite strains, with evidence that vaccination of three or more successive flocks can significantly reduce the occurrence of drug resistance in field parasite populations (Chapman and Jeffers, 2015). The relative risk posed by these virulent vaccines can be managed using a bioshuttle approach, where vaccination of chicks at day of hatch is followed by anticoccidial supplementation of grower and finisher diets (Kimminau and Duong, 2019). Challenges in vaccine management include ensuring effective vaccine recycling, especially for less immunogenic species such as *E. tenella* and *E. necatrix*, where multiple rounds of infection can be required to induce a robust protective immune response.

A wide range of alternatives to drugs and vaccines has been suggested to improve control of *Eimeria*. Examples include natural herbs and botanicals or their extracts, essential oils, organic acids, immunomodulators and complex carbohydrates, probiotics and prebiotics (Khater et al., 2020). Probiotic formulations based upon *Bacillus*, *Lactobacillus* or *Saccharomyces* are becoming increasingly popular, with multiple commercial providers.

Challenges and conclusions

Control of *Eimeria* and the disease coccidiosis remains a major ongoing challenge to poultry producers. Increasing public and legislative pressure to reduce the use of drugs in livestock production has prompted renewed interest in existing and novel vaccines, a range of diet-based alternatives and selective breeding of chickens for genetic resistance. It is likely that the range of drugs available to control *Eimeria* will be reduced in the future, voluntarily in some sectors and by law in others. As the industry migrates away from drugs, new challenges will emerge. Differentiation of vaccinal from virulent field *Eimeria* strains is a significant gap that affects management and application of live vaccines. The recent description of three new *Eimeria* species that infect chickens and are capable of escape from current vaccines indicates an immediate problem for vaccination-led control of coccidiosis, with new vaccine formulations anticipated (Blake et al., 2021). Future opportunities for genetic and genomic characterisation are becoming increasingly accessible as costs diminish and expertise more readily available. It is likely that the coming decade will see a notable evolution in anticoccidial control for poultry.

Histomonosis

Target populations, incidence and economic relevance

Histomonosis is a disease of poultry with worldwide occurrence. The disease is caused by the protozoan *Histomonas meleagridis*, a flagellated parasite (Tyzzer, 1920). Gallinaceous birds can be infected with the parasite and turkeys and chickens are the most affected hosts (Hess et al., 2015). In turkeys, histomonosis can cause severe morbidity and mortality, whereas in chickens clinical disease is less prominent (Tyzzer, 1934). Nevertheless, in both species the clinical outcome of infection can be variable, from absent clinical signs to high mortality.

Poultry production is economically affected by histomonosis as a result of retarded growth, loss in egg production and mortality. Animal welfare and economic constraints became more relevant after prophylactic and therapeutic drugs were banned for use in poultry in many countries worldwide for reasons of consumer protection (Liebhart et al., 2017). Until the 21st century, histomonosis could be controlled by antihistomonal drugs in Europe. However, the only applicable chemicals, nitroimidazoles and nitrofurans, were withdrawn in 1996 and 2003, respectively (CEC, 1995, CEC, 2002). In the USA, arsenicals were the last remaining compounds that could be used for control prior to a ban in 2015 (FDA, 2015). In many other countries similar regulations have been applied that now preclude prophylaxis and therapy against histomonosis. Consequently, the ban of drugs effective against histomonosis has resulted in an increase in the number of cases, some of them incurring high economic losses (Hess et al., 2015, Clark and Kimminau, 2017). Severe outbreaks of the disease in turkey flocks are characterized by distinct clinical signs and pathological lesions, whereas infected chickens show less pathognomonic changes (Liebhart and Hess, 2020). However, economic aspects in chicken production might be underestimated as indicated in a report investigating several outbreaks in chicken flocks, especially breeder and layer flocks (Dolka et al., 2015).

In the USA, more than 100 cases of histomonosis were reported in 2016. The economic cost incurred by *H. meleagridis* worldwide is yet to be calculated, but has been estimated that the economic relevance of the disease for poultry production is similar to that of coccidiosis (McDougald, 2005).

Host-pathogen interactions

Clinical signs and pathology

Histomonas meleagridis can cause clinical signs like apathy, ruffled feathers and drooping wings. In turkeys, diarrhoea and sulphur coloured faeces can be observed as characteristic signs of the disease. In chickens, parameters like growth, weight and egg production can be the only clinical outcome.

The clinical status of birds suffering from histomonosis reflects the specific pathological changes. The caeca are the primary infected organs showing necrosis and inflammation, typically with fibrinous exudate in the lumen (Figure 3). Following tissue destruction in this part of the intestinal tract, the parasite can reach the liver via the portal vein resulting in necrosis and inflammation as indicated in figure 3. In chickens, lesions in the liver are less common than in turkeys but the parasite can be distributed throughout several organs in both host species (Grabensteiner et al., 2006). However, the genotype of *H. meleagridis* has an impact on the severity of clinical signs and lesions, as outlined below.

Host immune responses

Variability in the clinical outcome of histomonosis in chickens and turkeys underlines differences in the immune response of each host species against *H. meleagridis*. In turkeys, which are more susceptible to histomonosis than chickens, it was shown that circulating antibodies do not have a protective effect against the disease (Clarkson, 1963, Bleyen et al., 2009b). Consequently, immune protection induced by exposure to killed histomonads was not successful (Bleyen et al., 2009b, Hess et al., 2008). These studies indicate that the systemic humoral immune response does not have a substantial impact on protection. The effect of the local humoral response against *H. meleagridis* is not elucidated, but increased IgM, IgY and IgA in the caeca and other parts of the intestine in chickens has been reported (Windisch and Hess, 2010).

In contrast, the cellular immune response has been found to be crucial against histomonosis based on several studies using attenuated *H. meleagridis* to induce protective immune responses in turkeys and chickens, as outlined below (see “Vaccination”).

Recently, it was shown that chickens mount a faster immune response following *H. meleagridis* infection than turkeys, resulting in earlier defence mechanisms that can restrict the parasite to the caeca of infected individuals (Powell et al., 2009). In another study, flow cytometry (FCM) analyses revealed that histomonosis caused more severe changes in B cells and T-cell subsets of turkeys than chickens that may induce immunopathogenic effects (Mitra et al., 2017). Differences in the cellular immune response of chickens and turkeys have been further investigated by determining cytokine producing cells using *in situ* hybridization (ISH) (Kidane et al., 2018). In this work, chickens showed a higher presence of IFN-γ producing cells in the caeca than turkeys that may influence the nature of the immune response. Studies on the mentioned immune traits against *H. meleagridis* have been summarized by Mitra and colleagues (Mitra et al., 2018).

In a recent study, it was concluded that *H. meleagridis* infection induces a type-1 differentiation of $CD4^+$ T cells, but also of non-$CD4^+$ cells, in chickens based on histomonad-specific immune cells (Lagler et al., 2019). Furthermore, FCM analyses revealed significant increments of IFN-γ-producing cells

within major T-cell subsets ($CD4^+$, $CD8\alpha^+$ and $CD3\varepsilon^+CD4^-CD8\alpha^-$) of the spleen and liver in infected turkeys compared to infected chickens (Lagler et al., 2021).

Impact on enteric dysbiosis

The growth of *H. meleagridis* is known to be highly dependent on the presence of live bacteria (Bilic and Hess, 2020). It has been shown that the parasite is unable to cause disease in gnotobiotic turkeys, requiring the presence of specific bacterial species including *Escherichia coli* (Doll and Franker, 1963, Bradley and Reid, 1966). A recent study on protection in turkeys revealed that co-cultivated bacteria like *E. coli*, *Staphylococcus aureus* and *Salmonella* Enteritidis influence the colonization of monoxenic attenuated *H. meleagridis* (Liebhart et al., 2013a).

In the chicken host, the consequences of co-infection with *H. meleagridis* and avian pathogenic *E. coli* (APEC) have been investigated on enteric pathology, microbiota and bacterial translocation (Abdelhamid et al., 2020). It was found that such a co-infection caused caecal typhlitis and severe dysbiosis defined by a severe reduction in microbial species richness and diversity, with a relatively higher abundance of the *Escherichia* genus, *Helicobacter* and *Bacteroides* revealed by 16S rRNA gene amplicon sequencing. Furthermore, lux-tagged APEC introduced into the caeca were tracked and found to be significantly increased and distributed outside of the intestine in co-infected birds, indicating the role of *H. meleagridis* to support *E. coli* in the pathogenesis of colibacillosis in chickens.

Histomonas meleagridis population dynamics

Molecular studies on histomonads/genotypes and differences in clinical signs and pathology

In recent years, several studies focused on molecular identification of *H. meleagridis*. Initially, the 18S rDNA sequence was determined and used as a target for taxonomic identification of *H. meleagridis* (Gerbod et al., 2001). Taxonomically, the parasite belongs to the order Tritrichomonadida and family Dientamoebidae, showing greatest genetic similarity to *Dientamoeba fragilis*, a parasite of humans and several other mammals. Subsequently, several studies have focused on genetic differences between isolates of *H. meleagridis* (van der Heijden et al., 2006, Hauck and Hafez, 2009, Munsch et al., 2009, Reis et al., 2009, Hauck et al., 2010, Hauck and Hafez, 2010, Gerhold et al., 2011, Lollis et al., 2011, Lynn and Beckstead, 2012).

Genetic differences in the ITS1-5.8S-ITS2 region have been detected in a clonal *H. meleagridis* line, highlighting the occurrence of sequence degeneracy between genomic copies and emphasising the requirement for appropriate interpretation of sequence analysis using this genetic region (Hauck et al., 2010). More recently, Multi-Locus typing using the 18S rRNA, α-actinin1 and rpb1 genes from different *H. meleagridis* isolates demonstrated the existence of two different genotypes (Bilic et al., 2014). Importantly, differences in the outcome of infection by genotypes 1 or 2 could be observed in a flock of turkeys by clinical and pathological outcomes, suggesting genotype-specific pathogenesis (Grafl et al., 2015). In contrast to genotype 1, which has been well investigated in several experimental studies (Hess et al., 2006a, Liebhart and Hess, 2009), turkeys naturally infected with genotype 2 show reduced involvement of the liver. However, infection with genotype 2 compromised growth and resulted in more than 30% mortality (Grafl et al., 2015). Experimental infection of turkeys with genotype 2 have confirmed clinical and pathological differences to genotype 1 (unpublished data).

Introduction to a flock and patterns of transmission

The introduction of *H. meleagridis* into a poultry flock can occur via the intermediate vector, *H. gallinarum* using earthworms as a paratenic host (Graybill and Smith, 1920, Lund et al., 1966). Following introduction, direct transmission from bird to bird is effective and appears to play an important role (Hess et al., 2006a, Liebhart and Hess, 2009). While cyst-like stages of *H. meleagridis* have been described (Zaragatzki et al., 2010), prolonged viability of the parasite in the environment has not been reported. *In vitro* cultivated histomonads can only survive for a few hours on different materials or in media like water and faeces (Lotfi et al., 2012). However, direct transmission between individuals within a flock is rapid, supposably below this threshold. Based on an experimental infection and the detection of *H. meleagridis* in faeces by qPCR, the basic reproduction number (R0) was estimated to be 8.4 (Landman et al., 2015). This finding might explain the rapid dissemination reported within flocks, recognising that the study detected *H. meleagridis* DNA and not infective histomonads. Furthermore, as outlined above for mortality and morbidity, other factors such as parasite genotype are likely to influence transmission.

Current methods of detection for *Histomonas meleagridis*

In recent years, diagnostic tools to detect *H. meleagridis* have improved in terms of sensitivity and specificity. However, older detection methods still remain widely used, depending on the specific diagnostic demand.

Histomonas meleagridis can be observed by microscopic examination, either in native samples from intestinal contents or in histological preparations (Tyzzer, 1934). The viability of histomonads in preparations from caecal content or following *in vitro* propagation is crucial, since morphology (Figure 4) and motility is characteristic for the parasite. In histological tissue samples the flagella cannot be observed due to morphological changes of tissue stages of the parasite. However, size, shape and the formation of a gap between the parasite and the host tissue indicate the presence of histomonads. Conventionally, Periodic Acid-Schiff (PAS) staining has been found to be most suitable to identify *H. meleagridis* in tissue sections (Kemp and Reid, 1966). However, the occurrence of other protozoans such as trichomonads or blastocysts in host birds may impede an accurate diagnosis (Hess et al., 2006b).

Several molecular detection systems have been established in response to challenges posed to microscopy, mainly focusing on the 18S rRNA gene using conventional PCR to detect parasite DNA (Hafez et al., 2005, Huber et al., 2005, Grabensteiner and Hess, 2006, Bleyen et al., 2007). Subsequently, qPCR assays have been developed to allow the detection and quantification of *H. meleagridis* in samples (Hussain et al., 2015, Landman et al., 2015). A LAMP assay has also been published, providing high sensitivity and specificity (Xu et al., 2014). Histological examination has been improved by access to reagents for specific staining of *H. meleagridis* based on genomic sequences (*in-situ* hybridisation; ISH) or antigen-antibody reactions (immunohistochemistry) in tissue sections (Figure 5) (Liebhart et al., 2006, Singh et al., 2008). For indirect detection, a sandwich ELISA and a blocking ELISA have been set-up to measure antibodies against *H. meleagridis* (Windisch and Hess, 2009, van der Heijden et al., 2010).

Monitoring of flocks

The introduction of *H. meleagridis* into poultry flocks and its spread from bird to bird can be monitored by direct or indirect detection systems, as described above. In experimental settings, necropsy and a

404 combination of diagnostic tools including PCR, histology and re-isolation of *H. meleagridis* from cloacal
405 swabs have been shown to give substantial results on the progression of infection (Grabensteiner et
406 al., 2006, Hess et al., 2006a). For detailed monitoring of flocks in the field the same methods should
407 be applied, as described in a survey of histomonosis outbreaks in turkey flocks (Sulejmanovic et al.,
408 2017). Additionally, the detection of specific antibodies can be used to identify infected birds by their
409 immune response (Grafl et al., 2011, van der Heijden and Landman, 2011), although the appearance
410 of antibodies can take at least two weeks in chickens and turkeys (Windisch and Hess, 2009). For
411 example, a combination of PCR applied to faeces and dust samples with serology confirmed the
412 infection of turkey hens and their resilience to histomonosis in barns equipped with both sexes
413 following high mortalities in toms (Sulejmanovic et al., 2019a).
414
415 Another approach has been to monitor flocks exclusively by examination of environmental samples
416 using PCR, as described in a recent study (Sulejmanovic et al., 2019b). Here, parasite DNA could be
417 detected in dust samples collected from 15 of 65 investigated turkey flocks. Nine of the flocks found
418 to be positive by PCR presented with no signs of histomonosis, indicating a high epidemiological value
419 for histomonad detection using DNA in dust samples when negative controls are robust.
420
421 **Current methods of control for *Histomonas meleagridis***
422 *Limitations of current prophylaxis and therapy*
423 The most effective drugs against histomonosis are nitroimidazoles, nitrofurans and arsenicals, all of
424 which have been used for therapeutic and/or prophylactic purposes (Liebhart et al., 2017). However,
425 as outlined above, these chemicals came under public and legislative pressure due to concerns around
426 consumer health and have been banned from use in poultry production in many countries. In their
427 absence, no effective prophylaxis or therapy is available. The antibiotic paromomycin has shown a
428 prophylactic effect against histomonosis in turkeys (Lindquist, 1962, Bleyen et al., 2009a), but in
429 several countries antibiotics are not licensed to be administered prophylactically. Further, application
430 of paromomycin after diagnosing histomonosis in commercial turkey flocks has not shown promising
431 results when compared to untreated flocks (Sulejmanovic et al., 2017). Biosecurity and hygiene are of
432 high importance to prevent the introduction and spread of the parasite in poultry flocks. However,
433 the value of such measures is limited based on reports of histomonosis outbreaks in breeder birds,
434 where high biosecurity can be presumed (Dolka et al., 2015, Aka et al., 2011).
435
436 **Recent experimental approaches to control for *Histomonas meleagridis***
437 *Plant derived substances*
438 The lack of available chemotherapeutics that are effective against histomonosis in poultry argued for
439 intensification of research into alternative substances with anti-histomonal effects (Liebhart et al.,
440 2017). In response, several plant-derived essential oils, extracts in ethanol and water, lypholisiates,
441 alkaloids and sesquiterpene lactones have been examined for this purpose. Several compounds have
442 been found to reduce or suppress the propagation of *H. meleagridis in vitro*, including essential oils
443 from cinnamon, lemon, rosemary, garlic and thyme, but confirmation *in vivo* remains to be shown
444 (Zenner et al., 2003, Grabensteiner et al., 2007, Hauck and Hafez, 2007, van der Heijden and Landman,
445 2008a, van der Heijden and Landman, 2008b). Similarly, ethanol and water extracts from *Thymus*
446 *vulgaris*, *Vitis vinifera*, *Olea europaea*, *Peganum harmala*, *Ginkgo biloba* and *Aesculus hippocastanum*,
447 the alkaloids saponin, harmane, harmalol, harmaline and harmine, as well as artemisinin, a

sesquiterpene lactone, have all shown promising results *in vitro* without equivalent results *in vivo* (Grabensteiner et al., 2007, Grabensteiner et al., 2008, Arshad et al., 2008, Thøfner et al., 2012).

Commercial plant-based products tested for an effect against histomonosis in turkeys include Enteroguard™ and Aromabiotic™, but neither protected against disease (van der Heijden and Landman, 2008a, van der Heijden and Landman, 2008b). Application of the product Protophyt™ could not prevent clinical signs and lesions of histomonosis in infected turkeys (van der Heijden and Landman, 2008b, Hafez and Hauck, 2006). Similarly, suggestions of protection against disease using Natustat™ in turkeys kept in commercial farms are currently unconfirmed by standardized infection experiments (Duffy et al., 2005).

Vaccination including data on immune response following vaccination

Vaccination as a strategy to prevent histomonosis was first investigated more than 80 years ago, but attenuation of virulent *H. meleagridis* was described to be inhomogeneous and administration routines for poultry flocks have not been developed (Tyzzer, 1934).

A prerequisite for a well-defined live-vaccine was to establish a monoclonal culture of *H. meleagridis* (Hess et al., 2006b). Following prolonged culturing (295 passages *in vitro*), the clonal parasite became attenuated and could be used as a vaccine, inducing protection against severe challenge (Hess et al., 2008). Attenuated histomonads were restricted to the caecal lumen and several *in vivo* passages did not lead to a reversion to virulence, confirming the safety of the vaccine candidate (Liebhart et al., 2011, Sulejmanovic et al., 2016). Cross-protection against different isolates of the homologous genotype 1 has been demonstrated (Sulejmanovic et al., 2016) and a pilot study indicated protection against the heterologous genotype 2 (unpublished data). In chickens, vaccinated layers were shown to be protected against a significant reduction in egg production caused by histomonosis (Liebhart et al., 2013b). Optimization of experimental vaccination against histomonosis could be achieved by administration of the vaccine via the oral route in day-old turkeys (Liebhart et al., 2010), and by establishing a monoxenic vaccine candidate (Ganas et al., 2012).

Beside attenuation *in vitro*, it has been shown that serial *in vivo* passage in turkeys reduces the virulence of histomonads whilst retaining the ability to protect turkeys from a subsequent severe challenge (Nguyen Pham et al., 2013). In contrast, application of killed histomonads does not result in protection for challenged turkeys (Hess et al., 2008, Bleyen et al., 2009b).

Investigations to define those immune mechanisms that are relevant for protection against histomonosis have included flow cytometry, demonstrating that vaccination with attenuated histomonads induced a lower cellular immune response than virulent histomonads, inducing protective immunity without an immunopathogenic effect (Mitra et al., 2017). Furthermore, vaccination of turkeys led to increased IFN-γ producing cells in the caeca to levels comparable to naïve chickens that are innately less affected to histomonosis (Kidane et al., 2018). In studies using intracellular cytokine staining, it was found that vaccinated turkeys produce significant more IFN-γ-producing cells by all major T-cell subsets of the spleen and liver compared to vaccinated chickens (Lagler et al., 2021). Based on these results it can be concluded that the vaccine causes more intense systemic immune responses in turkeys, whereas in chickens protection might be driven by the local immune response.

Challenges and conclusions

The absence of effective prophylactic and therapeutic options to control histomonosis urgently requires new and improved approaches. Recent studies have focused on a wide range of plant-derived substances, but it is clear that any future product will be subject to increasingly strict regulations designed to protect consumer health. New products must be carefully selected for their safety and independence from existing or proposed products that are used in human medicine. To date, plant-derived substances have not shown substantial effects against histomonosis, but these substances may be refined and other active components may yet prove effective. Cultured *H. meleagridis* are highly suitable for efficacy tests and can be used to improve screening capacity, but it should be mandatory to confirm positive results *in vivo*.

Studies focused on vaccine development have highlighted *in vitro* attenuated histomonads as a promising new approach. Work to develop live attenuated vaccines further will require strategies for up-scaling, storage, transportation and application under field conditions. Histomonads are highly sensitive to environmental conditions, demanding innovative solutions for vaccine application to address these challenges.

Dermanyssus gallinae – the poultry red mite

Target populations, incidence and economic relevance

Dermanyssus gallinae (the poultry red mite; PRM) is an obligatory blood feeding ectoparasite (Chauve, 1998). The PRM lifecycle includes five distinct stages: egg, larva, protonymph, deutonymph and adult (Figure 6.A), and can be completed within just seven days under optimal conditions (i.e. temperature: 20-25^{o}C, humidity >70%) (Koziatek and Sokół, 2015, Immediato et al., 2015, Maurer and Baumgartner, 1992). Consumption of blood is required for maturation of the protonymph, deutonymph and adult lifecycle stages, as well as development of viable eggs. PRM have been described from a broad host range, including horses, rodents and humans (Valiente Moro et al., 2009), but avian hosts are most common. PRM have been reported to infest at least 28 different avian species, most notably the domestic chicken but also canaries, pigeons and doves (Roy et al., 2009b). While all chickens can be targeted by PRM, laying and breeding stock are at greatest risk, primarily due to their extended flock duration compared to the faster turnaround time associated with broiler chickens, providing longer opportunities for infestation and mite replication. PRM spend the majority of their life cycle living separately from their hosts, sheltering in cracks and crevices, nests and cages (Fiddes et al., 2005) (Figure 6.B), emerging to feed when dark for approximately 30-90 minutes (Chauve, 1998).

The poultry red mite has a global distribution although occurrence is reported most frequently in Europe and Asia, where up to 90% of layer hen farms can be infested (Cencek, 2003, Sparagano et al., 2009, Hoglund et al., 1995, Guy et al., 2004, Marangi et al., 2012, Fiddes et al., 2005, Oh et al., 2019). Other mites such as the northern fowl mite (*Ornithonyssus sylviarum*) are commonly considered to be more important in North America. PRM are responsible for significant economic losses from the European poultry industry with estimates in excess of ~EU€ 230 million lost every year (Price et al., 2019). This cost has primarily been attributed to production losses (increased mortality, decreased egg production and quality, abbreviated laying cycle), higher feed conversion ratios, and the costs of control (Sparagano et al., 2009, Wojcik et al., 2000, Sleeckx et al., 2019). Costs estimated from

individual countries range from EU€ 3 million to EU€ 66.8 million for the UK, the Netherlands and Japan (Sparagano et al., 2009).

Host-pathogen interactions

Clinical signs, pathology and welfare

PRM infestation can impact on production parameters. Direct interaction between PRM and the host is usually restricted to feeding, when infestations in excess of 50,000 mites per hen are not uncommon (Kilpinen et al., 2005). Infestation levels of ~150,000 mites per hen have been found to result in increased restlessness, irritation, feather pecking and cannibalism, as well as anaemia and increased hen mortality (Kilpinen et al., 2005). Extended periods of infestation have also been linked with decreased body weight (Wojcik et al., 2000). Weekly mortality and laying rates, as well as egg weight, have all been shown to improve following effective anti-mite (acaricidal) treatment (Temple et al., 2020).

PRM infestation can also severely compromise hen welfare. Laying hens have been shown to change their resting and sleeping locations in response to infestation, possibly attempting to evade or reduce mite challenge (Maurer, 1993). Effective acaricidal treatment has been associated with reduced nighttime activity, including preening, head scratching and headshaking, in addition to severity of feather peaking and aggressive behaviour during the daytime (Temple et al., 2020). Measures of comb quality, including colour and the presence of wounds, were also improved. Physiological assessments have shown that indicators of stress, such as corticosterone levels, increase in hens exposed to PRM (Kowalski and Sokol, 2009).

Host immune responses

Dermanyssus gallinae demonstrate minimal host interference during feeding, incurring few significant immune responses (Harrington et al., 2010b). Humoral immune responses such as serum IgY and IgM increase with the occurrence and intensity of PRM exposure (Harrington et al., 2010b). There is some evidence for an early Th1 and pro-inflammatory cytokine response, but this is short lived and might be down-regulated after subsequent feeding (Harrington et al., 2010a). The limited nature of the immune response induced by host-mite interaction poses a major challenge to development of anti-PRM vaccines. Attempts have primarily focused on development of hidden antigen vaccines, targeting mite proteins such as cathepsin-D that are not naturally exposed to the hen but can inhibit mite feeding, development or replication when targeted by antibodies (Price et al., 2019).

Vector capacity

It has been suggested that PRM can serve as a vector for transmission of several viral and bacterial pathogens (De Luna et al., 2008). PCR has been used to detect specific pathogen nucleic acids as an indication of possible transmission, recognising that detection is not evidence of viable organisms or their transmission, including Newcastle disease virus, *Mycoplasma synoviae* and *M. gallisepticum* (Huong et al., 2014, Arzey, 1990). *Mycobacterium* species DNA has also been detected in PRM eggs and unfed larvae (De Luna et al., 2008). Transmission has been demonstrated for fowlpox virus and *Pasturella multocida* (Petrov, 1975, Shirnov et al., 1972), as well as *Salmonella* Enteritidis (Valiente Moro et al., 2009). In the latter study PRM carrying *Salmonella* Enteritidis were found to transmit infection between chickens and to persist after cleaning and disinfection, indicating a source of

transmission between individuals and flocks. Transovarial transmission was also documented, demonstrating vertical transmission between PRM generations (Valiente Moro et al., 2009).

Dermanyssus gallinae population dynamics

Little has been published describing population structure and dynamics for PRM. In a series of papers Roy and colleagues have suggested that *D. gallinae* may represent a species complex and not a single, discrete species. The complex may represent at least two morphologically indistinguishable, but genetically distinct cryptic species (Roy et al., 2010, Roy et al., 2009a, Roy and Buronfosse, 2011). Importantly, the seemingly true (i.e. *sensu stricto*) *D. gallinae* has been detected infecting chickens and a range of other avian hosts, while the *D. gallinae* L1 lineage may be specific to pigeons. Complementary genetics-led studies have revealed distinct *D. gallinae* genotypes circulating in wild and domestic host populations in Sweden (Brännström et al., 2008). Findings that suggest distinct PRM populations in domestic and wild avians are important, since they can inform on likely sources of infestation and the dissemination of unfavourable phenotypes such as acaricide resistance. More recent studies focusing on *D. gallinae* sampled from populations in domestic chicken environments demonstrated the presence of multiple lineages in Europe (Karp-Tatham et al., 2020). Multiple genetic types were discovered, representing three haplogroups with six sub-haplogroups. Considerable variation was detected within and between countries, possibly reflecting movement of poultry or contaminated equipment and variation in husbandry practices.

Current methods of detection for *Dermanyssus gallinae*

Adult PRM can be visible to the human eye, but accurate enumeration for purposes such as assessment of risk or efficacy of control requires low-magnification microscopy. Importantly, PRM are only located on their host during feeding, hiding in the environment for the majority of the time. A wide range of traps have been described to facilitate environmental sampling for PRM, including fabric, corrugated cardboard or plastic traps that seek to create an environment that attracts mites (Kirkwood, 1965, Nordenfors and Chirico, 2001). An automated mite counting technique has been described (Mul et al., 2015), although uptake has not been high. Positioning of mite traps is key, recognising the importance of PRM feeding and aggregation behaviour (Mul et al., 2015)

Current methods of control for *Dermanyssus gallinae*

Acaricides

Control of PRM is challenging. A wide range of organophosphates, carbamates, formamidines and pyrethroids have been used to control PRM in the past (Abbas et al., 2014, Beugnet et al., 1997, Chauve, 1998), but public and legislative pressure have combined to limit the availability of many products. The widespread development of acaricide resistance and the scarcity of new products has added further complications (Sparagano et al., 2014, Katsavou et al., 2020). Very few products remain available and licenced for use with poultry, exceptions including the fluralaner-based Exzolt® solution (MSD Animal Health) (Temple et al., 2020). Reports of acaricide residues in poultry and poultry products for human consumption have added further pressure, with examples including carbaryl in the skin and fat of chickens (Marangi et al., 2012) and, more recently, the scandal around fipronil residues in chicken eggs (Tu et al., 2019).

A major limitation to the use of acaricides has been the rapid emergence and dissemination of acaricide resistance (Marangi et al., 2009, Marangi et al., 2012). The emergence of genetic (i.e.

heritable) resistance to acaricides has commonly been mediated by point mutations in genes that encode proteins with key metabolic functions, contributing to metabolism of the acaricide before it can achieve its target or enzymatic detoxification (e.g. glutathione-S-transferases and P450 monooxygenases) (Wang et al., 2021, Wang et al., 2020).

Desiccant dusts

Alternatives to chemical control include desiccant, silica or inert dusts (Steenberg and Kilpinen, 2014). It is believed that desiccant and equivalent dusts desiccate and kill PRM and other arthropods, possibly due to cuticle abrasion and absorption of cuticular lipids (Ebeling, 1971), acting within 24 hours (Kilpinen and Steenberg, 2009). Examples of desiccant dusts include synthetic silica products and diatomaceous earth (Kilpinen and Steenberg, 2009). Comparison of a range of desiccant products revealed the importance of cation exchange, where increased capacity improved efficacy, and water absorption, emphasising the importance of a dry environment (Schulz et al., 2014, Kilpinen and Steenberg, 2009). Challenges associated with the use of desiccant dusts include health and safety provision for workers active in the area.

Alternatives for control of PRM

The scale of the challenge posed by PRM, and the paucity of the controls available, have prompted development of several alternatives. The use of high heat/low humidity conditions between flocks can reduce residual PRM presence, although the approach can be costly and inappropriate for some older or extensive poultry accommodation. Predatory mites such as *Cheyletus eruditus* have been found to feed on PRM, especially larvae (Maurer, 1993). Several predatory mite species are being developed for use in biocontrol strategies (Zriki et al., 2020). Entomopathogenic fungi have also been considered. Several fungal species have been identified with known efficacy against arthropod pests and are currently used in agriculture and forestry (de Faria and Wraight, 2007). Laboratory experiments of PRM susceptibility to fungi such as *Beauveria bassiana* and *Metarhizium anisopliae* have suggested utility, although the process can be relatively slow (Tavassoli, 2011). Several vaccines are in development for use against PRM, including vaccine candidates such as cathepsin-D (Price et al., 2019), but none are close to commercialisation (Bartley et al., 2017, Xu et al., 2020).

Conclusions

Control of parasites that target poultry remains a major challenge. These antigenically complex pathogens are commonly adept at evolving to escape conventional control based on husbandry and routine prophylaxis. Increasing public and legislative demands for the replacement of drugs and chemicals in livestock and poultry production is exacerbating the situation, with few or no effective products left available for control. Improved understanding of genetic diversity and population structure is beginning to support development of novel controls, revealing previously unknown new genotypes and, in some examples, species. Attempts to modify or develop new vaccines for the three parasite groups discussed here are ongoing, offering considerable promise for management of poultry flock health in the near future.

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Figures

Figure 1. *Eimeria brunetti* oocysts. Bottom left = unsporulated and uninfectious. Top and right = sporulated and infectious. Scale bar = 10 µm.

Figure 2. Example of *Eimeria* species and genus specific PCR assays. PCR amplicons resolved for four different *Eimeria* species-specific assays targeting the internal transcribed spacer 1 (ITS1) repeat region and one *Eimeria* genus-specific assay targeting the 18S rRNA locus (all as described by (Schwarz et al., 2009)). DNA templates used as indicated. The lack of cross-reactivity with the chicken host demonstrated by inclusion of an assay targeting the chicken glyceraldehyde-3-phosphate dehydrogenase locus (Blake et al., 2006).
E. ace = *E. acervulina*; *E. bru* = *E. brunetti*, *E. max* = *E. maxima*, *E. mit* = *E. mitis*, *E. nec* = *E. necatrix*, *E. pra* = *E. praecox*, *E. ten* = *E. tenella*.

Figure 3. Lesions in liver and caeca of a turkey caused by *H. meleagridis*

Figure 4. Cultured histomonads. Intracellular objects represent incorporated rice starch particles.

Figure 5. Immunohistochemistry for the detection and localization of *H. meleagridis* in tissues sections. (A) Section of caecum with histomonads in all layers of the organ. (B) Liver sample showing specifically stained histomonads in the parenchyma at a higher magnification.

Figure 6. *Dermanyssus gallinae*, the poultry red mite (PRM). (A) Examples of a *D. gallinae* protonymph (bottom, white colouration), two deutonymphs (right) and three adults. (B) A typical hiding place for *D. gallinae* within poultry accommodation. PRM spend most of their lives in the environment, only infesting chickens when taking a blood meal. Photographs taken by Eleanor Karp-Tatham.