Aus dem Institut für Tierernährung
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin
und
dem Institut für Ernährungsphysiologie ‚Oskar Kellner'
des Forschunginstituts für Nutztierbiologie Dummerstorf

Metabolic responses and diagnostic advancements in chickens exposed to mixed intestinal parasite infections

Inaugural-Dissertation
zur Erlangung des Grades eines
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an der
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vorgelegt von
M.Sc. Oyekunle John Oladosu
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My Parents:

Alice Temilade & Gabriel Olusegun Oladosu

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List of Abbreviations

^{1}H-NMR	^{1}H Nuclear magnetic resonance
AGP	Alpha (1)-acid glycoprotein
APG	Antigen per gram of faeces
AUC	Area under the curve
ELISA	Enzyme-linked immunosorbent assay
EPG	Eggs per gram of faeces
ESP	Excretory-Secretory products
FDR	False discovery rate
FEC	Faecal egg count
FID	Free induction decay
GC-MS	Gas chromatography–mass spectrometry
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LB	Lohmann Brown
LD	Lohmann Dual
ROC	Receiver operating characteristics
Th2	T helper 2
TMAO	Trimethylamine-N-oxide
WPI	Weeks post infection

1 Introduction

Intestinal parasite infections, particularly those caused by nematodes such as *Ascaridia galli* and *Heterakis gallinarum*, pose significant challenges to poultry production, manifested as reduced productivity and in extreme cases, mortality (Tomza-Marciniak et al., 2014). These parasites colonize the gut of chickens, where they can influence the microbiome and metabolism, thereby negatively impacting feed intake, feed conversion efficiency, nutrient absorption, utilization, growth rate, and overall chicken performance (Permin et al., 2006; Sharma et al., 2018a; Stehr et al., 2019a). Nematode infections can begin as early as within the first 4 weeks of chicken life, persisting for a significant duration. *A. galli* and *H. gallinarum* have a life cycle of 4-8 weeks, and prevalence of infections with these nematodes can be up to 69.5% and 95%, respectively (Kaufmann et al., 2011; Thapa et al., 2015). Despite the host's ability to expel most ascarids within the initial 3 weeks of infection, persistent reinfection particularly for *H. gallinarum*, occurs and poses a threat that lasts over an extended period (Stehr et al., 2018).

Nematode infections trigger the activation of immune responses notably the Th2 response to stimulate worm expulsion mechanism (Sorobetea et al., 2018). This response is said to be metabolically costly and thus may steer the diversification of nutrient resources to prioritize mounting defence at the expense of performance, particularly in genotypes of birds under genetic pressure for high productivity (Colditz, 2008; Rauw, 2012; Rauw et al., 1998). Highperforming birds have indeed shown more decreased production levels following nematode infections compared to low performing genotypes, highlighting the complex interplay between performance and defence (Stehr, et al., 2019b). However, the actual metabolic resources that is traded off between performance and immune response is not well understood and thus required further study to understand host-parasite interactions.

The exploration of host-parasite interactions and the development of effective intervention strategies depend on the ability to precisely identify and accurately quantify infections. Currently, nematode infection quantification involves methods such as faecal egg count (FEC), which counts the number of eggs per gram of host faeces (Gordon and Whitlock, 1939; Nielsen, 2021), and the measurement of worm-specific antibody in host serum or egg yolks (Daş et al., 2017). Each of these methods comes with its own set of limitations. Firstly, FEC can be a laborious and time-consuming process with low sensitivity (Daş et al., 2020). The measurement of antibody levels in serum is invasive, requiring blood collection, which may compromise the welfare of chickens (Daş et al., 2017). Additionally, the quantification of antibodies in egg yolk, while non-invasive, is applicable only to laying hens and cannot be

measured before the onset of laying or in broiler chickens. An alternative approach for quantifying nematode infections is the use of worm-specific antigens in host faeces (coproantigen), which has been reported effective for detecting and quantifying helminth infections in other species (Sykes and McCarthy, 2011; Elsemore et al., 2017; Lagatie et al., 2020). The noteworthy advantage of coproantigen diagnosis is its complete noninvasiveness, as samples are collected from faeces making it practical for use in all types of poultry production systems. However, as of now, there is no existing coproantigen method specifically designed for chickens.

1.1 Objectives of the study

1. The main objective of this thesis is to identify infection-induced alterations in plasma and liver metabolome of chickens to understand the metabolic cost of immune response to nematodes and to advance understanding of host-parasite interactions.
2. To develop a coproantigen ELISA diagnostics for the indirect quantification of nematode infections in chicken.
3. A further aim is to assess the faecal worm antigen excretion pattern and to compare the diagnostic performance of the coproantigen ELISA with existing diagnostic tools.

2 Literature

2.1 General epidemiology of helminth infections in the chicken host

Ascaridia galli and *Heterakis gallinarum* are economically significant helminth species in chickens, primarily due to their high prevalences (Shifaw, et al., 2021a). In a study investigating helminth prevalence in organic laying hens across Europe, *A. galli* exhibited an average prevalence of 69.5%, with an approximate burden of 10 worms per hen (Thapa et al., 2015). Conversely, *H. gallinarum* showed an average prevalence of 29%, with around 16 worms per hen. Variations in prevalence are reported across different European countries. For instance, in a mountain farming region in northern Italy, 95% of the studied population were infected with *H. gallinarum* and 63% with *A. galli* (Wuthijaree et al., 2017). Similar prevalences for both parasites have been reported in other European studies. Prevalence of *H. gallinarum* was 98% and *A. galli* was 88% in organic production systems in Germany (Kaufmann et al., 2011). About 64% prevalence for *A. galli* and 72.5% for *H. gallinarum* were reported in a free-range/organic system in Denmark (Permin et al., 1999), while in the UK, prevalence report was 89% for *H. gallinarum* and 84% of free-range laying hens examined were infected with *A. galli* (Sherwin et al., 2013).

Both *A. galli* and *H. gallinarum* are intestinal ascarid species with high genetic similarities and are transmitted through the faecal-oral route (Ramadan and Znada, 1992; Nadler et al., 2007). Both ascarids follow a direct life cycle (Figure 1) wherein embryonated eggs become infective in the soil or litter. Upon ingestion, these eggs, containing L3 larvae, can infect the hosts (Herd and McNaught, 1975). A rather intriguing addition to this cycle is the role of the eggs of *H. gallinarum* as vectors for another parasite—*Histomonas meleagridis*, a protozoon responsible for causing blackhead diseases in poultry and damaging the liver and or caeca (Tyzzer, 1920; McDougald and Fuller, 2005; Hess et al., 2015).

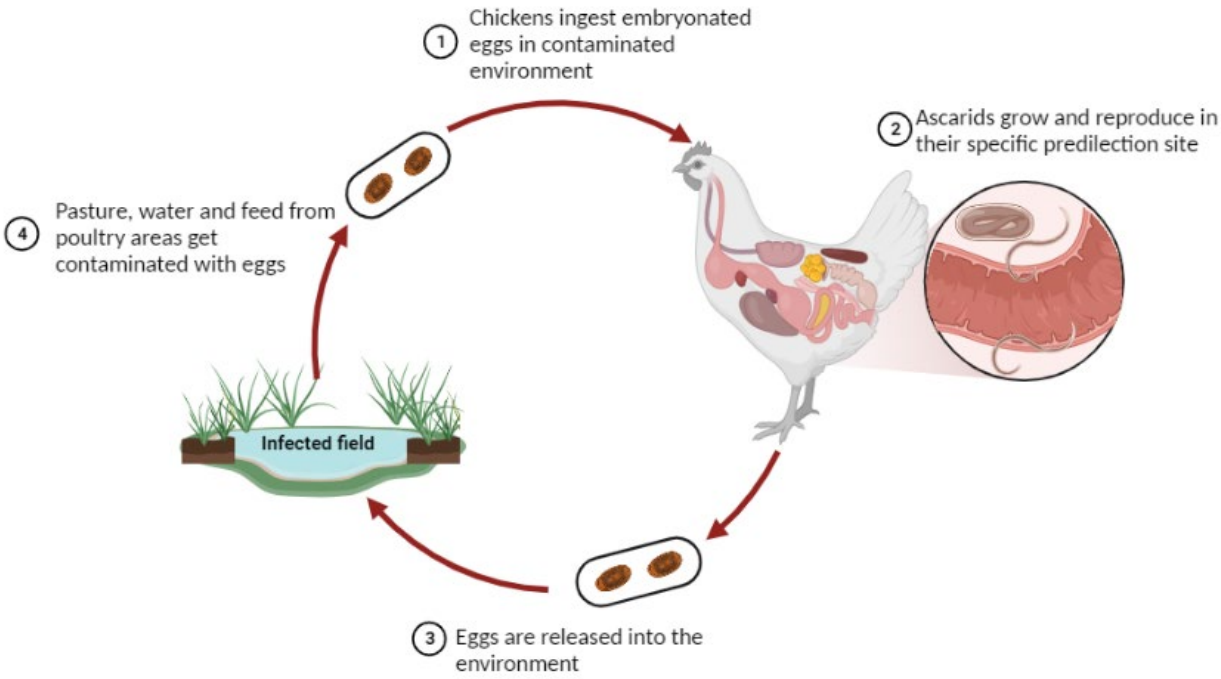

Figure 1 *Illustration of the direct life cycle of ascarids. Created with biorender.com*

Both species of nematode can exert patho-physiological effects on their chicken hosts. Direct impacts on host physiological functions, such as growth and laying activity, primarily result from diminished nutrient availability induced by nematode-related impairments in nutrient absorption and utilization as a consequence of compromised feed intake and conversion efficiency (Hurwitz et al., 1972a; Schwarz et al., 2011; Stehr, et al., 2019a).

Concurrently, immune responses which entail a high metabolic cost are triggered particularly at the early phase of infection when ascarid larvae penetrate the intestinal mucosa (Colditz, 2008; Dalgaard et al., 2015). Upon entry *A. galli* larvae induce damages to the intestinal wall (Luna-Olivares et al., 2012) and alter the intestinal morphology (Marcos-Atxutegi et al., 2009) which may further contribute to the impairment of nutrient absorption. Impaired protein utilization has been reported in *A. galli*-infected chickens, linked to reduced proteolytic enzyme activity in the jejunum (Hurwitz et al.,1972b). In addition, *A. galli* infections negatively impact energy metabolism, nitrogen retention and the transport of alanine and glucose (Walker and Farrell, 1976; Schwarz et al., 2011).

2.2 Factors associated with prevalence of nematode infections and worm development

The prevalence of nematodes observed in European countries may result from the shift in production systems to non-cage systems following the ban on cages in the EU by Council Directive 1999/74/EC (EC, 2003; Sharma et al., 2019; Shifaw et al., 2021a). Non-cage production systems are favoured for animal welfare reasons; however, birds with access to free-range areas, despite having better plumage and possibility to show more natural behaviours (Grafl et al., 2017), are more predisposed to nematode infections due to the ease of transmission, posing a significant concern for modern production systems (Grafl et al., 2017; Shifaw et al., 2021a).

The scavenging habits and prolonged contact of non-cage chickens with an environment likely contaminated with nematode eggs provide suitable conditions for the development of nematode infections. Not only is the prevalence of nematodes high in a free-range system, but the intensity of infection is also a cause for concern. One study demonstrated that the infection intensity of *A.galli* and *H.gallinarum* was five and three times respectively higher in an organic production system with outdoor access than in conventional farms (Wuthijaree et al., 2017). This is attributed to the avoidance of anthelminthics in organic production system for reasons associated with environmental protection, food safety and consumer well-being. Despite the prevalence of nematodes and their associated pathological effects on non-caged chickens, this production system remains as a valuable choice at least in developed countries. Thus, it is highly unlikely that this production system will be discontinued, especially as consumers of poultry products now demand a high level of welfare standards for farmed animals (Gorton et al., 2023). Maintaining a production system that meets consumer demand would be of economic importance. At least 15% of laying hens in Europe are now kept in freerange, and 7.1% in organic holdings (European Commission, 2022). Free-range egg production is on the rise in other developed countries, accounting for 57% of total egg production in the UK, 30% in New Zealand, and contributing to 47% of all retail sales with a sales value of 56% in Australia (Egg Producers Federation New Zealand, 2018; Australia Egg, 2019; Department for Environment Food & Rural Affairs, 2021). Therefore, the demand for free-range products is expected to increase, driven by the public demand for improved animal welfare standards.

Dealing with the occurrence of nematode infections requires a detailed understanding of the development of worm infections and the factors influencing worm establishment. Similarly, study of hosts interaction with the worms extends our understanding of hosts' resistance, susceptibility and tolerance to worm infection. In figure 2 host resistance, tolerance, and susceptibility are clearly defined in the context of this thesis. While we established that helminth

infections are inherently prevalent in organic and freerange farming systems, the factors influencing the transmission and establishment of nematode infection in poultry are much more complex. The effects of the production system alone cannot explain the varying prevalence of nematode infections in poultry between and even within a production system. Other environmental factors, as well as parasite and host-related factors, contribute to the susceptibility of nematode infections.

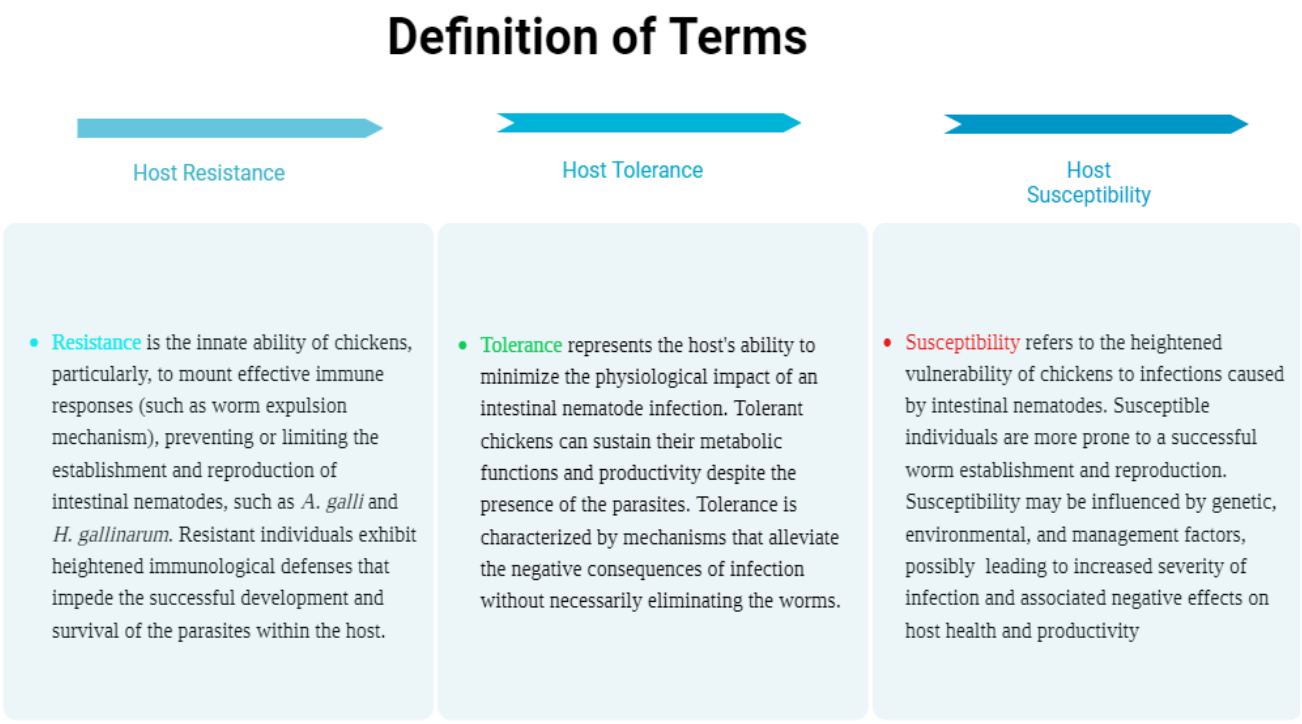

Figure 2 *Definition of key terminologies related to infection. Created with biorender.com*

2.2.1 Environmental factors driving exposure to infections

Environmental factors, including diet and climatic conditions, are among the most important influencers of natural helminth infections (Skallerup et al., 2005). Supplementation with a highfibre diet, such as maize silage and insoluble non-starch polysaccharides has been linked with higher worm burdens and faecal egg count for *A.galli* in experimentally inoculated hens in comparison to those receiving a standard diet (Daş et al., 2011, 2012; Shrestha et al., 2013). Ascarid-infected chickens fed insoluble non-starch polysaccharides have increased feed intake by 8% yet had a lower final body weight compared to those that were fed concentrate diet. But this fibre rich diet also likely altered the birds' gut to favour the establishment of *A. galli* leading to higher prevalence and worm burdens (Daş et al., 2012). Climatic conditions have also been shown to influence the dynamics of parasites infections. A study found a high prevalence of intestinal helminths during raining seasons in Tanzania suggesting the role of temperature and humidity on worm infection dynamics (Magwisha et al., 2002). Worm eggs did not become infective in temperate regions where temperature fall below 15°C in winter

(Tarbiat et al., 2015). Conditions for egg development (i.e. embryonation) may improve in spring and summer, albeit at a slower pace in northern Europe, as evidenced by the case of eggs of *Ascaris suum* (Larsen and Roepstorff, 1999). Additionally, due to adverse environmental factors like direct exposure to sunlight, ascarid eggs in pastures can be susceptible to high mortality (Brown, 1927). Predicting which environmental factors are most crucial in the context of transmission and dynamics of nematodes infection remain challenging (Thapa et al., 2015).

2.2.2 Parasite related factors driving host susceptibility

The age and dose of infective eggs, as well as the presence of other worm species also contribute to the successful establishment of nematode infection in chickens. Evidence supports that infection dose has positive relationship on worm establishment in the host (Feyera et al., 2022). The authors demonstrated differences in the worm burdens between birds that were infected with low (100) and high (900) doses of *A. galli* eggs. This underscores a positive relationship, indicating that higher doses lead to increased worm burdens. It is crucial to consider the temporal dynamics of worm expulsion. Previous research, such as that by Stehr et al. (2018), highlights that most worms are expelled between 31- and 36-days post-infection for *A. galli* and *H. gallinarum* respectively. Consequently, the worms found in birds at after this period are likely a result of naturally occurring re-infections. This explains time-related changes in worm burdens, and further introduces a confounding factor, as re-infection may remove the effects of the first circle of infection on worm burden. Thus, the relationship between infection dose and worm establishment is not only dose-dependent but also influenced by subsequent re-infections, adding a layer of complexity to our understanding of these dynamics.

The presence of co-pathogens during nematode infection also affects the susceptibility of the worms. In some studies, *A. galli* alone would likely produce no clinical symptoms, but with coinfection, noticeable clinical signs become apparent. An example is the high depression and mortality observed when chickens were infected with both *Pasteurella multocida* and *A. galli* (Dahl et al., 2002). Infections with a single species of nematode are rare in natural conditions. Under natural circumstances, there is likely to be an incidence of multiple helminth infections rather than a single infection. Only about 17% of a studied population of chickens had a single helminth infection; 70% were infected with mixed helminth infections. Of these, 31.4% were infected with two (2) species, 35.4% with three (3) species, and about 20% with four (4) species (Wuthijaree et al., 2017). This implies that infection with nematodes may contribute to hosts' susceptibility to other pathogens and vice versa (Mabbott, 2018; Magalhães et al., 2020). The presence of *H. meleagridis* alongside *H. gallinarum* would further compound the effects on chicken health and productivity (Daş et al., 2021).

2.2.3 Host-related factors driving resistance to worms

The genetic background and physiological status of the bird also determine the resistance and extent of infection intensity (i.e. resistance). These factors deal with host's capacity to activate an effective immune response for the expulsion of worms. Worm expulsion is regarded as the primary effective immune-regulatory mechanism by which the host responds to helminth infections (Dold and Holland, 2011). This process of worm expulsion depends on complex immuno-physiological mechanisms involving goblet cells' secretion of mucus, granulocytes and epithelial cells' release of neutralizing proteins and the production of mucins. Mucins are critical in disrupting the motility of worms to enhance expulsion. Following expulsion, inflammation is receded, and the tissue is repaired. This process is thought to be partly coordinated by the type 2 cytokines involving several immune cell types (Sorobetea et al., 2018).

Therefore, different factors related to the chicken host can cause variation in their susceptibility to nematodes, ranging from production purpose, age, physiological status to genetic pressure for performance. One study evaluating susceptibility to nematodes in chickens raised in a semi-intensive system showed that broilers have a smaller number of worms compared to layers (da Silva et al., 2018). In another study, birds with extremely different growth rates and production purposes showed differences in their resistance to nematode infections (Stehr et al., 2019b). Genotypes of birds selected for high performance showed the least resistance given a higher worm burden for *A. galli* infection compared to low-performing genotypes. This difference suggests that host genotype and their production performance may play a key role in infection resistance (Stehr et al., 2019b) while also supporting the evidence that broilers selected for high productivity have consequential impairment in their ability to withstand physiological and immunological responses (Koenen et al., 2002; Rauw, 2012) as required for effective worm expulsion. Similarly, another study investigated tolerance to nematode infections in laying hens with contrasted capacity for laying performance (Stehr et al., 2019a). High-performing and low-performing hens were inoculated with eggs of *H. gallinarum* and *A. galli* and were monitored for productivity for 18 weeks. Both genotypes studied had a significantly lower laying rate, per capita egg mass and egg weight. However, compared to 14 weeks in low-performing hens, the decline in the laying rate in high-performing birds occurred as immediate as 3 weeks post-infection, which is the onset of immune activation to worm infections (Stehr et al., 2018).

The diminished capacity of high-performance-selected chickens to contend with challenges related to fitness may be linked to the allocation of nutrient resources within a specific environment (Glazier, 2008; Rauw, 2012). This association arises from the consequence of

selecting for heightened production, wherein available resources are prioritized towards production, thereby compromising other essential traits, such as immunity to pathogens (Rauw, 2012).

2.3 Allocation of nutrient metabolites during nematode infections

The allocation of nutrient resources to different physiological processes appears to be a fundamental evolutionary mechanism that enables an organism's survival (French et al., 2007). Each physiological process requires those nutrient resources which when allocated to one process (e.g reproductive) cannot be re-allocated to other events (e.g immune functions) (Hayward et al., 2019). Accordingly, there may be no equilibrium in the allocation of resources, and it depends on the genetic disposition of an organism or its immediate physiological needs in such a way that resources allocation are prioritized towards the most consuming physiological process. In livestock production, the trade-off that has been of most interest is between immune function and performance (e.g., muscle growth and reproduction) as both traits are negatively associated (Bishop and Stear, 2003; Klasing, 2007; Greer, 2008). A meta-analysis of studies investigating such trade-offs also revealed that genetic selection for high performance is the driver of the high cost of defence against nematode infections in chickens (Most et al., 2011). The authors suggested that augmented allocation of energy for growth through genetic selections led to the scarcity of resources for the maintenance of immune functions. On one hand, reduction in feed intake is a known immediate effect of ascarid infection, which may create a condition of scarcity of nutrient resources. On the other hand, during infection, inflammatory cytokines can also impair distribution of resources allocated for growth. The activation of innate immune response occurring through the toll-like receptors to produce pro-inflammatory cytokines can alter protein metabolism, increases oxygen usage, and the utilization of glucose and glutamine (Colditz, 2008).

Both innate and adaptive immunity can be triggered during exposure to nematode infection. Innate immunity is activated as the foremost defence mechanism against pathogens but it is not specific to any pathogens. It provides a rapid, non-specific response to a wide range of pathogens (Schachner et al., 2023). In chickens, the metabolic cost of mounting innate immune response is proposed to be higher than that of the adaptive immune system because the adaptive immune is only activated to protect during re-entry of the pathogens (Klasing, 2007; Kim and Lillehoj, 2019). Conversely, the cost of developing an adaptive immunity is higher than the innate response (Klasing, 2007). The innate response is exceptionally expensive in chicken because selection for rapid growth and egg production dampens the acute phase of the response (Klasing, 1998).

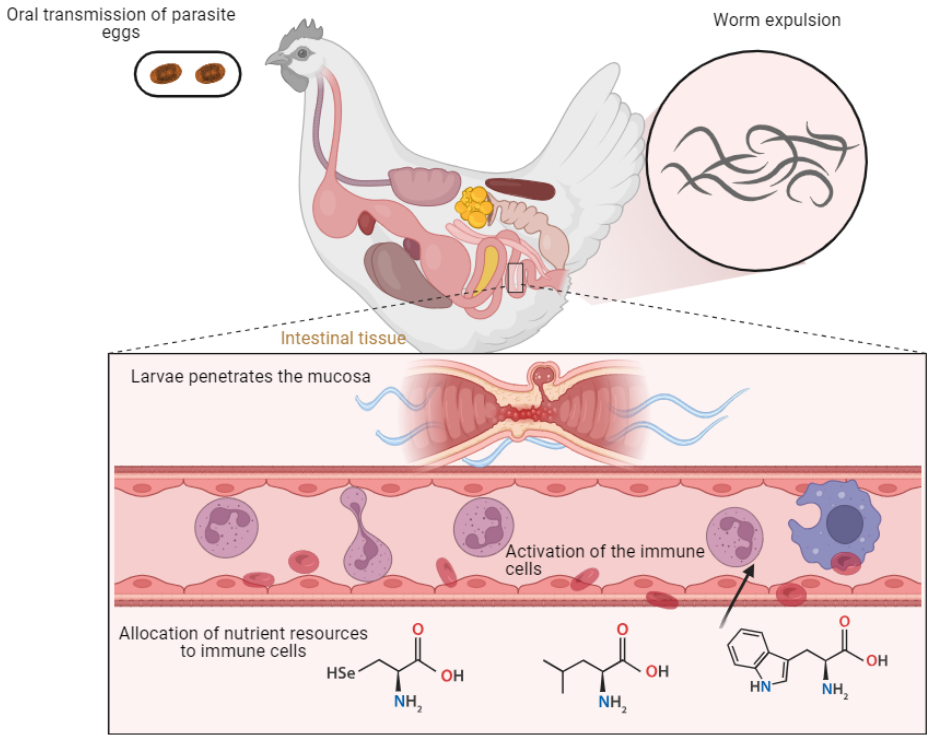

Figure 3 *Illustration of the allocation of nutrient resources for the activation of immune response during nematode infections. Image was created with biorender.com*

To further demonstrate that defence against infection is costly, it has been shown that the immune activation following the induction of lipopolysaccharides (LPS) increases the energy required for maintenance in nursery pigs (Huntley et al., 2017; Kvidera et al., 2017). This is potentially due to the shift of metabolism from oxidative phosphorylation to aerobic glycolysis to enhance the distribution of glucose for leukocytes demand (Kvidera et al., 2017). The costs and the benefits of mounting immunity have also been reviewed in small ruminants (Greer, 2008). The cost of immunity was reportedly greater than the benefit unless if the animals have sufficient opportunity to recover the nutrients invested during infection. Maintenance of immunity against *Trichostrongylus colubriformis* in sheep required a 16% reduction in nutrient utilization and that led to up to 15% production loss (Greer, 2005, 2008).

The production cycle in chicken is significantly shorter to allow such adequate recovery of metabolic loss as a consequence of defence against nematode infection. In fact, the benefits of the costly defence to the mild effects of ascarids infection in chicken is not well justified. Perhaps the most obvious benefit of the mounting an immune response against helminth in chicken is the rapid expulsion of worms (Stehr et al., 2018). However, elevation in antibody—a common immune response to ascarids infection does not correspond to lower worm burden in chicken (Rahimian et al., 2017; Daş et al., 2018). Moreover, even though infected birds could expel most of the worms, birds can be re-infected with higher worm burdens in the long-term questioning the effectiveness of the costly worm expulsion in chicken (Stehr et al., 2018).

These studies, taken together showed that initiating a defence response to nematode infection in poultry and other livestock species is indeed costly. We know at least—given the differences between high and low performing chickens—that host performance level is partly responsible for the alteration of chicken metabolome during nematode infection. But the exact mechanism by which parasite interacts with the host, particularly high performing host to alter the metabolome is not known in chicken. Studies in ascarid infections in chicken have focused on such interaction like modulation of the Th2-coordinated immunity including cytokines and antibody release (Norup et al., 2013; Pleidrup et al., 2014; Dalgaard et al., 2015). Studies in alteration of the metabolome can provide a biochemical signature and the physiological state of the chicken during infection (Whitman et al., 2021). Investigating the ways whereby parasites induce changes in the host metabolome can further our understanding of host-parasite interaction in chicken beyond our current knowledge. Based on the research investigating parasites interaction with the host from other species, it can be inferred that intestinal helminth can alter host metabolome through alteration of the gut microbiome, secretion of excretory/secretory products and uptake of nutrients from the intestine (Midha et al., 2021; Miller et al., 2019; White et al., 2018).

2.3.1 Parasites alter the gut microbiome

Numerous microbes of different genera populate the gut of poultry where they interact with epithelium and feed (Pan and Yu, 2014). Through their fermentative competencies, they can release short-chain fatty acids such as propionate, acetate, and butyrate. The microbes also encode enzymes that enable them to play roles in the metabolism of tyrosine, tryptophan, and phenylalanine (Aggrey et al., 2019).

Nematodes can directly interact with the gut microbiome (Midha et al., 2021). Although the mechanisms of interaction are somewhat obscure, there are different accounts available in the literature. On the one hand, it is thought that gut microbiome can be needed to enhance the establishment and survival of parasites in the gut as demonstrated in mice infected with *Trichuris muris* (White et al., 2018). After the first infection, *T. muris* were able to acquire distinct gut microbiota from their host even when embryonated eggs contained no microbes. The alteration in the microbiome created a less favourable intestinal environment for the establishment of a second *T. muris* infection, suggesting that a favourable intestinal microbiota might be required for the survival of *T. muris* (White et al., 2018).

On the other hand, helminths including ascarids penetrate rapidly into the mucosal of host organisms where they must also compete for nutrients with microbiota. They produce excretory-secretory molecules with antimicrobial properties that can inhibit the activities of gut

bacteria permitting successful competition for nutrients (Midha et al., 2018; Rausch et al., 2018). As a result, helminth infections are linked to a reduction in the abundance and consistency of gut microbial species (Paerewijck et al., 2015).

Given the role of the microbiome in nutrient fermentation and nutrient absorption, it is logical to assume that alteration of the microbiome diversity will indirectly alter the composition of essential metabolites in the gut. In chickens infected with *Eimeria acervulina*, microbial associated metabolites were significantly altered at different time points indicating that parasitic infection may have modified gut microbial composition in chicken consequently altering their metabolome (Aggrey et al., 2019). The microbiome associated metabolites that were altered in infected chicken include histidine products (imidazole lactate and imidazole propionate), phenylalanine and tryptophan with distinct metabolic signatures associated with inflammation and oxidative stress chemicals. In the same study, infection significantly decreased levels of butyrate—a product of microbial fermentation (Aggrey et al., 2019).

In another related study, pigs infected with *A. suum* had reduced microbial activities in the gut with corresponding shift in the metabolic capability of the microbiome in the colon (Wang et al., 2019). They reported alterations in up to 30 metabolic pathways including amino acid and carbohydrate metabolism associated with microbial diversity.

Alterations in the metabolites due to compositional changes of the microbiota induced by parasites could negatively affect the quality of the cellular and humoral response to nematode infection which also depends on rich metabolites (Williams et al., 2021). The consequences of which could amplify susceptibility to other pathogenic infections. Alteration in the intestinal metabolome induced by enteric *Heligmosomoides polygyrus* infection enhanced co-infection with salmonella (Reynolds et al., 2017). They revealed that the metabolites that were required to mask the expression of virulence genes in bacteria have been downregulated in the nematode-infected host. Therefore, salmonella could induce infection in the absence of such metabolites (Reynolds et al., 2017).

2.3.2 Parasites secrete excretory-secretory products

Parasites secret excretory-secretory products (ESPs) that may interact with the host immune cells to encourage their survival and establish infections. These ESPs can be an exogenous source of metabolites to the host that enhances the migration of parasites to penetrate the host tissue (Yeshi et al., 2020). The release of such molecules could change the composition and structure of intestinal layers and can also interact directly with the host intestinal tissues

(Williams et al., 2021). However, the specific roles of these parasite-derived metabolites have inadequate attention so far.

ESPs of parasites contain an excess of biomolecules such as proteins, lipids and peptides that have pharmacological functions (Wangchuk et al., 2019). Arora et al., (2020) showed that compounds in the pseudocoelomic fluid from *A. suum* suppress the induction of Th1 and Th17 responses by down-regulating genes in the TL4 signaling pathways.

2.3.3 Parasites depend on their host for nutrient

Parasites' survival also depends on the host-derived biosynthetic pathways as they may lack some necessary metabolic pathways to synthesise amino acids, fatty acids and nucleosides (Wu et al., 2021). For instance, cryptosporidium has incomplete gluconeogenesis pathways and must therefore depend on the host for acquisition of sugar as its source of energy. Analysis of the glycolysis/gluconeogenesis pathways revealed that host genes with no homolog in the parasite genome were upregulated while those genes with homologs in the parasite genome were downregulated (Xu et al., 2010). As reported recently (Miller et al., 2019), *C. parvum* and *C. hominis* influenced the host's biosynthesis pathway of various amino acids, ketones and Coenzyme A. Many of the products in the pathway were significantly altered during infection including glycine, valine adenosine derivatives (ATP, ADP) creatine and taurine (Miller et al., 2019).

Although the exact mechanisms are complex and not well elucidated, in Miller and colleague's report, it was suggested that parasites can derive creatine from their host by directly or indirectly inducing biosynthetic pathways such as host mitochondrial activities because creatine and also taurine are very closely associated with mitochondrial activities during infection (Miller et al., 2019). A later study has also shown that metabolic pathways for purine and amino acid metabolism including valine, glycine, aspartate, glutamate, serine alanine and threonine metabolism are missing in *C. parvum* (Xu et al., 2010). An incomplete or missing metabolic pathway is a common feature of many helminths likely to be a result of genomic evolution. The reason being that pathogenic organisms may have lost genes associated with the biosynthesis pathway while extending their dependencies on the host for nutrients (McCutcheon and Moran, 2007).

Finally, pathogens could be facing a resource allocation trade between virulence and their proliferation activities inside the host as their survival depends on the ability to take in nutrients from the host cell, thus they interfere with the host cell metabolism to re-orientate metabolic resources for their purpose (Peyraud et al., 2016).

2.4 Role of nutrient metabolites in immune development

The nutrients required by livestock species for the development of various non-immune functions including growth and reproduction has been well described. However, the intricate link between nutrition and immunology remains convoluted. Many studies investigating nutrients and immunity majorly report that deficiency in certain nutrients impairs the host ability to respond to infection (Klasing, 2007). While this is informative, it does not provide a satisfactory understanding of the role of each specific nutrient metabolite required for different immune functions. Even when there is no reduction in feed intake and feed efficiency during infection, there has been a noticeable reduction in the immune response. In the absence of protein deficiency but limited access to energy, nematode infected mice did not reduce worm burden, instead, there was a reduction in lymphocyte proliferation, Th2 cytokine production, IgE, parasite specific IgG1 and eosinophils (Koski et al., 1999). This implies that deficiency or availability of nutrients is not sufficient to understand what exactly is needed for immune development.

All cells require appropriate metabolites inadequate amount for optimal functioning. These demands can be provided via exogenous sources mainly derived from diet, gut microbiome, medications and the environment or endogenous sources through enzymatic activities as encoded in the genome (Childs et al., 2019). Metabolites from diets can be transported for immune cell functioning, however, in the case of nutrient deficiency, they can also be mobilised from body reserves (Childs et al., 2019). During infection, the immune system becomes activated and requires greater energy expenditure (Klasing, 2007). As suggested by Klasing (2007), it is important to understand the specific nutrient metabolites required by the immune system to function, the stage of the infection where their requirement increase and how well the immune cells compete for nutrients against non-immune cells. Answering these questions can further our understanding of the interaction between host and parasites as well as the metabolic trade-off that may exist between immune functioning and performance. Therefore, the complexity in the mechanism of interaction between pathogens, metabolites and immune cells requires further investigation. Here, we summarize the findings from different studies that have reported the specific role of nutrient metabolites in activating/developing immune functions across different species.

The essential nutrients for the immune system appear to be amino glucose, acids, fatty acids, and vitamins D (Newsholme, 2021). The increase in nutrients needed for immune function during infection may be due to the elevation in the livers' production of protective accessory proteins—a process that requires lysine and cysteine in greater quantity (Klasing, 2007). Similarly, arginine is needed as a substrate for generating nitric oxide—(a cytotoxic

immunoregulatory mediator) by the macrophage. The macrophage can also be induced by vitamins D to synthesize antimicrobial peptides including cathelicidin (Newsholme, 2021) While vitamins A and zinc regulates cell division and are essential for a proliferative response within the immune system (Childs et al., 2019). Glutamine also influences the proliferation of immune cells, tissue repair and recognition of pathogens. This is because glutamine; activates transcription factors, are needed as an energy substrate for leukocytes and are particularly required for the expression of lymphocytes and the production of cytokines including IFN-y, TNF-a and IL-6 (Curi et al., 2005).

2.5 Methods of assessing nematode infections in chicken host

An accurate and precise method, which can detect early infection, and is non-invasive, simple to carry out is crucial for reducing the spread of infections and reducing their impacts on host animal health and welfare. Diagnostic accuracy refers to the degree of proximity between a test's measurement and the actual value of a given sample while precision is a measure of discrepancy between repeated measures on the same sample (Nielsen, 2021). In the context of nematode infection, accuracy signifies how closely a determined measurement aligns with the true count of worms in a specific sample and precision indicates how reliable this the measurements can repeatedly give true outcome.

Accurate, precise, and early detection can also help us to offer selective therapeutic by selecting the group of animals that particularly needs deworming or treatment. Consequently, reducing the use of anthelminthics and also reduce production losses. The gold standard for quantifying infection intensity in experimental infection rely on actual worm burden whereby visible adult worms from the cecum and intestine (duodenum, jejunum, ileum) are counted manually (Nielsen, 2021). To do this, intestine content is rinsed through a proper metal sieve and counted by a magnified microscope (Daş et al., 2017). Indirect assessment of nematode infections in chicken is currently based on FEC (Pouillevet et al., 2017; Nielsen, 2021; Shifaw et al., 2021b), detection of anti-parasite antibodies (IgY) in egg yolk and in the serum (Norup et al., 2013; Daş et al., 2017; Sharma et al., 2018b).

2.5.1 Faecal egg counting techniques

Methods of faecal egg counting are established coproscopical methods for indirect assessment of parasite infection in poultry based on quantifying nematode eggs in the faeces. Two prominent techniques, McMaster and Mini-FLOTAC, have been extensively employed and modified over time. The choice between different techniques becomes pivotal given the varying accuracy, sensitivity, and precision of these methods (Shifaw et al., 2021b). The McMaster method developed nearly 100 years ago (Gordon and Whitlock, 1939) remains the

most widely used technique for parasite egg counting (Daş et al., 2020). Numerous modifications have been introduced, impacting factors like flotation solution, density, excreta weight, flotation time, centrifugation, counting chambers, and multiplication factors (Shifaw et al., 2021b). While the McMaster technique is criticized for high variability and poor sensitivity when egg counts is low, it remains popular due to its simplicity, equipment reusability, cost-effectiveness, and short processing time. In contrast, the FLOTAC, is an alternative with improved sensitivity, accuracy, and precision (Cringoli et al., 2010). FLOTAC incorporates a centrifugal flotation system, but some drawbacks is that it is slightly more complex and requires special centrifuge device. The Mini-FLOTAC, a simplified version, eliminates the need for centrifugation but extends the processing time compared to McMaster (Cringoli et al., 2017). Mini-FLOTAC has demonstrated higher sensitivity, accuracy, and precision in various mammalian host-parasite studies, presented as a more user-friendly option. However, its slightly longer processing time may be a consideration. In summary, the choice between McMaster and Mini-FLOTAC depends on the specific requirements and priorities of the diagnostic task, considering factors such as simplicity, cost, and processing time.

Faecal egg counting techniques are non-invasive and can be easily implemented in field conditions. However, FEC methods can be laborious, time-consuming and have low sensitivity (Johnson et al., 1996). Host resistance, variation in worms' fecundity, diurnal fluctuations in eggs shed, uneven distribution of worm eggs in faeces amongst others, can limit the consistency of egg counts for helminth diagnosis (Wongrak et al., 2015; Daş et al., 2017; Daş et al., 2019).

Also, the presence of matured female worms is unavoidably required in the host for egg shedding. Shedding of eggs in faeces takes about 4-8 weeks post-infection as this represents the time required for *A. galli* worms to mature. As a result, *A. galli* eggs are sometimes not detectable in faeces until 8 weeks post-infection (wpi) making it difficult to detect infection in broilers birds with a shorter production cycle (Ruhnke et al., 2017). In addition, Daş et al., (2017) observed no correlation between worm burden (infection intensity) and FEC, suggesting that FEC may be inadequate in some cases to quantify worm burden for *A. galli* even if based on well-mixed 24 h faecal samples.

2.5.2 Plasma and egg yolk antibody ELISA

Infection proxy such as antibody quantification using ELISA assay is also an increasingly preferred method of quantifying nematode infection in poultry (Sharma et al., 2018b). Antibodies can be identified in the serum and egg yolk within the first two weeks after infection. Greater than 80% of *A. galli* infected birds were already correctly categorized as infected by 2

wpi using antibody ELISA indicating that the assay is suitable for early diagnosis of nematode infections (Daş et al., 2017).

A disadvantage of using serum antibodies is the need for trained technicians or veterinarians to bleed the chickens to obtain plasma. Moreover, bleeding animals is an invasive procedure that is not animal welfare friendly. Conversely, quantifying antibodies from egg yolk is noninvasive and relatively easy to assess but can only be assessed in laying hens (Daş et al., 2017). In practice, FEC appears to provide better quantitative information than antibody ELISA, given its higher correlation with infection intensity albeit insignificant (Daş et al., 2017).

ELISA antibody test (plasma and egg yolk) correlated positively with worm burden (i.e infection intensities) (Daş et al., 2017). Nevertheless, antibody ELISA assay using both serum and egg yolk detects mainly the magnitude of animals' response to infection rather than the intensity of infection. The presence of antibodies in yolk and serum does not necessarily signify current infection (Dao et al., 2019).

Quantifying specific worm excretory-secretory (ES) antigen in faeces may however provide detailed information about current infection (Allan et al., 1992). A coproantigen ELISA test showed higher sensitivity compared to antibody ELISA and FEC in assessing *Hepatica fasciola* infection ((Villa-Mancera et al., 2016).

2.5.3 Coproantigen ELISA

In coproantigen analysis, the faecal antigen is captured and tested on a parasite-specific antibody coated on a microtiter plate in an ELISA assay (Johnson et al., 1996). Coproantigen ELISAs have been used for different parasite species including strongyloides, trematodes, haemonchus and teladorsagia (Allan et al., 1992; Johnson et al., 2004; Sykes and McCarthy, 2011; Elsemore et al., 2014).

The usage of specific antibodies raised against ESP derived antigens of the respective parasite offers satisfactory performance than utilising antibodies against the total somatic antigen. Accordingly, cross-reactivity among parasite antigens may be an important challenge when assessing specific parasitic infections using antigens from heterologous parasites. Daş et al., (2017) showed that chickensh does not differentiate between somatic antigens of *A.galli* and *H. gallinarum* and releases similar antibodies indicating cross-reactivity of antigens. In practice, this offers a unique opportunity to quantify two nematode infections of economic importance at the same time.

An advantage of developing a coproantigen ELISA test is that it can be converted into a rapid immunochromatographic dipstick test. It can be used rapidly, is sensitive and also relatively easy to use (Johnson et al., 1996; Sykes and McCarthy, 2011). The coproantigen ELISA test has been described to have a sensitivity of up to 100% for nematode quantification, albeit with low specificity. Nonetheless, it was found to be superior to FEC in the diagnosis of *Capillaria phillippinensis* infections (Khalifa et al., 2020). Lagatie et al., (2020) recently developed a coproantigen based ELISA for the detection of *A. lumbricoides* in humans, the sensitivity was 91% with a specificity of 95%. The authors used ABA-1—a protein associated with ascarids with a molecular weight of 14kDa. Their results showed both qualitative measure and quantitative measure for infection intensity by measuring the nanogram of antigen ABA-1 per gram of faeces. Such a promising result suggests that a coproantigen ELISA test is possible and practical for the assessment of nematode infections in chickens.

3 Publications in peer-reviewed journals

This section presents three peer-reviewed articles, each corresponding to a specific study objective.

The first article describes the experimental procedures conducted to address objective 1 of this thesis. It focuses on the alterations in plasma and liver metabolites during nematode infections, with the aim to comprehend the potential metabolic resources inherent to infection.

In the second article, the development of a coproantigen ELISA for characterizing infections is described. It discusses the methodology and findings associated with this novel diagnostic approach, which provides insights into its specificity and sensitivity.

The third and final paper addresses the last objective of the study, with a particular emphasis on comparing traditional helminth diagnostic systems with the novel coproantigen. Additionally, it explores the patterns and repeatability of antigen excretion in laying hens, shedding light on the temporal dynamics of infection.

3.1 ^{1}H-NMR based-metabolomics reveals alterations in the metabolite profiles of chickens infected with ascarids and concurrent histomonosis Infection

Authors: **Oyekunle John Oladosu**, Banny Silva Barbosa Correia, Beatrice Grafl, Dieter Liebhart, Cornelia C. Metges, Hanne Christine Bertram & Gürbüz Daş

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Contributions:

OJO: Conceptualization, data curation, data analysis, ^{1}H-NMR-Metabolomics, data interpretation, writing—original draft.

BSBC: Methodology, ^{1}H-NMR—Metabolomics, data curation, data analysis, data interpretation, writing—review & editing.

BG: Methodology, serology analysis, writing—review & editing.

DL: Methodology, serology analysis, writing—review & editing.

CCM: Conceptualization, funding, writing—review & editing; HCB: Methodology—interpretation, supervision, writing—review & editing.

GD: Conceptualization, project administration, funding, interpretation, supervision, writing—review & editing. All authors read and approved the final manuscript.

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Gut Pathogens

RESEARCH

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^{1}H-NMR based-metabolomics reveals alterations in the metabolite profiles of chickens infected with ascarids and concurrent histomonosis infection

Oyekunle John Oladosu[1], Banny Silva Barbosa Correia[2], Beatrice Grafl[3], Dieter Liebhart[3], Cornelia C. Metges[1], Hanne Christine Bertram[2] and Gürbüz Daş[1*]

Abstract

Background Gut infections of chickens caused by *Ascaridia galli* and *Heterakis gallinarum* are associated with impaired host performance, particularly in high-performing genotypes. *Heterakis gallinarum* is also a vector of *Histomonas meleagridis* that is often co-involved with ascarid infections. Here, we provide a first insight into the alteration of the chicken plasma and liver metabolome as a result of gastrointestinal nematode infections with concomitant histomonosis. ^{1}H nuclear magnetic resonance (^{1}H-NMR) based-metabolomics coupled with a bioinformatics analysis was applied to explore the variation in the metabolite profiles of the liver (N = 105) and plasma samples from chickens (N = 108) experimentally infected with *A. galli* and *H. gallinarum* (+*H. meleagridis*). This was compared with uninfected chickens at different weeks post-infection (wpi 2, 4, 6, 10, 14, 18) representing different developmental stages of the worms.

Results A total of 31 and 54 metabolites were quantified in plasma and aqueous liver extracts, respectively. Statistical analysis showed no significant differences ($P > 0.05$) in any of the 54 identified liver metabolites between infected and uninfected hens. In contrast, 20 plasma metabolites including, amino acids, sugars, and organic acids showed significantly elevated concentrations in the infected hens ($P < 0.05$). Alterations of plasma metabolites occurred particularly in wpi 2, 6 and 10, covering the pre-patent period of worm infections. Plasma metabolites with the highest variation at these time points included glutamate, succinate, trimethylamine-*N*-oxide, myo-inositol, and acetate. Differential pathway analysis suggested that infection induced changes in (1) phenylalanine, tyrosine, and tryptophan metabolism, (2) alanine, aspartate and glutamate metabolism; and 3) arginine and proline metabolism (Pathway impact > 0.1 with FDR adjusted P-value < 0.05).

Conclusion In conclusion, ^{1}H-NMR based-metabolomics revealed significant alterations in the plasma metabolome of high performing chickens infected with gut pathogens—*A. galli* and *H. gallinarum*. The alterations suggested upregulation of key metabolic pathways mainly during the patency of infections. This approach extends our understanding of host interactions with gastrointestinal nematodes at the metabolic level.

Keywords Blackhead disease, Gastrointestinal infections, Helminths, Infection-induced metabolite alterations, Liver, Metabolome, Plasma, Poultry, Spectroscopy

*Correspondence:
Gürbüz Daş
gdas@fbn-dummerstorf.de
Full list of author information is available at the end of the article

Background

Infection with gastrointestinal nematode species including *Ascaridia galli* and *Heterakis gallinarum* in chickens is of importance, particularly since these species are re-emerging across Europe due to non-cage housing systems that ease completion of parasite life cycle [1]. The direct effects triggered by these species are associated with impaired host performance [1–3]. These effects are manifested through reduced feed intake, reduced nutrient absorption, eventually leading to potential economic losses [3–6]. Infected birds initiate a rapid immune response within the first weeks of infection through the production of antibodies and activation of the cell-mediated responses that are associated with an effective expulsion of worms [7, 8]. Infection effects are likely dependent on the performance level of the host, such that the performance (e.g., per capita egg mass) of high-performing layer genotypes is considerably penalized during the period of exerting a strong immune response against mixed-nematode infection [3]. Thus, a possible trade-off in the utilisation of metabolic resources required to maintain performance level while developing an effective immune response can be anticipated in nematode-infected chickens.

The reduction in the host's feed intake [3] and subsequent body weight loss [9] are indicative of the potential changes in the physiology of infected animals. Presently, investigations on the underlying pathophysiological changes induced by gastrointestinal nematode infections in avian species are limited. Studies have focused on such interactions as the activation of the T helper 2 (Th2) immune response including cytokines production [8, 10–12]. However, gut parasites may also interact with the host in other ways, e.g., an interaction between ascarids and endogenous metabolism could be hypothesized. As nematode infections impair host animal performance, the induction of immune responses to expel worms may suggest an interchange in the allocation of metabolic resources between immune function and performance traits [13]. Thus, identifying infection-related alterations in metabolism over time may improve our understanding of host–pathogen interactions and provide insight into potential physiological trade-offs.

Both *A. galli* and *H. gallinarum* often co-infect their common chicken host and have similar direct life cycles, whereby chickens ingest embryonated eggs containing the second or third larval stage from the litter, soil, or contaminated feed and water. The eggs are then hatched in the small intestine within 24 h of ingestion [14, 15]. The larvae of *A. galli* migrate into the superficial mucosal layer of the host small intestine [16] while *H. gallinarum* larvae migrate and develop in the cecum, although with an associated but short tissue phase [17]. Both parasites mature in the lumen of the small intestine and cecum respectively and have a pre-patent period (i.e., time from egg ingestion to the laying of first eggs by adult worms) of between approximately 4–8 weeks [18, 19] after which re-infection can occur, likely earlier with *H. gallinarum*.

Heterakis gallinarum is known to be the main vector for the protozoon *Histomonas meleagridis* by harbouring the eggs. Thus, *Heterakis*-infected hens may additionally be exposed to *H. meleagridis,* which can cause severe liver injury to their host [20, 21]. Because of the central role of the liver in metabolism of nutrients and detoxification [22, 23], gastrointestinal nematode infections may further influence liver functions, particularly when histomonosis (syn. blackhead disease) is co-involved in the mixed infections. Depending on the developmental stages of the parasites, they can influence the trade-off between performance and immune defence by altering the metabolism of the host [24–28].

^{1}H nuclear magnetic resonance (^{1}H-NMR) spectroscopy is a high-throughput analytical method that can be coupled with multivariate analysis to investigate the metabolome of hosts through either biofluids or tissue examination [29]. It could lead to the development of infection signatures through the discovery of novel proxies/biomarkers and, thus enhancing integrated control strategies to improve chicken health and productivity. Given this potential, we hypothesise that the use of ^{1}H-NMR based-metabolomics can reveal alterations in the metabolite profile of chickens that may be representative of the trade-off between performance and immune function during mixed nematode infections. In this study, we explore the infection-induced changes in a high-performing commercial line of laying hens. We applied the ^{1}H-NMR spectroscopy technique to study the changes in the liver and plasma metabolome at different periods of ascarid infection representing different developmental stages of the worms.

Results

Confirmation of ascarid infection in chickens

Infection with both ascarids was confirmed based on the recovery of larval and mature stages of both *A. galli* and *H. gallinarum* from the small intestine and cecum of hens necropsied at different time points after infection with embryonated eggs (Fig. 1). All experimentally infected hens harboured worms, whereas no worms were recovered from the non-infected hens, confirming infection-free status of these control hens.

Involvement of histomonosis in the infections

One of the infected hens necropsied at 6th week post-infection (wpi) showed the classical target-like liver lesion associated with histomonosis. Also, the plasma

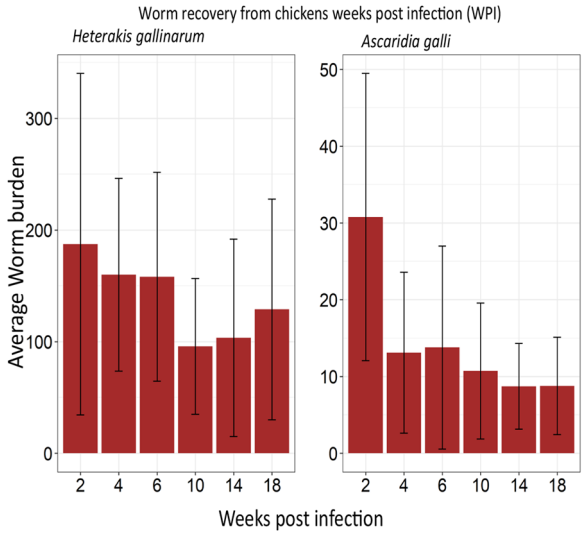

Fig. 1 Average worm burdens of hens with *Heterakis gallinarum* and *Ascaridia galli* at different time points. Error bars indicate standard deviation

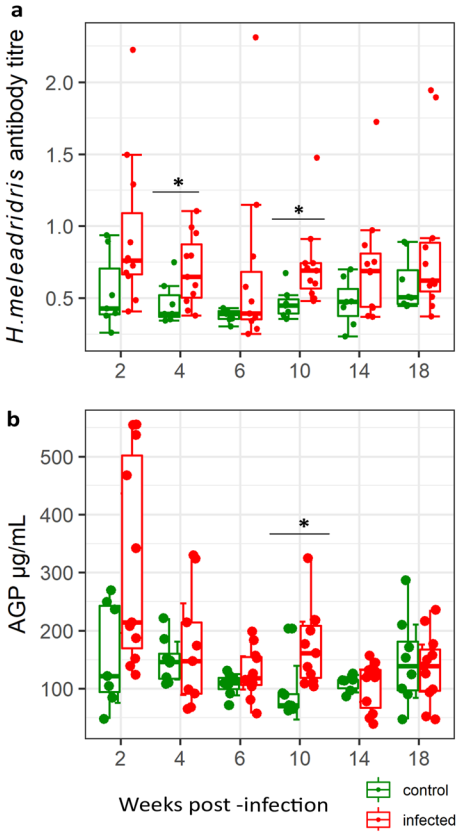

Fig. 2 Plasma antibody titres against *Histomonas meleagridis* (**a**) and concentration of plasma alpha (1)-acid glycoprotein (AGP) (**b**) in uninfected (green dots) and infected (red dots) hens throughout experimental weeks. Each dot in the boxplot represents an individual hen. The vertical line inside the boxplots shows the sample median, while the lower and upper end of the box represents the 25th and 75th quantiles respectively. Significant ($P < 0.05$) differences are marked with (*)

H. meleagridis antibody titres were quantified in detail from wpi 2 until the end of the experiment to monitor the co-infection with *H. meleagridis.* About 65% of all the ascarid infected hens were above the predetermined cut-off to be considered positive for histomonosis infection. Moreover, anti-histomonas antibody titre was significantly ($P < 0.05$) increased in wpi 4 and 10 in infected hens (Fig. 2a), suggesting the presence of *H. meleagridis* in the mixed infections. The concentration of alpha (1)-acid-glycoprotein (AGP) in plasma was also quantified to further determine signs of possible tissue inflammation due to infection. The result showed that the effect of infection was significant ($P < 0.05$) for the level of AGP at wpi 10 (Fig. 2b). There were no differences in the level of both anti-*H. meleagridis* antibody titres and AGP concentration between infected and uninfected control hens after wpi 10 ($P > 0.05$).

Infection-induced alterations in liver and plasma metabolites profiles

A total of 57 individual metabolites were identified by the Chenomx database in both plasma and liver. In plasma, 31 of the metabolites were present (Additional file 1: Table S1), whereas 54 metabolites were found in the liver samples (Additional file 1: Table S2). The metabolite identifications were verified by the match factor of the NMR spectral signals given by Chenomx software, and by assignment of the signals of 2D spectra. Examples of these assignment details are shown in supporting information (Additional file 4: Figure S2–S10). Univariate analysis with student *t*-test and fold change analysis across the six wpi time-points was used to explore infection-induced changes in the concentration of liver and plasma metabolites. None of the 54 metabolites identified in the liver samples showed significant differences between the infected and uninfected-control groups (Additional file 2: Table S2). However, as shown in Fig. 3, 20 plasma metabolites had significantly (FDR adjusted $P < 0.05$) higher concentrations with fold change (FC) > 1 (Additional file 1: Table S1) in infected samples than samples of the control group. The metabolites glutamate (FC = 1.747), succinate (FC = 1.743), trimethylamine N-oxide (FC = 1.432) and alanine (FC = 1.242) were among the most altered metabolites.

Time-dependent changes in plasma metabolite concentration due to infection

One-way-ANOVA and volcano plot analysis were further performed within each wpi to identify the most

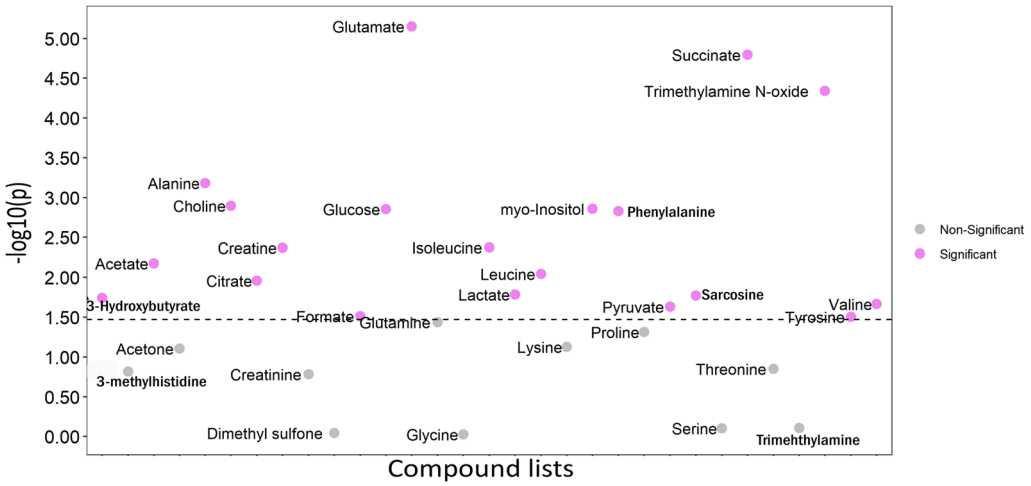

Fig. 3 Results of student t-test of plasma metabolite concentrations in nematode infected hens across all wpis. Scatterplot showing compounds selected by two-sample t-tests with an FDR-adjusted *p*-value threshold of 0.05. Pink dots show significant metabolites while the grey dots show non-significant metabolites. Y axis represents the (−)log10 *p*-value

important plasma metabolites discriminating infected and non-infected hens at different time points (Fig. 4 & Additional file 3: Figure S1). The analysis identified key time points with significant elevation of specific metabolites in infected hens as compared to non-infected group. Thus, results showed infection-induced changes in plasma metabolites in wpi 2, 6 and 10, while no differences were observed in the concentration of plasma metabolites in wpi 4, 14 and wpi 18. Additional file 3: Figure S1 shows significant differences of metabolite concentration between infected and control groups in wpi 2, 6 and 10. Selected metabolites with significant differences between infected and control hens in at least two time points are visualised in Fig. 4. Citrate, glutamate, succinate, creatinine, leucine, isoleucine, myo-inositol, sarcosine and acetate were the metabolites showing the largest changes in infected samples in wpi 2. In wpi 6, trimethylamine *N*-oxide (TMAO), myo-inositol, creatinine, choline, valine, phenylalanine, acetate, alanine, pyruvate, isoleucine, and succinate were the most altered metabolites. The metabolites most altered in wpi 10 included glutamate, TMAO, 3-hydroxybutyrate, pyruvate, and glucose. At all the time points, metabolites showed significantly higher concentrations in infected compared with non-infected hens. No differences were observed in the concentration of plasma metabolites in wpi 4, 14 and wpi 18.

The hierarchical clustering (Additional file 5: Figure S11) further suggested infection-induced considerable alterations of metabolites at time points up to wpi 10. The pattern of the top 15 significant (based on *t*-test) plasma metabolites within each wpi is presented in Additional file 5: Figure S11.

Differential alteration of metabolic pathways induced by mixed nematode infection

Figure 5 shows the results of metabolic pathway analysis of significant plasma metabolites. Thirty-three metabolic pathways were found to be involved in the differentiation of control and infected hens. Sixteen pathways had impact values > 0, nine of which had a pathway impact > 0.1 with *P*-value < 0.05 (FDR adjusted). The relevant pathways included (1) Phenylalanine, tyrosine, and tryptophan metabolism; (2) D-Glutamine and D-glutamate metabolism; (3) Phenylalanine metabolism; (4) Alanine, aspartate and glutamate metabolism; (5) Pyruvate metabolism; (6) Arginine and proline metabolism; (7) Tricarboxylic acid cycle; (8) Tyrosine metabolism; (9) Glycolysis/Gluconeogenesis. All 9 pathways were found to contain metabolites that are mainly involved in amino acid and energy metabolism, and which were significantly altered by infection. Three of the identified metabolites including pyruvate, lactate, and glucose were involved in the glycolysis/gluconeogenesis pathway. Similarly, for the alanine, aspartate, glutamate metabolism, the metabolites involved (alanine, citrate, glutamate, succinate, pyruvate, and glutamine) were significantly elevated due to infection. A significant alteration of the citrate cycle was indicated by the infection-induced increase in the concentration of pyruvate, glutamate, alanine, succinate, and citrate. Pathway analysis of significant metabolites across all the weeks post-infections is shown in Additional file 2: Table S3.

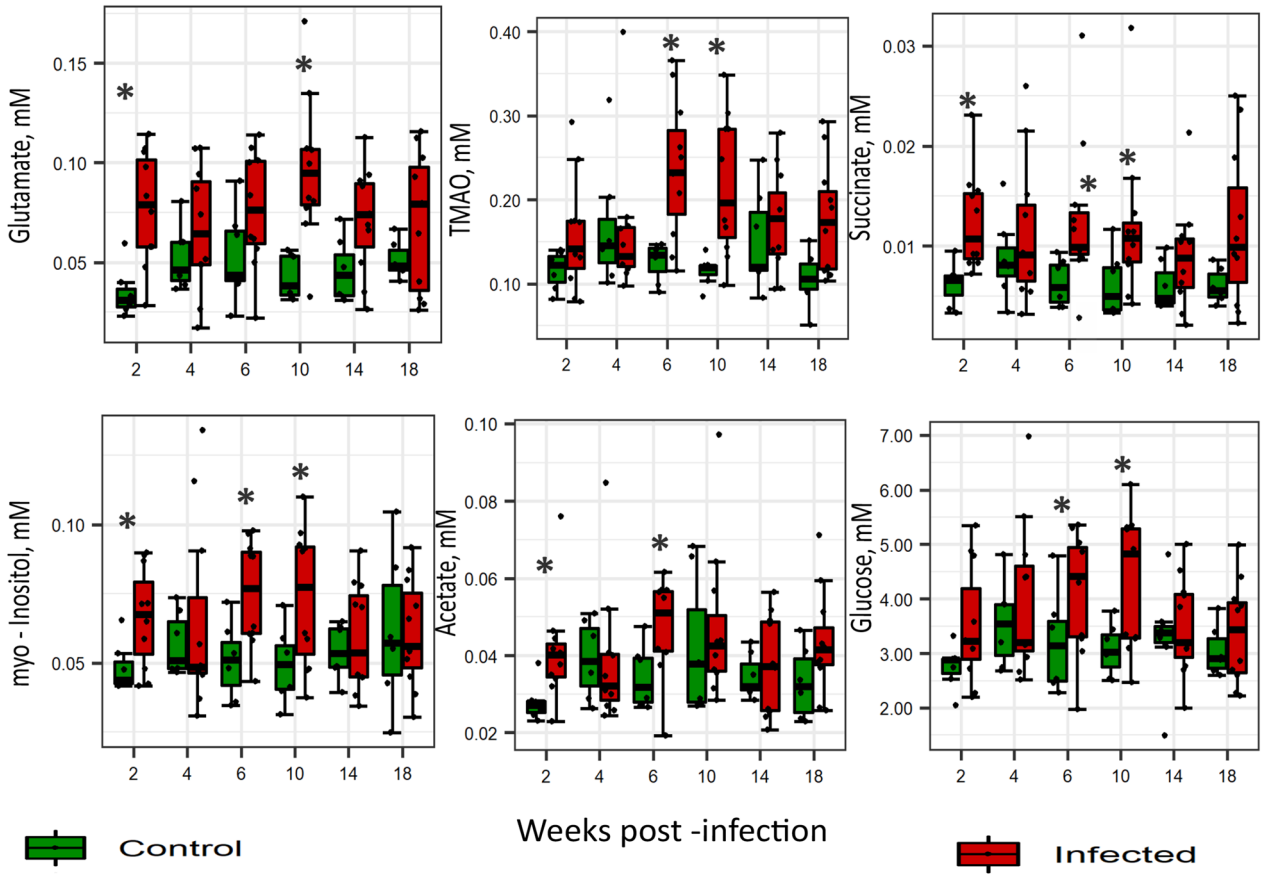

Fig. 4 Univariate analysis of selected metabolites showing time—dependent difference between infected (red dots) and control group (green dots). Each dot on the boxplot represents an individual hen. The vertical line inside the boxplots shows the sample median, while the lower and upper end of the box represents the 25th and 75th quantiles respectively. Significant ($P < 0.05$) differences are marked with (*)

Discussion

This study provides insight into the metabolic alterations that occur in chickens infected with mixed ascarid species. The results presented here show the metabolomic changes induced by mixed infections with two of the most common gastrointestinal nematode species, namely *A. galli* and *H. gallinarum*, as well as concurrent histomonosis infection in a high-performing laying hen genotype. The changes in the plasma metabolites quantified at different time points during infection are an indication of the host-responses occurring at the different developmental stages of worms, particularly during larval penetration of host mucosa (2 wpi), shedding of eggs (>4 wpi), patency (wpi 4–10) and re-infection (wpi > 10).

We also found that the infection-induced metabolic changes are manifested to a greater extent in the plasma metabolome than the liver metabolome, likely indicating that the plasma metabolome is a better indicator of ascarid infection in chickens. The plasma metabolome showed clear and significant differences in the metabolite profiles of the infected and control groups. Amino acids and sugars were the most strongly altered metabolites and these metabolites were significantly increased in infected hens. One possible reason that could be attributed to the increase of plasma amino acid concentration in infected hens is discrepancy between endogenous protein secretion and re-absorption in the small intestine. Previous studies identified that infection with *A. galli* in chickens was resulted in a large net secretion of nitrogen with a reduced apparent protein absorption in the duodenum of infected chicks [4]. Similarly, reduced activities of proteolytic enzymes (chymotrypsin and trypsin) were observed in the jejunum of ascarid infected chicks [30]. Although, the authors also reported a later reabsorption of protein in the jejunum which may compensate for the losses in the duodenum. Their studies were performed at one time point when worms were at adult stage, hence could not capture the complex dynamics of infection involving both larvae and matured worms. Another possible reason for the increased plasma amino acid concentrations could be the elevated liver protein turnover associated with the acute phase protein response, which

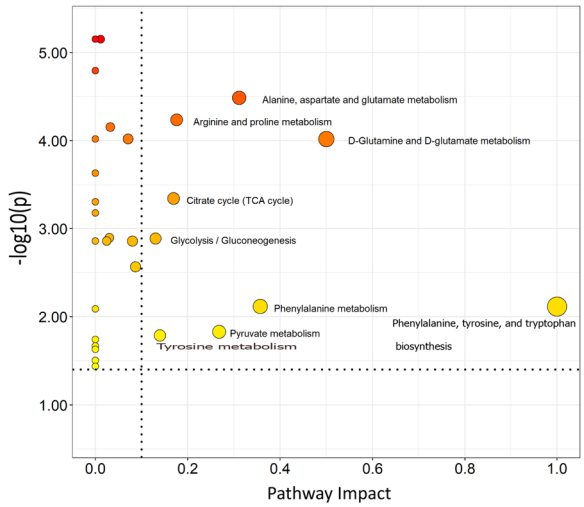

Fig. 5 Pathway map. Shows the pathway map of significant metabolites in plasma of laying hens infected with mixed *Ascaridia galli* and *Heterakis gallinarum,* named according to the Additional file 1: Table S1. Size of cycle corresponds to pathway impact with greater size representing greater pathway impact. The darker the colour the greater the −log10(p) values. Pathways with pathway impact > 0.1 and FDR adjusted P-value < 0.05 are labelled and are considered relevant

occurs as a consequence of infection [31]. Nevertheless, the mechanism of alteration in protein metabolism induced by nematode infections remains elusive and warrants further investigations.

Amino acids are the building blocks for proteins underlying poultry growth and egg formation [32]. A differential upregulation of crucial amino acid pathways such as arginine and proline metabolism and alanine, aspartate, glutamate metabolism was detected in association with the mixed nematode infection. The availability of arginine in the host influences the regulation of the host's defence mechanism by supporting the activation of T cells and macrophages. Arginine and proline play vital roles in collagen synthesis, contributing to tissue repair and enhancing the function of the intestinal mucosa [33, 34]. Therefore, the upregulation of arginine and proline metabolic pathway may have been a metabolic response to the intestinal inflammation associated with gut infections. Mon et al., 2020 observed such upregulation of arginine and proline metabolism as the most significantly enriched pathway during *Salmonella enteritidis* infection in chickens [35]. They postulated that this upregulation was possibly a metabolic response to the intestinal inflammation caused by *Salmonella* infection.

The plasma glucose concentration remained relatively stable during the initial infection of the hens with mixed nematode species as there were no significant differences between the infected and uninfected groups. However, after a period of re-infection (i.e., 10 wpi), the infected groups exhibited a significantly higher blood glucose level in the plasma. This suggests a potential stress response that influence glucose concentration even though chickens are known to maintain relatively stable blood glucose level [36]. Similar observation was made in chickens infected with *Eimeria acervulina*, where the serum glucose levels were unchanged between infected and uninfected chickens [37]. Collectively, the present findings indicate no conclusive effects of nematode infection on circulating plasma glucose levels.

Another notable alteration due to ascarid infection was the significant increase in the level of plasma TMAO, although it did not seem to contribute to the regulation of any biosynthetic pathways. In mammals, TMAO is formed through liver oxidation of trimethylamine (TMA) by flavin monooxygenase enzyme, and TMA is a product of gut microbial metabolism of compounds such as choline and carnitine [38, 39]. The increased concentrations of both choline and TMAO in this study aligns with this understanding. As TMAO formation is related to both gut microbiota and diet, it has also been shown to be associated with metabolic disorders, systemic inflammation, and cardiovascular disease [39]. In broilers, woody breast myopathy was found to be associated with higher levels of plasma TMAO [40]. Although the role of TMAO in poultry health and diseases is not well understood, it might provide a potential proxy for the identification of parasite-infected individuals. Nonetheless, further studies are necessary to establish the role of TMAO in poultry health.

Other metabolites including sarcosine, creatine, isoleucine, leucine 3- hydroxybutyrate and myo-inositol were also significantly elevated in the plasma due to the mixed infection. Overall, these significant increases reflect considerable metabolic changes that likely indicate adaptation of host metabolism to support physiological demand during gastrointestinal infection. For example, myo-inositol has been associated with immune barrier functions and its deficiency in young grass carp decreases the intestinal immune functions [41]. Whether or not similar role is achieved in poultry remains to be investigated. Similarly, branched chain amino acids play a crucial role in supporting the effective functioning of both the innate and adaptive immune systems and ensuring the integrity of the intestinal mucosa [42]. Given that a robust adaptive immune activation is crucial for an effective worm expulsion [7], we postulate that drastic changes in the branched chain amino acids is likely a metabolic response to the activation of adaptive immune system but, the specific mechanisms and implications of these plasma metabolic alterations in the context of poultry gut infections have not been fully described.

Although a higher number of metabolites could be identified and quantified applying ^{1}H-NMR spectroscopy on aqueous liver extracts than on plasma samples, none of the metabolites measured in the liver indicated infection-induced differences. The reason for this lack of changes is elusive. The liver is understandably not the main site of infection for both *A. galli* and *H. gallinarum* but given that liver is a highly metabolic active organ [22, 43] it was hypothesised that liver metabolome could reflect infection-induced changes in host metabolism. The liver metabolome was also studied to further understand concurrent histomonosis infection with ascarid infection. *Heterakis gallinarum* infections are known to predispose their hosts to *H. meleagridis*—a protozoon that potentially causes liver damage in poultry, although with moderate effects in chickens compared to turkeys [44, 45]. Thus, the ascarid-infected hens were additionally investigated for concurrent infection with *H. meleagridis.* One of the hens necropsied in wpi 6 indeed showed typical histomonosis lesion on the liver, otherwise there was no indication of severe liver damage for all hens during post-mortem examination. The antibody against *H. meleagridis* was significantly higher in infected birds in certain wpi (4, 10) and 65% of the infected hens were tested positive for histomonosis based on the cut-off of the serology test [52]. These findings suggest the involvement of concurrent infection with *H. meleagridis.* To gain more insight into the consequences of the mixed infection on liver inflammation, we further measured an acute phase protein, AGP, which is an indication of inflammation [46, 47]. The concentration of AGP as measured in this study was significantly higher in infected birds at the key time points representing worm establishment and patency. Despite this indication of the possible presence of *H. meleagridis,* there was no evidence of severe liver damage during post-mortem examination. This may partly explain the lack of significant changes in the liver metabolome.

To the best of our knowledge no previous studies have examined the alteration of chicken metabolite profiles during nematode infections. While other studies have reported metabolic changes induced by other pathogens, e.g., *Eimeria acervulina* [37], *Salmonella enteritidis* [35], this study is the first to evaluate the metabolic changes in chickens infected with mixed gastrointestinal nematode species. Future studies should be conducted for broader knowledge, potentially by integrating the application of other metabolomics approaches based on different extracts and other omics technologies (e.g., genomics, transcriptomics, proteomics). Furthermore, a detailed analysis of intestinal tissues as the site of nematode infection could also reveal novel and interesting features that could extend our understanding of the pathogenesis of intestinal parasite infections.

Conclusions

^{1}H-NMR based-metabolomics approach revealed significant alterations in the plasma metabolome of high performing chickens infected with *A. galli* and *H. gallinarum.* The alterations were dependent on both the presence and the patency of infections. Infection upregulated key metabolic pathways. This approach spearheads our understanding of ascarid-host interactions at metabolic level.

Methods

Sample collection and study design

A total of 108 laying hens of the Lohmann Brown Plus genotype (LB, N = 108) was used for the infection experiment with *A. galli* and *H. gallinarum.* The laying hens used in this work originated from a previous study [3], where we evaluated the tolerance and resistance of laying hens of different genotypes to nematode infections. We particularly selected this high-performing genotype, as it is more sensitive to the effects of nematode infections. The hens were 24 weeks of age at the start of the experiment as described below. The total number of uninfected control hens was 42 while 66 hens were infected. The pens of infected and non-infected control hens were kept separately in two different rooms to prevent cross-contamination. In each room the LB hens were kept in 3 separate pens in a stocking density of maximum 6 hens per m^2. Each hen was given a wing-tag to enable repeated measurements on the same individuals over time. The hens were fed a commercial diet (ad libitum), containing 11.2 MJ metabolizable energy, 170 g crude protein and 3.6 g calcium per kg feed [3]. The climatic conditions were optimally regulated using an automatic system to ensure similar lighting, temperature, and aeration across the pens within and between the rooms.

The infection experiment lasted for 18 weeks post infection (wpi), and randomly selected hens from each infection group were necropsied at wpi 2, 4, 6, 10, 14, and 18. Two weeks prior to necropsy hens were randomly selected from their pens (i.e., all pens were sampled with at least one hen), and transferred to individual cages (W 40 × L 45 × H 50 cm). The cages provided equipment for ad libitum water and feed intake of the hens. In each wpi, 11 infected and 7 control birds were necropsied to assess the worm burden as a direct measure of infection intensity. The hens were killed after 3-h feed withdrawal by stunning using a bolt shoot followed by bleeding to death.

Immediately after bleeding to death, blood and liver samples were collected from each bird. Blood was

collected in potassium-EDTA treated tubes (Kabe Labortechnik GmbH, Nümbrecht-Elsenroth, Germany) and centrifuged for 20 min at 2500×*g*. The resulting supernatant was stored at − 20 °C for later analysis. The livers were macroscopically examined for typical signs of histomonosis [48]. The liver samples collected from the larger lobe (i.e., right) were snap frozen and stored at − 80 °C until use.

Experimental infection procedures and diagnosis of infections

The ethics committee for animal experimentation from the Mecklenburg-Western Pomerania State Office for Agriculture, Food Safety and Fishery, Germany, gave approval for the experiment (Permission number AZ.: 7221.3-1-080/16). The experimental procedure for infections followed the guidelines provided by the World Association for the Advancement of Veterinary Parasitology for poultry [49]. Animal handling, care, and necropsies were done by trained and authorised staff members according to the animal welfare rules. Infection material for the experiment was collected from female worms residing in intestines of free-range hens that were naturally infected with ascarids according to the procedure detailed in [50]. At 24 weeks of age, each bird was orally administered 0.4 mL containing a total of 1000 embryonated eggs of two nematodes (*A. galli* and *H. gallinarum*) using a 5-cm oesophageal cannula. The control hens were given a placebo containing 0.4 ml of 0.9% NaCl.

Determination of worm burden

Worm burdens were quantified from the hens that were necropsied at wpi 2, 4, 6, 10, 14, and 18. The hens were denied access to feed for 3 h prior to necropsies to partly empty the gastrointestinal tract (GIT). The GIT was removed immediately post-mortem, and the small intestine and caecum, the predilection sites of *A. galli* and *H. gallinarum*, respectively, were separated. The procedure for opening the intestine has been given in details in [3]. The total number of each of the *A. galli* and *H. gallinarum* worms present in the small intestine and caeca were recorded separately for the respective locations.

Measurements of plasma anti—Histomonas antibody and alpha (1)-acid glycoprotein

Because *H. gallinarum* is a natural vector for transmission of *H. meleagridis* [45] concomitant histomonosis infections cannot be excluded when the birds are infected with *H. gallinarum*. This also applies to experimental *H. gallinarum* infections, unless heterakis-infected birds are treated against histomonosis [51]. Thus, antibody titres against *H. meleagridis* were measured using an Enzyme Linked Immunosorbent Assay (ELISA) (52) to elucidate whether *H. meleagridis* was involved in the mixed infection. Briefly, ELISA plates were coated with rabbit anti-Histomonas serum at 1:10,000 dilution in carbonate buffer. The plates were treated with blocking buffer after an overnight incubation at 4 °C and a previous wash with PBS-Tween 20 (0.05 per cent PBST). Prior to the next washing step, diluted *H. meleagridis* antigen was added to each well and left for 1 h at room temperature. The plasma samples were then diluted (1:500) with blocking buffer and incubated for another 1 h at room temperature. Each plate included positive and negative control sera obtained from chickens infected experimentally with *H. meleagridis*. Goat anti-chicken IgG-horseradish peroxidase (Southern Biotech, Birmingham, AL, USA) was added for 1 h before another wash. A tetramethylbenzidine substrate solution (TMB; Calbiochem, Merck, Vienna, Austria) was used for 15 min in the dark. The optical density was measured at a wavelength of 450 nm. A predetermined the cut-off OD value of 0.54 nm suggested by Windisch & Hess, 2009 [52] was applied to differentiate birds tested for negative and positive histomonosis.

As *H. meleagridis* can induce damage on the caecal and liver tissues, an acute-phase protein, alpha (1)-acid glycoprotein (AGP) was measured in plasma samples using a commercial ELISA according to the manufacturer's protocol (Life Diagnostics, West Chester, USA, Catalogue number: LD-AGP-5).

Sample preparation for ^{1}H-NMR spectroscopy

The plasma samples were thawed on ice and filtered. Prior to filtering, empty filter tubes were initially rinsed with 500 µL of distilled water and centrifuged 3 times for (10,000 × 24 °C × 10 min). An aliquot of 500 µL each of plasma samples was transferred to filter tubes and centrifuged (14,000 × 4 °C × 120 min). For each filtered sample, 350 µL of phosphate buffer (deuterium oxide phosphate buffer 0.10 M, pH = 7.4 containing 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid, sodium salt (TSP d4) 0.04% and 0.04% of sodium azide) was added to an Eppendorf tube and 350 µL of filtered plasma was added. The tubes were mixed for 2 min at 350 rpm at a table mixer and 600 µL of the solution was transferred to a 5 mm NMR tube.

The frozen liver samples were extracted prior to NMR analysis. The samples were first lyophilized, and 20 mg of lyophilized tissue was weighed, 300 µL of ice-cold methanol (MeOH) was added to samples and whirl mixed thoroughly. Samples were placed on ice for 10 min. A 300 µL of ice-cold water was then added to samples, mixed thoroughly, and placed in 4-degree refrigerator overnight for separation.

Samples were then centrifuged for 30 min × 1400 at 4 °C. The upper MeOH phase was transferred to a

new Eppendorf tube and dried for approximately 3 h. Extracted samples were re-dissolved in 575 µL of phosphate buffer (deuterium oxide phosphate buffer 0.10 M, pH = 7.4) and 25 µL of D_2O with 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid, sodium salt (TSP d4) 0.05% were added, 550 µL was transferred to NMR tube containing.

^{1}H-NMR spectrum acquisition

NMR spectroscopy was conducted at 310 K on a 14 T Bruker Avance III spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a 5 mm TXI probe head with gradients, automated tuning, and matching accessory (ATMATM), BCU-I for the regulation of temperature, and SampleJet robot cooling system set to 5 °C as a sample changer. The ^{1}H NMR spectra were acquired using NOESY pre-saturation pulse sequence (Bruker 1D noesygppr1d pulse sequence), 64 K data points, spectral width of 20 ppm, acquisition time of 2.75 s, a recycle delay of 4 s, a relaxation delay of 5*T1 (19 s for blood, 26 s for liver), and 64 scans. 2D NMR experiments (JRES, ^{1}H-^{13}C HSQC, and ^{1}H-^{1}H COSY) were performed on selected samples.

The free induction decays (FIDs) were multiplied by a 0.3 Hz exponential function prior to Fourier transform. Phase and baseline corrections were carried out, and the reference standard Trimethylsilylpropanoic acid (TMSP-d4) signal was adjusted to δ 0.00. The 1D spectra were assigned using Chenomix database values, and the 2D NMR spectra.

For quantification of individual metabolites, processed spectra were imported into the Chenomx NMRSuite VX software and metabolite peaks were quantified relative to the area of TMSP-d4 signal. The Chenomx library considers metabolite information such as number of protons and molecular weight. The processing method was set with pH 7.4 and with TMSP-d4 concentrations of 0.41 mM in plasma samples, and 0.1 mM in liver samples.

Statistical analysis

Statistical analyses of AGP and histomonas antibody titers data were based on log transformation to correct for heterogeneity of variance and to produce approximately normally distributed data. Transformation was done using a natural logarithm function [Ln (y) = ln (y + 1)]. The data were analysed with one-way ANOVA using the R software version 4.1.2 [53].

For the metabolomics data, statistical significance was determined using student t-test with FDR adjustment. Data from all wpi were either pooled or analysed separately within each wpi to examine the effects of infections on metabolite concentrations. One sample with outlier metabolite results was removed from the plasma metabolite data. Differences were considered significant when $p < 0.05$ and a tendency for significant difference was declared when $0.05 < p \leq 0.10$.

MetaboAnalyst software (http://www.metaboanalyst.ca) was employed for univariate, hierarchical clustering and pathway enrichment analysis of the metabolomics data, and analyses were conducted separately for plasma and liver data, respectively. A hierarchical clustering heatmap showing group averages with Euclidean distance measures and ward linkage was constructed to explore the patterns among the metabolites between the two groups within each wpi. Pareto-scaling normalisation (i.e. normalised based on mean-centered and divided by the square root of the standard deviation of each variable) was applied to metabolite data before analysis. For pathway analysis, data were cross-listed with the pathways in the *Gallus gallus* Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway library. A global test was selected for the enrichment analysis method, while topology analysis was based on relative-betweenness centrality and scatter plot (testing significant features) was chosen for visualisation method.

Abbreviations

^{1}H-NMR	^{1}H Nuclear magnetic resonance
KEGG	Kyoto Encyclopaedia of Genes and Genomes
AGP	Alpha (1)-acid glycoprotein
Th2	T helper 2
FID	Free induction decay
FDR	False discovery rate
ELISA	Enzyme linked immunosorbent assay

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13099-023-00584-7.

Additional file 1: Table S1. Univariate analysis of plasma metabolites across all the weeks post-infection. **Table S2.** Univariate analysis of liver metabolites across all the weeks post-infection

Additional file 2: Table S3. Pathway analysis of significant metabolites across all weeks post-infections

Additional file 3: Figure S1. Volcano plot analysis (fold change > 1 and p-value < 0.05) of plasma metabolites at showing significantly higher metabolites in infected hens in wpi 2, 6 and 10.

Additional file 4: Figure S2. NMR spectral signal of Trimethylamine-N-oxide from database shown in one-dimensional experiment (1D) data (A) and in two-dimensional (2D) experiment data (B); and from plasma sample of nematode infected hens during wpi 18 shown in 1D experiment (C) and in 2D experiment (D); X axis represents the ^{1}H chemical shift in ppm. The blue colour represents the fitting obtained from Chenomx database, the black line represents the real sample, and the Match Factor is given in percentage (C). Frequency 1 (F1 ppm) represents the ^{13}C chemical shift in ppm, while the frequency 2 (F2 ppm) represents the ^{1}H chemical shift in ppm (B, D). **Figure S3.** NMR spectral signal of Dimethyl sulfone from database shown in one-dimensional experiment (1D) data (A) and in two-dimensional (2D) experiment data (B); and from plasma sample of nematode infected hens during wpi 18 shown in 1D experiment (C) and in 2D experiment (D); X axis represents the 1H chemical shift in ppm. The blue colour represents the fitting obtained from Chenomx database, the black line represents the real sample, and the Match Factor is given

in percentage (C). Frequency 1 (F1 ppm) represents the 13C chemical shift in ppm, while the frequency 2 (F2 ppm) represents the 1H chemical shift in ppm (B, D). **Figure S4.** NMR spectral signal of Myo-inositol from database shown in one-dimensional experiment data (1D) (A) and in two-dimensional (2D) experiment data (B); and from plasma sample of nematode infected hens during wpi 18 shown in 1D experiment (C) and in 2D experiment (D); X axis represents the 1H chemical shift in ppm. The blue colour represents the fitting obtained from Chenomx database, the black line represents the real sample, and the Match Factor is given in percentage (C). Frequency 1 (F1 ppm) represents the 13C chemical shift in ppm, while the frequency 2 (F2 ppm) represents the 1H chemical shift in ppm (B, D). **Figure S5.** NMR spectral signal of Serine from database shown in one-dimensional experiment data (1D) (A) and in two-dimensional (2D) experiment data (B); and from plasma sample of nematode infected hens during wpi 18 shown in 1D experiment (C) and in 2D experiment (D); X axis represents the 1H chemical shift in ppm. The blue colour represents the fitting obtained from Chenomx database, the black line represents the real sample, and the Match Factor is given in percentage (c). Frequency 1 (F1 ppm) represents the 13C chemical shift in ppm, while the frequency 2 (F2 ppm) represents the 1H chemical shift in ppm (B, D). **Figure S6.** NMR spectral signal of 3-Methylhistidine from database shown in one-dimensional experiment (1D) data (A); and from plasma sample of nematode infected hens during wpi 18 shown in 1D experiment (B); X axis represents the 1H chemical shift in ppm. The blue colour represents the fitting obtained from Chenomx database, the black line represents the real sample, and the Match Factor is given in percentage (B). **Figure S7.** NMR spectral signal of 1,7-Dimethylxanthine from database shown in one-dimensional experiment (1D) data (A); and from liver sample of nematode infected hens during wpi 18 shown in 1D experiment (B); X axis represents the 1H chemical shift in ppm. The blue colour represents the fitting obtained from Chenomx database, the black line represents the real sample, and the Match Factor is given in percentage (B). **Figure S8.** NMR spectral signal of 3-Methylxanthine from database shown in one-dimensional experiment (1D) data (A) and in two-dimensional (2D) experiment data (B); and from liver sample of nematode infected hens during wpi 18 shown in 1D experiment (C) and in 2D experiment (D); X axis represents the 1H chemical shift in ppm. The blue colour represents the fitting obtained from Chenomx database, the black line represents the real sample, and the Match Factor is given in percentage (C). Frequency 1 (F1 ppm) represents the 13C chemical shift in ppm, while the frequency 2 (F2 ppm) represents the 1H chemical shift in ppm (B, D). **Figure S9.** NMR spectral signal of Oxypurinol from database shown in one-dimensional experiment (1D) data (A) and in two-dimensional (2D) experiment data (B); and from liver sample of nematode infected hens during wpi 18 shown in 1D experiment (C) and in 2D experiment (D); X axis represents the 1H chemical shift in ppm. The blue colour represents the fitting of Chenomx database, the black line represents the real sample, and the Match Factor is given in percentage (C). Frequency 1 (F1 ppm) represents the 13C chemical shift in ppm, while the frequency 2 (F2 ppm) represents the 1H chemical shift in ppm (B, D). **Figure S10.** NMR spectral signal of TMAO from database shown in one-dimensional experiment (1D) data (A) and in two-dimensional (2D) experiment data (B); and from liver sample of nematode infected hens during wpi 18 shown in 1D experiment (C) and in 2D experiment (D); X axis represents the 1H chemical shift in ppm. The blue colour represents the fitting of Chenomx database, the black line represents the real sample, and the Match Factor is given in percentage (C). Frequency 1 (F1 ppm) represents the 13C chemical shift in ppm, while the frequency 2 (F2 ppm) represents the 1H chemical shift in ppm (B, D).

Additional file 5: Figure S11. Hierarchical clustering analysis of plasma metabolites. The heat map of the top 25 most significant plasma metabolites between control and infected groups in at all wpis. The patterns of each compound (shown in each row) were categorized by Ward's clustering algorithm and Euclidean distance metrics. Increased and decreased metabolite concentration are given in red and blue, respectively.

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Author contributions
OJO: Conceptualization, data curation, data analysis, ^{1}H-NMR-Metabolomics, data interpretation, writing—original draft. BSBC: Methodology, ^{1}H-NMR—Metabolomics, data curation, data analysis, data interpretation, writing—review & editing; BG: Methodology, serology analysis, writing—review & editing; DL: Methodology, serology analysis, writing—review & editing; CCM: Conceptualization, funding, writing—review & editing; HCB: Methodology—interpretation, supervision, writing—review & editing; GD: Conceptualization, project administration, funding, interpretation, supervision, writing—review & editing. All authors read and approved the final manuscript.

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Data availability
Data have been made available in open access repository (DOI: https://doi.org/10.5281/zenodo.10108935).

Declarations

Ethics approval and consent to participate
The ethics committee for animal experimentation from the Mecklenburg-Western Pomerania State Office for Agriculture, Food Safety and Fishery, Germany, gave approval for the experiment (Permission number AZ.: 7221.3-1-080/16).

Consent for publication
Not applicable.

Competing interests
Authors declare that they have no competing interest.

Author details
^{1}Research Institute for Farm Animal Biology (FBN), Institute of Nutritional Physiology 'Oskar Kellner', Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany. ^{2}Department of Food Science, Aarhus University, Agro Food Park 48, 8200 Aarhus N, Denmark. ^{3}Clinic for Poultry and Fish Medicine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine, Vienna, Austria.

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Authors: **Oyekunle John Oladosu**, Mark Hennies, Matthias Gauly, Gürbüz Das

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Oyekunle John Oladosu:	Conceptualization, Data curation, Formal analysis, Writing – original draft. Mark Hennies: Conceptualization, Methodology, Writing – review & editing.
Matthias Gauly:	Conceptualization, Funding, Writing – review & editing.
Gürbüz Daş:	Conceptualization, Funding, Project administration, Investigation, Supervision, Writing – review & editing.

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A copro-antigen ELISA for the detection of ascarid infections in chickens

Oyekunle John Oladosu [a], Mark Hennies [b], Matthias Gauly [c], Gürbüz Daş [a,*]

[a] Institute of Nutritional Physiology 'Oskar Kellner', Research Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany
[b] TECOdevelopment GmbH, Marie-Curie-Str. 1, 53359, Rheinbach, Germany
[c] Free University of Bozen - Bolzano, Faculty of Science and Technology, Universitätsplatz 5, 39100 Bolzano, Italy

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ABSTRACT

A reliable method of diagnosing the most prevalent helminth infections in chickens is vital for developing effective control strategies. *Ascaridia galli* and *Heterakisgallinarum* are phylogenetically close nematode species that can elicit the development of cross-reactive antibodies in chickens. Therefore, an enzyme-linked immunoassay (ELISA) based on *Ascaridia galli* antigens in faeces of chickens to detect and quantify infections with both *A. galli* and *H. gallinarum* was developed. The ELISA utilised polyclonal antibodies that were obtained from rabbits immunised with soluble antigens isolated from *A. galli*. In two separate experiments, chickens were kept as uninfected controls or were orally infected with either 100 or 1000 of embryonated eggs of *A. galli* or *H. gallinarum*. Faecal samples were collected after 28–30 weeks post-infection. The ELISA was then used to quantify the concentration of soluble worm antigens in faecal samples, i.e., the amount of antigen per gram faeces, APG. The APG from infected chickens was significantly higher than non-infected groups in both experiments (P 0.001). Both 100 and 1000 infection dose groups were not significantly different (P = 0.999) in the experiment with *H. gallinarum,* whereas in the experiment with *A. galli,* APG was significantly higher in the 1000 infection group (P 0.001). A receiver operation characteristics (ROC) analysis that evaluates the qualitative performance of diagnostics tests was used to calculate the assay parameters within each mono-infection experiment. The result showed that the assay had a high diagnostics accuracy with an area-under-curve (AUC) of 0.99 in detecting infection in *A. galli* infected chickens and a moderate-high accuracy (AUC = 0.89) in birds infected with *H. gallinarum.* The diagnostic sensitivity and specificity of the assay at the optimal cut-off point equivalent to Youden index were 93% and 100% for detecting infections in *A. galli* experiment and 85% and 92% in *H. gallinarum* experiment, respectively. The correlation between faecal antigen concentration and all worm burden parameters was positive but generally low (r < 0.33), which provided less information about infection intensities. Nonetheless, these results indicate that a reliable and accurate qualitative diagnosis of the two most prevalent intestinal nematodes in chickens can be achieved using a non-invasive copro-antigen ELISA assay.

1. Introduction

Ascaridia galli and *Heterakis gallinarum* are two economically important gastrointestinal nematode species that are common globally (Thapa et al., 2015; Shifaw et al., 2021). They are more widespread than other common helminths of chicken like *Capillaria spp.* and *Raillietina spp.* (Shifaw et al., 2021). These species have pathogenic effects that are associated with damage to intestinal tissue, reduced nutrient absorption and utilisation and impaired growth and laying performance (Dahl et al., 2002; Daş et al., 2011, 2012; Sharma et al., 2019; Stehr et al., 2019). Consequences of infection, especially the level of mortality have been shown to be associated with infection level (Hinrichsen et al., 2016).

The gold standard technique for examining the severity of nematode infections is to directly count the number of worms present in the intestine of the infected host post-mortem (Permin & Hansen, 1998). As this method requires necropsy, microscopic counting of parasite eggs in host faeces has been the classical method of indicating the presence and severity of infections (Nielsen, 2021).

Although, faecal egg counts (FEC) indeed had moderate to strong correlations with worm burdens in chickens (Thapa et al., 2015; Daş et al., 2017; Sharma et al., 2018; Feyera et al., 2022) the results are not consistent and not always reliable for several reasons. Egg shedding through faeces can only occur if infected hosts harbour matured female worms, whereas immature stages and male worms do not (at least

* Corresponding author.
E-mail address: gdas@fbn-dummerstorf.de (G. Daş).

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directly) contribute to the egg shedding. The uneven distribution of eggs in host faeces, diurnal fluctuations in eggs shed, predilection site of the parasite species in the gastrointestinal tract and variation in worms' fecundity can also impede the reliability of egg counts for diagnosing infection (Wongrak et al., 2015; Daş et al., 2019).

The efforts to find an alternative reliable method to FEC led to the development of an enzymatic linked immunosorbent assay (ELISA) that measures anti-ascarid antibody in plasma and egg-yolks of infected hosts (Daş et al., 2017; Sharma et al., 2018). While the assay provided a better qualitative diagnosis than FEC, it was not a better tool to determine the severity of infections. Moreover, plasma antibody ELISA requires both an authorised field expert and an invasive technique to obtain the blood samples, while egg yolk antibody ELISA can only be performed for matured female chickens, leaving male and young chickens out of the scope. Hence, a non-invasive alternative to FEC that can capture all groups of birds and different developmental stages of worms is still missing.

An ELISA that quantifies the concentration of parasite antigens in hosts' faeces (copro-antigens) is a non-invasive method that could also be used for evaluating the infection status of the living host given the presence of excretory/secretory products of parasites in the host intestine (Allan & Craig, 2006). Copro-antigens have been used in the evaluation of parasitic infections with considerably higher accuracy than FEC in cattle, sheep, pigs, red deer, horses, and dogs (Palmer et al., 2014; Kajugu et al., 2015; French et al., 2016; Martínez-Sernández et al., 2016; Lagatie et al., 2020), but its' performance in diagnosing chickens infected with either *A. galli* or *H. gallinarum* has not been reported.

Given that chickens develop cross-reactive antibodies (Daş et al., 2017) to these two phylogenetically close nematode species (Nadler et al., 2007; Wang et al., 2016), a copro-antigen ELISA coated with a specific antibody against one species could be sensitive to antigens of other related species. Therefore, this study was designed with three objectives *i)* to evaluate the potential of an ELISA to capture worm antigens in host faeces and to discriminate between nematode infected chickens and non-infected ones *ii)* to test whether an ELISA that uses anti-*A. galli* polyclonal antibody could also measure antigens of *H. gallinarum* in host faeces and *iii)* to evaluate if copro-antigen ELISA would provide adequate information about infection intensities.

2. Materials and methods

2.1. Sample description

This study utilised faecal samples collected from two independent nematode infection experiments in which laying hens (Lohmann Selected Leghorn, LSL) were infected with different doses of embryonated eggs of either *A. galli* or *H. gallinarum* and were left infected for a long period to allow reinfections with the same species. The aim of the experiments from which data were collected was to evaluate the diagnostic accuracy of ELISA using plasma and egg yolk samples in comparison with faecal egg count (FEC), hence, the data for worm burden, antibody response and the faecal egg counts from these two independent experiments have been previously published (Daş et al., 2017; 2018). However, the analysis of copro-antigen using samples from these experiments has not been reported and is the core idea newly presented in this study. Readers are therefore referred to previously published articles for details about the experimental animals and infection procedures (Daş et al., 2017; 2018). Briefly, in each experiment, the LSL chickens were divided into three groups. One group was kept as uninfected control, and the birds of the other two groups were either experimentally infected with 100 or 1000 embryonated/infective eggs of either nematode species. The birds used in this experiment were purchased from commercial hatchery at one-day of age and reared under strict conditions free of helminth. The birds were reared in a floor husbandry system with wood shavings as the litter material and were fed commercial diets *ad libitum*. The birds were 16 and 4 weeks old at the time of infection in *A. galli* and *H. gallinarum* experiments, respectively. Analysis of pooled faecal samples on infection day showed no gastrointestinal helminth infection during the pre-experimental period. The infection-free status of the birds at the beginning of the experiment was also confirmed by assessing plasma anti-ascarid antibody levels (Daş et al., 2018). Approval for experimental infection was provided by the ethics committee of the Lower Saxony State Office for Consumer Protection and Food Safety, Germany (Permission no: AZ:33.9–42502–04–12/0707). Post-mortem parasitological examination was done at 28 and 30-weeks post-infection (wpi) in *A. galli* and *H. gallinarum,* respectively, according to the recommended practice for experimental infections of chickens (Yazwinski et al., 2003). Worms were classified as either larvae or matured worms based on general morphology as reported in a previous study (Daş et al., 2017). The average length of the worms was estimated by measuring 20 randomly selected worms (10 worms per sex) in each bird at necropsy. Prior to post-mortem evaluation, the birds were transferred to individual cages for 24 h to collect daily total faeces, which included both intestinal droppings and the caecal excreta. The daily total faeces were homogenized thoroughly and stored at − 20 °C. The number of faecal samples used to determine antigen concentration in both experiments is shown in Table 1.

2.2. Preparation of the copro-ELISA for the detection of ascarids antigen in host's faeces

2.2.1. Antigen isolation from A. galli

To isolate soluble antigens from worms, thawed intact worms were initially washed thrice with phosphate-buffered saline (PBS), followed by one wash with 70% ethanol and lastly with another PBS wash. Homogenisation of the worms was done in a mortar after which one part of the worm was extracted using two parts of a basic buffer containing 35 mM BisTris, 25 mM Tris, pH 9 for 5 min. This was then followed by centrifugation at 17,000, 4 °C for 15 min. The supernatant representing the soluble antigen was collected and used as standard.

2.2.1.1. Rabbit immunisation. The soluble antigen was used for immunization of a rabbit. For this, the rabbit was immunized three times at 2 weeks interval with 25 μl of antigen (soluble worm extract), 175 μl PBS, and 200 μl Montanide adjuvant ISA 206 (immunoGlobe Antikörpertechnik GmbH, Himmelstadt. Germany). After initial test bleeding of ~ 5 ml serum, additional booster was given. The rabbit was exsanguinated (min. 35–45 ml serum) after three times of large bleeding (~ 15 ml serum) at two weeks interval. Permission for immunization of the rabbit was obtained from the relevant state authority ('Government of Lower Franconia', AZ 55.2–2531.01–57/11).

2.2.2. Faecal sample preparation

A total of 50 mg of the homogenized, defrosted faeces from each individual chicken was weighed and ten parts of sample buffer (0.12 M NaCl, 0.02 M Na2HPO4, 0.01 M EDTA, 0.2% BSA, 0.1% gelatine hydrolysate, 0.05% Tween 20) was added to 1 part of each of the weighed faecal samples. The mixture was intensively vortexed and left to settle for 10–20 min. The supernatant (100 μl) was collected and pipetted into the assay wells.

2.2.3. ELISA procedures

A 96-well microtiter plate (Costar 2592, Corning, USA) was used for the sandwich ELISA.

The plates were coated with 100 μl of anti-Ascaridia polyclonal rabbit antibody at a concentration of 10 μg/ml in coating buffer (0.05 M NaHCO3; pH 9.6). Antibodies were purified by using Protein A Sepharose Fast Flow according to the protocol specified by the manufacturer (Cytiva, USA). After overnight incubation at 4 °C, the wells were blocked with washing buffer (10% PBS, 0.05% Tween 20) for 1 h and

Table 1
Mean, median and range of worm counts, EPG and APG in both *Ascaridia galli* and *Heterakis gallinarum* experiments.

Experiment		Experimental Group								
		0-dose			100-dose			1000-dose		
		Mean	Median	Range	Mean	Median	Range	Mean	Median	Range
A. galli	Larvae (n/ bird)	0	0	0	2.9[a]	2.0	11.0	10.5[b]	5.0	79.0
	Mature (n/ bird)	0	0	0	34.2[a]	29.0	105.0	39.4[a]	28.0	114.0
	Total (n/ bird)	0	0	0	37.1[a]	34.0	109.0	49.8[a]	45.0	114.0
	EPG	0	0	0	526.5[a]	200.0	3250.0	1802.8[a]	450.0	15900.0
	APG(μg/g faeces)	0.02[a]	0.02	0.03	0.12[b]	0.09	0.28	0.32[c]	0.17	1.82
H.gallinarum	Larvae (n/ bird)	0	0	0	85.4[a]	62.0	262.0	82.7[a]	64.0	420.0
	Mature (n/ bird)	0	0	0	410.2[a]	386.0	970.0	453.5[a]	418.0	1086.0
	Total (n/ bird)	0	0	0	495.6[a]	440.0	1134.0	536.2[a]	482.0	1309.0
	EPG	0	0	0	472.8[a]	349.0	2351.0	334.7[a]	199.0	1650.0
	APG (μg/g faeces)	0.05[a]	0.04	0.11	0.10[b]	0.10	0.14	0.12[b]	0.09	0.97

Means in the same row with different superscript are significantly different ($P < 0.05$). Statistical analyses of the data are based on analysis of variance using log transformed data (Log(y + 1)). Whereas means, median and range presented are based on untransformed data. n = 41 in 100 dose group in both experiments and n = 31 in 1000 dose group in *A. galli* experiment and 41 in 1000 dose group in *H. gallinarum* experiment. Number of samples in 0 dose group is 9 and 25 in *A. galli* and *H. gallinarum* experiments, respectively. Note that 0-dose group was excluded from the statistical comparisons with 100 and 1000 dose groups for worm burden and EPG data.

then washed five times with 350 μl washing buffer. Plates were stabilised by using 300 μl of 20% sucrose solution for 1 h. After decantation, the plates were dried at room temperature for 3–4 days and stored with silica gel at 4 °C until use.

Initial run of the ELISA was done multiple times (up to 30x) to establish optimal antibody and biotin conjugate concentrations, and to determine optimal buffer and assay conditions. For the first step after optimisation, 10 μl of chicken serum from non-infected animals were pipetted in each well to saturate the polyclonal antibodies and reduce the unspecific binding by exclusion of these antibodies. 100 μl standards of 400, 200, 100, 50, 25, and 0 ng soluble antigen/ml were added to the assay wells in duplicate while controls and infected samples were in single determination. The assay was then incubated at room temperature (20 – 25 °C) for 4 h on a microplate shaker (500 rpm).

After incubation, the plate was washed three times with 350 μl of washing buffer (10% PBS; 0.05% Tween 20) before adding a biotinylated anti-Ascaridia polyclonal rabbit antibody (TECODevelopment, GmbH, Germany). The antibody (Protein A purified) was conjugated to Biotinamidohexanoic acid Nhydroxysuccinimide ester (Sigma). The biotin-antibody conjugate was diluted in biotinylated antibody buffer (70 ng/ml in sample diluent containing 5% normal rabbit serum and 0.5% casein). To each well, 100 μl of diluted biotin antibody conjugate was added, followed by 30 min incubation at room temperature on a shaker (500 rpm). After three washing steps with 350 μl of diluted washing buffer, 100 μl of enzyme conjugate (Streptavidin-PolyHRP20 conjugate, SDT, Baesweiler, Germany) at 200 ng/ml in PBS containing 0.05% Tween 20 was incubated for 30 mins on a plate shaker. After an additional 5 washes, the TMB substrate (TMBS, SurModics, MN, USA) was added for and left for 30 min on a plate shaker, followed by termination with 100 μl of 1 M hydrochloric acid and an OD measurement at 450 nm with a 650 nm reference filter using an Emax Plus Microplate reader (Molecular Devices, USA) with software SoftMax Pro 6.5.1 (Molecular Devices, USA). The conversion of OD to concentration (in ng/ml) via the standard curve was done using a 4-parameter logistic (4-PL) curve-fit of the SoftMax Pro software (Molecular Device, USA). The equation for the 4-PL model is:

$$y = \frac{a - d}{1 + \left(\frac{x}{c}\right)^{b}} + d$$

Where; y = the measured OD.
x = the concentration.
a = the minimum value.
d = the maximum value.
c = point of inflection.
b = slope of the curve.

Antigen concentration in faeces was then estimated as the amount of ascarid antigen per gram of faeces (APG, μg/g faeces) based on the standard curve for 400, 200, 100, 50, 25, 0 ng soluble antigen/ml. The assay read out (ng/ml) was multiplied by our dilution factor of 11 (as 10 parts of buffer was added to 1 part of weighed faeces) and results were further converted to μg/g.

The intra- and inter-assay coefficient of variation (CV) for this assay were 5.7% and 5.3% respectively. The CV determination was based on three independent assays performed with 5 random samples in 5 duplicates.

2.2.4. Faecal egg counts

A random fresh sub-sample (4 g) was obtained from thoroughly mixed daily faeces and analysed with the McMaster egg counting technique (MAFF, 1986). A saturated NaCl solution (specific gravity = 1.2) was used as the flotation liquid and minimum detection level of egg counting technique was set to 50 eggs per gram faeces (EPG).

2.3. Statistical analysis

Statistical analyses of total worm count, larva counts and APG data were based on log transformation of the data to correct for heterogeneity of variance and to produce approximately normally distributed data. Transformation was done using a natural logarithm function [Ln (*y*) = ln (y + 1)]. Differences in APG and worm burden between infection-dose groups within each experiment were analysed with one-way ANOVA using the function in the R programme (R Core Team, 2021).

Furthermore, a receiver operation characteristics (ROC) analysis was performed to assess the diagnostic accuracy of the copro-antigen ELISA using APG data. The ROC analysis is a commonly used method valid for evaluating the qualitative performance of a diagnostic test (Nielsen, 2021). The ROC calculates the area-under-the-curve (AUC), non-arbitrary threshold value, diagnostic sensitivity, and specificity of the assay (Swets, 1988). The ROC curve is a plot of the true positive rate in function of the false positive rate for all possible cut-off points. The AUC is an overall summary of the ROC curve across all cut-off points, and it indicates the probability of discriminating between infected and uninfected populations.

The ROC analysis was done using the pROC package in R programming software (Robin et al., 2011) for each mono-infection experiment, separately. For the analysis, samples from birds that harbour worms (i.e. gold standard) in both initial-infection dose groups in each experiment were considered an infected case (i.e., positive case) while the control birds (all free of worms) were classified as control case (i.e., negative

case). In the 'roc' function of the software, infection status (i.e., control vs infected) were set as the response, while the assay outcome (APG data) was set as the predictor. The function sets a cut-off as the mean of two consecutive values in the APG data. At each of these cut-offs, the diagnostic sensitivity was estimated as the true positive/(true positive + false negatives) value while diagnostic specificity was calculated as true negative/(true negatives + false positive) value. It is important to note that the test specificity here implies a differentiation between infected and uninfected birds and not differentiation between the two parasites as we examined birds from two independent mono-species infections with their own uninfected-control counterparts. The minimum sample size required for a valid ROC analysis was calculated using Medcalc statistical software version 20.023 (MedCalc, 2020) with a pre-set significance level of 0.05, maximum AUC of 0.99 and group ratio of 1. The optimal values for diagnostic sensitivity and specificity were selected according to the criteria value corresponding to the Youden index (J) (Youden, 1950; Ruopp et al., 2008). This represent the cut-off point where the difference between the true positive rates and false positive rate is at maximum. The ROC curve showing plots of diagnostic sensitivity and specificity values at all cut off point was generated, and the AUC was estimated.

Test accuracy of the assays was interpreted based on the range of the AUC value and are classified as follows; low accuracy ($0.5 < AUC \leq 0.7$), moderate accuracy ($0.7 < AUC \leq 0.9$) or high accuracy ($AUC > 0.90$) (Hanley and McNeil, 1982).

The correlation between APG and worm burden parameters (total worm burden, total worm length, total larva count) was estimated by Pearson correlation analysis using R software. Analysis was based on log-transformed data except for total worm length. Total worm length was calculated by multiplying the average worm length by the number of worms and was done separately for each worm sex. The raw data used for all analyses in this study are stored in a repository (DOI: 10.5281/zenodo.705607).

3. Results

3.1. Effects of mono-species infections and infection dose on copro-antigen concentration

The overall fixed effect of infection on APG was significant ($P < 0.001$) in both *A. galli* (Fig. 1) and *H. gallinarum* (Fig. 2) experiments. APG was also significantly different between the two infection doses in chickens that were infected with *A. galli*. Groups infected with 1000 *A. galli* eggs had a significantly ($P < 0.001$) higher concentration of APG than birds infected with only 100 eggs. Whereas, in the experiment with *H. gallinarum* infected birds, APG concentration was not significantly different ($P = 0.937$) between the two infection doses.

3.2. Effects of initial-infection dose on the worm burden

All but one of the experimentally infected birds harboured worms at the end of the experimental period. As confirmed through parasitological examination of the intestines and FEC, uninfected control birds remained nematode-free until the end of respective experiments. As shown in Table 1, the effect of the initial infection dose was not significant ($P > 0.05$) on the total worm burden in both experiments where birds were initially infected either with *A. galli* or *H. gallinarum* and then exposed to re-infection with the same species. In addition, larva count did not differ significantly between the two infection doses ($P = 0.850$) in chickens infected with *H. gallinarum*, however, it was significantly higher ($P = 0.004$) in chickens infected with 1000 instead of 100 *A. galli* eggs.

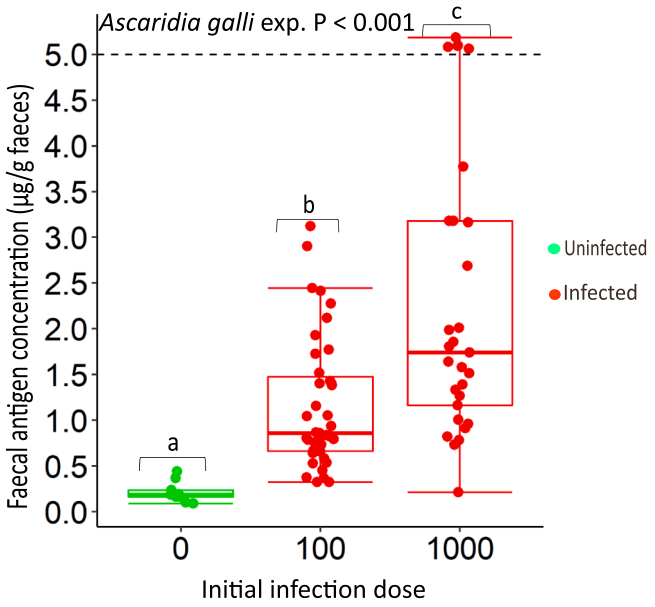

Fig. 1. Ascarid-antigen concentrations per gram faeces of chickens in *Ascaridia galli* experiment (n = 81). Birds were either kept as uninfected (●) or infected (●) with 100 or 1000 eggs of *A. galli*. Necropsy was performed at 28 weeks post-infection to examine worm burden and faecal samples collected to measure antigen concentration. Dots above the dashed line (≥ 5.0 µg / g) are shown on an extended reduced scale [reduced y-axis = (y * 0.01 + 5)] to depict a focused picture of the whole data. The figure represents raw data, but the statistical comparisons are based on the log-transformed data.

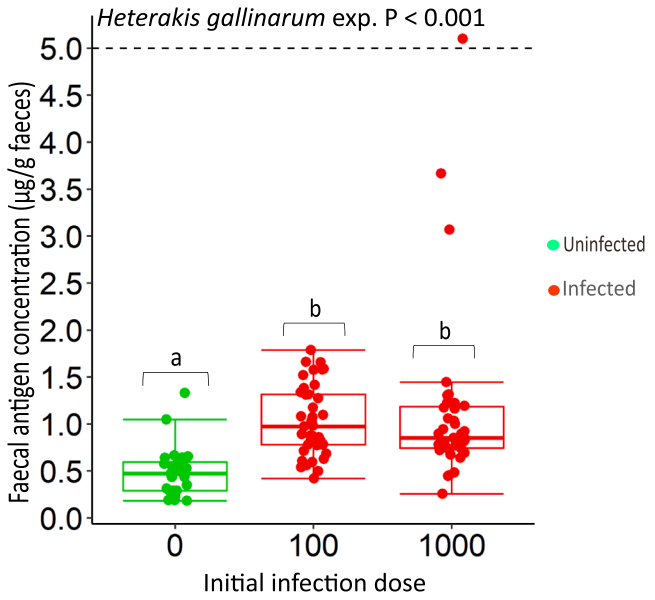

Fig. 2. Ascarid-antigen concentrations per gram faeces of chickens in *Heterakis gallinarum* experiment (n = 107). Birds were either kept as uninfected (●) or infected (●) with 100 or 1000 eggs of *H. gallinarum*. Faecal samples to measure antigen concentration were collected at 30 weeks post infection when birds were slaughtered for parasitological examination. Dots above the dashed line (≥ 5.0 µg / g) are shown on an extended reduced scale [reduced y-axis = (y * 0.01 + 5)] to depict a focused picture of the whole data. The figure represents raw data, but the statistical comparisons are based on the log-transformed data.

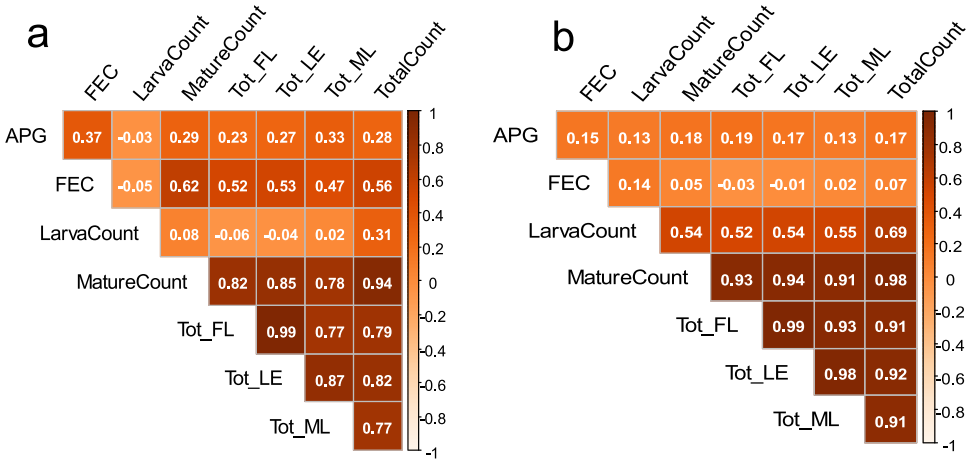

Fig. 3. Plots of correlation coefficients of faecal antigen concentration (APG) and faecal egg count (FEC) with worm burden parameters in *Ascaridia galli* experiment (a) and *Heterakis gallinarum* experiment (b).
Tot_FL:Total Female worm Length; Tot_LE: Total worm length; Tot_ML: Total male worm length. MatureCount: Total number of adult worm count; LarvaCount: Total larvae count; FEC: Faecal egg count; APG: Antigen concentration per gram faeces.

3.3. Correlation between APG and worm burden

All correlations are presented in Fig. 3. The correlation (r) between APG and all worm burden parameters in each experiment was positive albeit with generally low coefficients ($r < 0.33$). For example, in chickens infected with *A. galli,* the correlation between APG and total worm burden yielded a coefficient (r) of 0.28 that was nevertheless significantly different from zero ($P < 0.050$). A higher correlation coefficient was calculated between APG and total length of male worms ($r = 0.33$, $P < 0.050$) than length of female worms ($r = 0.23$, $P = 0.381$). None of the correlations of APG with the respective worm burden parameters was significant in *H. gallinarum* infected animals ($P > 0.05$). APG was positively correlated with EPG ($r = 0.37$, $P < 0.05$) in *A. galli* experiment. EPG had considerably higher correlation coefficient ($r \geq 0.47$) with worm burden parameters compared to APG in *A. galli* experiment. In this experiment, correlation between FEC with total worm count had coefficient of $r = 0.56$, while it was $r = 0.62$ for EPG and counts of mature *A. galli* worms.

3.4. Qualitative diagnostic performance of the copro-antigen ELISA assay

We applied a ROC analysis to investigate the qualitative performance of the ELISA for quantifying APG. As shown in Figs. 4 and 5, the results showed that the assay had a high overall diagnostic accuracy (AUC = 0.99) when used to identify chickens that were infected with *A. galli*. In chickens infected with *H. gallinarum,* the diagnostic accuracy of the assay was moderately high (AUC = 0.89). The assay could identify all truly negative chickens in the *A. galli* experiment with 100% diagnostic specificity while in the *H. gallinarum* experiment, the assay falsely classified 8% of the uninfected chickens at the optimal cut-off value (Fig. 5b). The diagnostic sensitivity of the assay was 93% for detecting infections in *A. galli* experiment and 85% for detecting infection in *H. gallinarum* experiment. In addition, the assay had a negative predictive value (npv) of 90% and a positive predictive value (ppv) of 100% in detecting infections in *A. galli* experiment whereas in *H. gallinarum* experiment, the assay had npv of 65%.and ppv of 97%.

Due to the lack of significant differences in the total worm burdens of the birds between the two initial infection dose groups (i.e., 100 vs. 1000 eggs) in either infection experiment, we pooled APG data of all birds that harboured worms from both dose groups as an infected case (i.e., positive case) for the analysis. However, we present the diagnostics performance of the assay for each infection dose separately in the Supplementary Figure 1. In the experiment with *A. galli* the ELISA test had AUC of 0.98 for the 100-dose group and 0.99 in 1000-dose group. The AUC for the ELISA was 0.88 for both 100 and 1000 infection group in the *H. gallinarum* experiment.

4. Discussion

This is the first study to provide insight into the use of worm antigens in host faeces to detect and quantify ascarids infections in chickens. For this, we developed a copro-antigen ELISA that accurately differentiated between non-infected chickens, and chickens infected either with *A. galli* or *H. gallinarum* at different doses. A differentiation between *A. galli* and

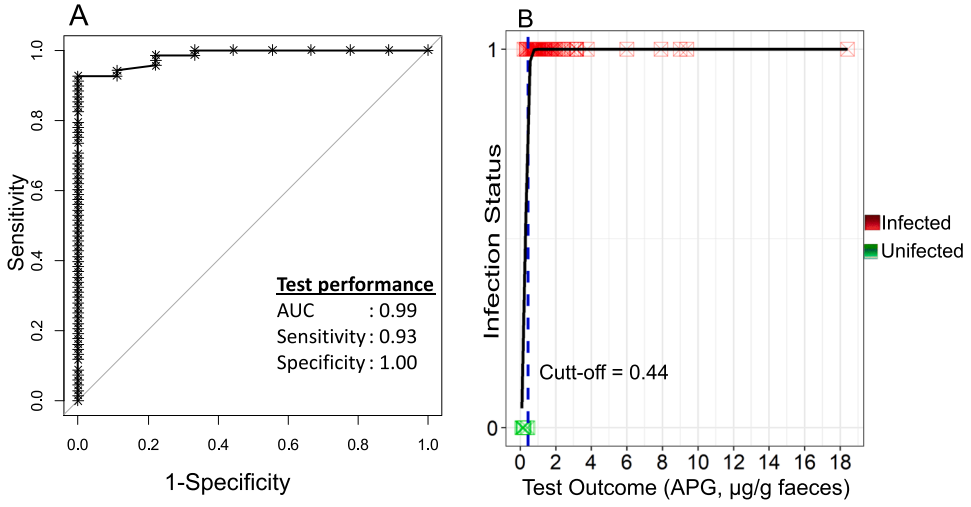

Fig. 4. A). ROC curve based on known infection status of chickens (n = 76) in *Ascaridia galli* experiment. The diagonal line corresponds to the half of the maximum area under curve (i.e., AUC = 1.0). The farther the location of the ROC curve is from the diagonal line, the higher the total test accuracy analyses. B). A logistic plot of the binary infection status (1 = infected, 0 = control) with continuous predictor, APG. Vertical dotted line shows the optimal cut-off point representing Youden Index.

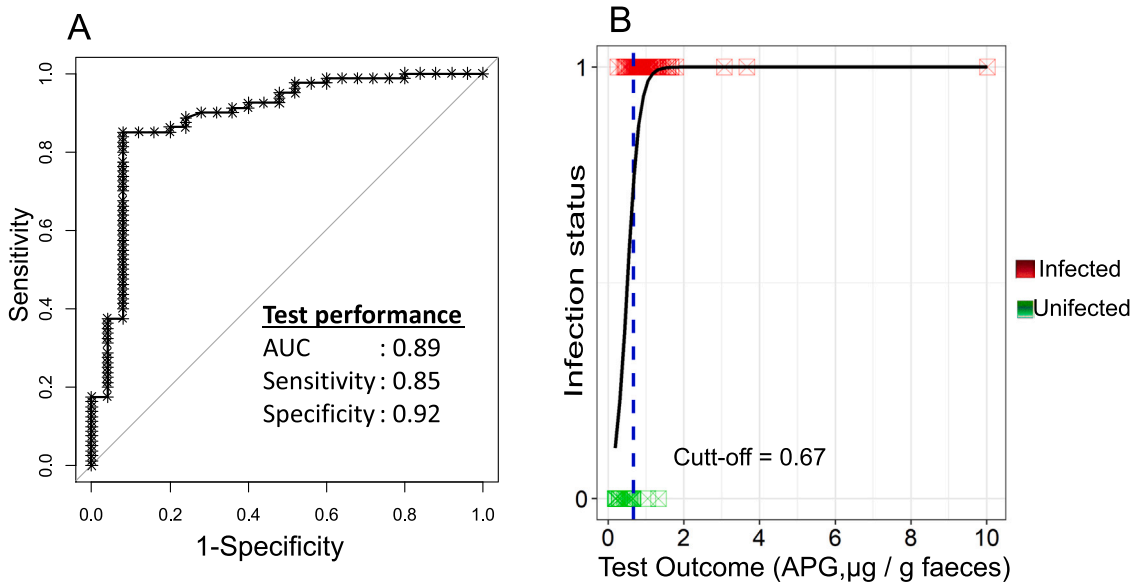

Fig. 5. A). ROC curve based on known infection status of chickens (n = 105) in *Heterakis gallinarum* experiment. The diagonal line corresponds to the half of the maximum area under curve (i.e., AUC = 1.0). The farther the location of the ROC curve is from the diagonal line, the higher the total test accuracy analyses. B). A logistic plot of the binary infection status (1 = infected, 0 = control) with continuous predictor, APG. Vertical dotted line shows the optimal cut-off point representing Youden Index.

H. gallinarum infected chickens was however not tested. Given the significant difference in APG between infected and non-infected groups obtained in this study, we conclude that measurement of worm antigens present in host faeces allows our method to distinguish (Figs. 1 and 2) noninfected birds from birds infected with either nematode species. We could, however, not find any linear relationship between infection dose and severity of infection at the end of our experiment, likely because of reinfections that eliminate impact of primary infections (Das et al., 2018). In addition, dose-dependency in establishment rate of the nematodes might have led to similar worm burdens as earlier reported by (Permin et al., 1997), where infection with 100, 500 or 2500 eggs of *A. galli* had no significant effect on the worm burden after 8 wpi. In contrast to these findings, Feyera and colleagues (2022) showed evidence of a significant difference in worm burdens between chicks infected with low dose (100) and high (900) of *A. galli* eggs (Feyera et al., 2022). The authors reported that there was a minimal possibility for re-infection by wpi 8–10 when necropsy was done. Whereas, in the current study, the parasitological examination was done at 28–30 wpi—enough time for reinfection to occur as it would in a natural condition. Previous research suggests that the majority of worms from initial experimental infection are expelled up to 31- and 36-days post-infection for both *A. galli* and *H. gallinarum* respectively (Stehr et al., 2018). Thus, most of the worms found in the birds at 28–30 wpi must have resulted from naturally occurring re-infections, which might have eliminated the effect of the initial infection dose on worm burden in the current study. This offers the possibility of comparing our test in a similar situation as in natural infection conditions.

It is important to note that even though there was no effect of infection dose on total worm burden, the 1000-dose group had higher larvae count in *A. galli* infected birds.

Similarly, this group had significantly higher antigen concentrations than the 100-dose group. This might suggest that the higher larvae count may have contributed to the higher antigen concentration obtained for the 1000-dose group suggesting that antigens could already be captured in faeces of the hosts containing only immature worms. This assumption could however not be confirmed in the present study as there was no significant correlation between faecal antigen concentration and larvae counts. An earlier report suggested that copro-antigen can be measured in dogs infected with *Echinococcus granulosus* and *Taenia pisiformis* weeks prior to patency and are independent of egg output (Allan & Craig, 2006). This present study did not, however, evaluate the detection of ascarids antigen prior to patency as faecal samples for APG analyses were available only at the end of the experiment. Thus, further studies are encouraged to quantify worm antigens in chicken faeces before patency and during infection.

We had previously provided the evidence that chickens develop cross-reactive antibodies (Daş et al., 2017) against the two closely related nematode species used in this study (Nadler et al., 2007; Wang et al., 2016), which allows for the use of one assay to diagnose two different nematode species without separation between the two infections. Therefore, in the present study, we used soluble antigens of *A. galli* as standards in a sandwich ELISA coated with anti-ascarid polyclonal rabbit antibody to quantify antigen concentration in the faeces of both *A. galli* and *H. gallinarum* infected chickens. Such an assay would be useful in the field where both nematode species are known to co-infect chickens, and mono-infection rarely occurs (Kaufmann et al., 2011; Thapa et al., 2015). The evaluation of the assay showed a high diagnostic accuracy, sensitivity, and specificity in *A. galli* infected chickens (Figs. 4 and 5) while, the assay showed slightly lower diagnostic specificity and sensitivity for detecting infection in *H. gallinarum*. This limitation, at least for experimental purposes, could be overcome with further improvement to the assay especially by limiting variability in the proteins targeted. This can be achieved by exploring more specific worm proteins that could yield a better sensitivity/specificity profile for each of the two worm species.

Until now, no published report on the use and accuracy of a copro-antigen ELISA assay in detecting *A. galli* and *H. gallinarum* infection in poultry is available, although its use is widely explored in other species. For example, Jara and colleagues reported a high accuracy (AUC = 0.995)—comparable to our result—for a copro-antigen ELISA in the detection of *E. granulosus* soluble antigens in dogs, where they used an anti-*E. granulosus* polyclonal rabbit antibody (Jara et al., 2019). They reported a higher diagnostic sensitivity and specificity of 96.5% and 98% respectively than what was obtained in this study. Similarly, a copro-antigen ELISA which measured a specific ABA-1 *Ascaris lumbricoides* antigen was used to detect ascarids infection in pigs and humans (Lagatie et al., 2020). The assay had a specificity of 95.3% and sensitivity of 91.5% which is also similar to what we found in the current

study. Our results are therefore within the range of other established methods used for other species.

Another objective of this study was to assess the performance of copro-antigen ELISA in indicating intensity of infection. We found positive but low correlations between APG and total worm burdens in both experiments. Our data were obtained from a single necropsy time point wpi 28/30 which does not allow to capture solely larvae antigens given that the majority of larvae expelled in a few weeks (Stehr et al., 2018).

Generally, a copro-antigen test has several advantages over classical parasitological diagnostics methods (i.e., FEC). FEC has been used as the standard proxy for adult female worm burden and infection intensity (Sharma et al., 2019; Nielsen, 2021) and it is known to correlate significantly with worm burden, especially for *A. galli* (Thapa et al., 2015; Daş et al., 2017; Feyera et al., 2022). However, FEC is prone to false negatives and does not correctly represent the dynamics of worms as worm eggs are only shed by matured female worms. By contrast, copro-antigen ELISA is not dependent on the presence of eggs or worm sex (Allan & Craig, 2006; Elsemore et al., 2014). Furthermore, worm antigens in host faeces are quite stable for days at temperatures ranging from − 80–35 °C (Allan & Craig, 2006).

5. Conclusion

A single non-invasive copro-antigen ELISA for the detection and quantification of infection in chickens infected with either *A. galli* or *H. gallinarum* was developed. Taken together, the findings suggest that a copro-antigen ELISA can reliably detect infections with either species of nematode studied. But the correlation between the antigen concentration in faeces and actual worm burden is rather low and further improvement would be required to evaluate the use of copro-antigen ELISA to quantify infection intensities.

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CRediT authorship contribution statement

Oyekunle John Oladosu: Conceptualization, Data curation, Formal analysis, Writing – original draft. **Mark Hennies:** Conceptualization, Methodology, Writing – review & editing. **Matthias Gauly:** Conceptualization, Funding, Writing – review & editing. **Gürbüz Daş:** Conceptualization, Funding, Project administration, Investigation, Supervision, Writing – review & editing.

Conflict of interest

The authors have read the journal's policy and have the following competing interests: MH is an employee of TECODevelopment GmbH. This does not alter our adherence to Veterinary Parasitology policies on sharing data and materials. There is no patent associated with this research to declare. Other authors have no competing interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2022.109795.

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3.3 Pattern and repeatability of ascarid-specific antigen excretion through chicken faeces, and the diagnostic accuracy of coproantigen measurements as compared with McMaster egg counts and plasma and egg yolk antibody measurements in laying hens

Authors: **Oyekunle John Oladosu**, Mark Hennies, Manuel Stehr, Cornelia C. Metges, Matthias Gauly & Gürbüz Daş

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Contributions:

OJO conducted the statistical analysis of all the data.

OJO and GD interpreted the data.

OJO wrote the original draft of the manuscript.

GD, CCM and MG conceived the study.

GD and MS induced the experimental infections and performed the experiments with chickens.

GD, CCM, MS, MG and MH reviewed the draft manuscript.

All authors read and approved the final manuscript.

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Parasites & Vectors

RESEARCH

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Pattern and repeatability of ascarid-specific antigen excretion through chicken faeces, and the diagnostic accuracy of coproantigen measurements as compared with McMaster egg counts and plasma and egg yolk antibody measurements in laying hens

Oyekunle John Oladosu[1], Mark Hennies[2], Manuel Stehr[1], Cornelia C. Metges[1], Matthias Gauly[3] and Gürbüz Daş[1*]

Abstract

Background A coproantigen enzyme-linked immunosorbent assay (ELISA) has recently been proposed for detecting ascarid infections in chickens. The excretion pattern of ascarid antigens through chicken faeces and the consistency of measurements over the course of infections are currently unknown. This study evaluates the pattern and repeatability of worm antigen per gram of faeces (APG) and compares the diagnostic performance of the coproantigen ELISA with a plasma and egg yolk antibody ELISA and McMaster faecal egg counts (M-FEC) at different weeks post-infection (wpi).

Methods Faecal, blood and egg yolk samples were collected from laying hens that were orally infected with a mix of *Ascaridia galli* and *Heterakis gallinarum* eggs ($N=108$) or kept as uninfected controls ($N=71$). Measurements including (a) APG using a coproantigen ELISA, (b) eggs per gram of faeces (EPG) using the McMaster technique and (c) ascarid-specific IgY in plasma and in egg yolks using an ascarid-specific antibody ELISA) were performed between wpi 2 and 18.

Results Time-dependent significant differences in APG between infected and non-infected laying hens were quantified. At wpi 2 ($t_{(164)}=0.66$, $P=1.00$) and 4 ($t_{(164)}=-3.09$, $P=0.094$) no significant differences were observed between the groups, whereas infected hens had significantly higher levels of APG than controls by wpi 6 ($t_{(164)}=-6.74$, $P<0.001$). As indicated by a high overall repeatability estimate of 0.91 (CI = 0.89–0.93), APG could be measured consistently from the same individual. Compared to McMaster and antibody ELISA, coproantigen ELISA showed the highest overall diagnostic performance (area under curve, AUC = 0.93), although the differences were time-dependent. From wpi 6 to 18 coproantigen ELISA had an AUC > 0.95, while plasma IgY ELISA showed the highest diagnostic

*Correspondence:
Gürbüz Daş
gdas@fbn-dummerstorf.de
Full list of author information is available at the end of the article

performance in wpi 2 (AUC = 0.95). M-FEC had the highest correlation with total worm burden, while APG had highest correlations with weights and lengths of *A. galli*.

Conclusion Ascarid antigen excretion through chicken faeces can be measured with high accuracy and repeatability using a coproantigen ELISA. The antigen excretion increases over time, and is associated with worm maturation, particularly with the size of *A. galli*. Our results suggest the necessity of complementary use of different diagnostic tools for a more accurate diagnosis of infections.

Keywords Diagnosis, ELISA, Helminths, IgY, Nematodes, Repeatability

Background

The promotion of practices that improve the welfare of laying hens is increasing the use of non-cage housing systems. When outdoor access is provided, laying hens can better express their natural behaviour and have less fear and stress in a free-range system [1]. As a consequence of keeping hens in non-cage housing systems, gastrointestinal nematodes—in particular *Ascaridia galli* and *Heterakis gallinarum* with oral–faecal transmission routes—have become widespread and are associated with production losses even with minimal or absence of clinical signs [2–7]. In such systems, laying hens are in closer contact with excreta, allowing the oral–faecal transmission of helminth infection [3]. Curbing the spread of the infections and reducing their impact on hen productivity is largely dependent on early and accurate diagnosis.

There are several important criteria to consider when choosing a method of diagnosing helminth infection in livestock. The first is to correctly identify and differentiate all infected and non-infected animals (i.e., qualitative diagnosis). Early detection of helminth infection can prevent the spread of infection within and between flocks. It could also be crucial for employing a targeted flock treatment, which might be cost-effective and could mitigate the development of drug resistance among parasites [8]. The second criterion is for the diagnostic tool to be capable of assessing infection intensity (i.e., quantitative diagnosis) by establishing a significant correlation between its measurement outcome and the actual worm burden of the host animal. Estimating infection intensity is important to poultry and other livestock because the effects of helminth infection on the productivity, health and welfare of animals are likely greater with higher worm burden (e.g., [9]). A quantitative diagnosis is also essential when testing for the efficacy of anthelminthics, which currently relies on the reduction of faecal egg counts (FEC) or worm counts through necropsy [10].

Another criterion for diagnosing helminth infection is to correctly identify specific parasite species causing the infection. However, species-specific identification may not always be a priority in the context of livestock farming, especially because multiple species simultaneously co-infect the host [2, 4, 11], and broad-spectrum anthelminthics are often used in practice to control different intestinal helminths [12]. Under natural conditions, *A. galli* and *H. gallinarum* are known to co-infect the chicken host [11], and a single diagnostic assay is considered sufficient to detect the co-infections by both species [13]. Hence, priority should be accorded to diagnostic methods which have both high qualitative and high quantitative value for assessing nematode infection in chickens.

The gold standard for assessing the intensity of nematode infection is to count the number of worms in different developmental stages in the host intestine, but this requires post-mortem examination [14]. As an indirect method, the presence and intensity of ascarid infection in chickens is generally accomplished by microscopic counting of parasite eggs in faeces, i.e., faecal egg counts (FEC) [15]. McMaster and MiniFLOTAC egg counting techniques are commonly used for quantification of nematode eggs in the faeces of different host species. As shown in two independent studies on chicken ascarids, McMaster is more accurate and faster than MiniFLOTAC, even if the latter has higher precision and sensitivity at low faecal egg counts [16, 17]. Nevertheless, dependence on worm fecundity and infection intensity amongst other challenges can impair the reliability of FECs to assess nematode infections [18, 19]. Therefore, the measurement of ascarid-specific immunoglobulin Y (IgY) in host plasma and egg yolks has been suggested as an alternative diagnostic method to FECs [13, 20]. Recently, we introduced a coproantigen enzyme-linked immunosorbent assay (ELISA) that can quantify soluble ascarid antigens in the faeces of the chicken host with high qualitative diagnostic accuracy [21]. Daş et al. [22] showed that the development of humoral response against *A. galli* in chickens is time-dependent, and larval stages are more strongly associated with antibody stimulation than the adult stages. Such time- and developmental stage-associated changes may also be expected for worm antigen excretion. The test performance of both antibody- and antigen-measuring ELISAs has not yet been compared. Moreover, both ELISAs have so far been separately evaluated only at a single necropsy time point in patent infections [13, 20, 21]. Thus, the evaluations could not account

for time-dependent variation in the production of antibodies in plasma or in egg yolks as well as in the excretion of antigens through hen faeces. In addition, there is at present no report on the pattern of worm antigen excretion throughout different phases of infections, i.e., whether worm antigens in host faeces can be consistently measured on the same individual over time.

Therefore, the first objective of this study was to assess the faecal worm antigen excretion pattern over an 18-week period to address time-dependent changes in antigen excretion due to progressing patency and re-infections. This also included estimating the repeatability of faecal antigen concentration measurements in host faeces within and between different weeks post-infection (wpi). The second objective was then to compare the diagnostic performance of the coproantigen ELISA with different diagnostic tools, including faecal egg counts and the measurement of anti-ascaridia antibody in plasma and egg yolk. The ability of the diagnostic methods to estimate the intensity of infection at different wpi was then evaluated.

Methods

Ethics statement

The ethics committee for animal experimentation from the Mecklenburg-Western Pomerania State Office for Agriculture, Food Safety and Fisheries, Germany, gave approval for the experiment (permission number AZ.: 7221.3-1-080/16). The experimental procedure for infections followed the guidelines listed by the World Association for the Advancement of Veterinary Parasitology for poultry [23]. Animal handling, care, pen and cage housing, stunning, killing and necropsies were performed by trained and authorized staff members according to the ethical permission and animal welfare rules.

Experimental design and sample collection

Blood, egg yolk, and faeces samples collected from a total of 179 laying hens of the Lohmann Brown Plus genotype (LB, $N=109$) and Lohmann Dual genotype (LD, $N=70$) were used for this study. The laying hens used in this work originated from a previous study [24], where we evaluated the tolerance and resistance of laying hens of different genotypes to nematode infections. The hens were obtained from a research farm (Farm for Education and Research in Ruthe, University of Veterinary Medicine Hannover) as 17-week-old pullets and were randomly allocated into two adjacent rooms each containing six pens. In each room, the hens were kept in three pens per genotype (i.e., three pens for each of the LB and LD genotypes). At the beginning of the experiment, the number of hens kept in the same pen ranged from 8 to 25, with an adjustment for stocking density of a maximum six hens/m^2. Each hen was given a wing tag to enable repeated measurements on the same individuals over time.

At 24 weeks of age, the hens in six pens of the first room ($N=108$) were experimentally infected with *A. galli* and *H. gallinarum*, while the hens in the six pens of the next room ($N=71$) were kept as uninfected controls. A consort diagram presenting the number of hens per genotype and infection status, necropsy time points and sampling schemes is given in Fig. 1. At wpi 0, 2, 4, 8, 12 and 16, a total of 29–34 hens were randomly selected from their pens (i.e., all pens were sampled with at least one hen), and transferred to individual cages where they remained for 2 weeks prior to individual egg collection and quantitative faeces sampling (i.e., 24-h sampling). The cages (W 40 × L45 × H 50 cm) with a wire mesh bottom were placed on a faeces collection plate that enabled quantitative daily faeces collection from individual hens. The cages provided equipment for ad libitum water and feed intake of the hens. After an adaptation period of 10 days in the cages, daily individual faeces (g/24 h) were quantified from each hen repeatedly for four consecutive days prior to slaughter. The daily faeces were homogenized thoroughly and subsamples for antigen measurements were stored at −20 °C. After 2 weeks of captivity in the cages, i.e., at wpi 2, 4, 6, 10, 14 and 18, all the caged hens were killed by stunning using a bolt shoot followed by bleeding to death. At each time point, starting from 2 to 14 wpi, 18 infected and 11 control hens were killed for necropsy, while the remaining 18 infected and 16 control hens were killed at wpi 18 (Fig. 1). Immediately after killing, slaughter blood was collected in potassium-ethylenediaminetetraacetic acid (EDTA)-treated tubes (Kabe Labortechnik GmbH, Nümbrecht-Elsenroth, Germany), and the hens were necropsied to assess worm burden as a direct measure of infection intensity. The blood samples were centrifuged for 20 min at 2500×*g*, and the resulting supernatant was stored at −20 °C for later analysis. Individual eggs were collected from each hen during the last day of captivity or at slaughter. Sampled eggs were opened to collect the egg yolks. A subsample of egg yolk (250 µL) was collected and diluted with 1.5 ml of purified water (pH = 2.5) and homogenized using a vortex mixer. The egg yolks were stored at −20 °C until analysis. A total of 179 blood and egg yolk samples and 716 (i.e., 179 hens × 4 days) faecal samples were therefore recorded from all laying hens throughout the experimental period.

Wood shavings were used as the litter materials in the pens. On the day of the infection, the litter was renewed and thereafter left in the pen for 18 weeks to allow subsequent natural infection to occur. All hens were fed a commercial diet (ad libitum), containing 11.2 MJ

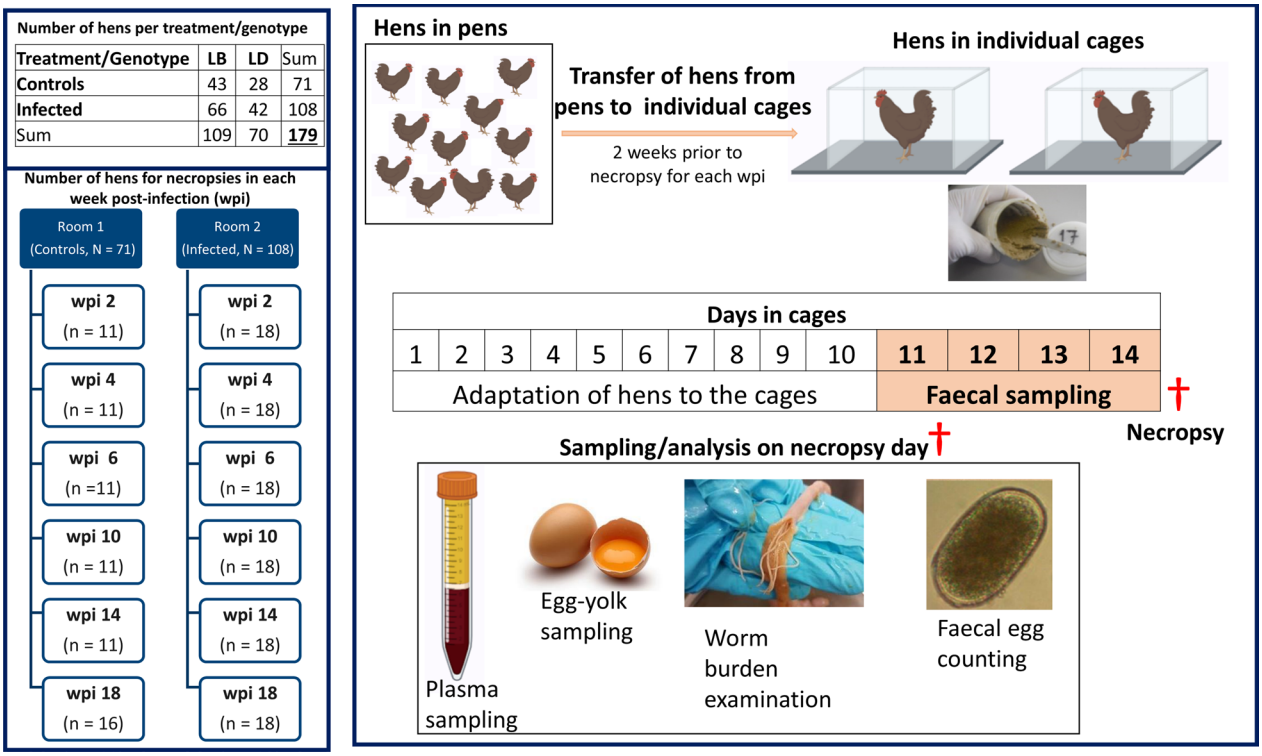

Fig. 1 A diagram presenting experimental flow with time-specific necropsies, pen and cage housing schemes, and the number of hens sampled for faeces, blood and egg yolk

metabolizable energy, 170 g crude protein and 3.6 g calcium/kg feed for laying hens [24]. The climatic conditions in the rooms were controlled using an automatic system to ensure constant temperature, light and aeration across all the pens.

Experimental infection procedures and measurements of infection intensity

Infection material for the experiment was collected from female worms residing in the intestines of free-range hens that were naturally infected with ascarids. The procedure for worm recovery and isolation from chicken intestines and the embryonation of eggs of *A. galli* and *H. gallinarum* have been described previously [25]. For the embryonation of *A. galli* eggs, 0.1% potassium dichromate ($K_2Cr_2O_7$) was used as the incubation medium, whereas intact *H. gallinarum* females were kept in formalin (0.5%) at room temperature for approximately 4 weeks. Eggs of *H. gallinarum* were isolated from the worms 1 day prior to infection, as described in Stehr et al. [25]. For the preparation of infection material to be given to the hens, the embryonated eggs of both species were rinsed in a 36-µm sieve and collected in 0.9% NaCl in two separate egg pools (i.e., for *A. galli* and *H. gallinarum* separately) at room temperature. The egg pools were assessed to determine the percentage of eggs that were fully embryonated [26]. After adjustment of the concentrations of the embryonated eggs in the 0.9% NaCl solution (i.e., 500 eggs of *A. galli* or *H. gallinarum* in 0.2 ml NaCl), a total of 1000 embryonated eggs of both species in 0.4 ml of NaCl was given to each hen. The hens were inoculated with the infective eggs of the two parasites using a 5-cm oesophageal cannula in a single dose (i.e., 500 *A. galli* + 500 *H. gallinarum* eggs). The uninfected control hens were given an oral placebo containing 0.4 ml of 0.9% NaCl.

Worm burden

Worm burden was quantified from laying hens that were necropsied at wpi 2, 4, 6, 10, 14 and 18. The hens were subjected to fasting for 3 h prior to necropsy to partly empty the gastrointestinal tract (GIT). The GIT was removed immediately after the necropsy, and the small intestine and caecum, the predilection sites of *A. galli* and *H. gallinarum*, respectively, were separated [24]. The jejunum and ileum were then opened longitudinally to wash the intestinal contents through a sieve (mesh size: 36 µm and 100 µm at 2–6 wpi and 10–18 wpi, respectively). After removal of intestinal content, the jejunum was rinsed under running lukewarm tap water while

simultaneously squeezing the tissue through a pair of fine pencil-pincers to remove the lumen worms attached to the tissue walls. Tissue larva recovery was done only in the jejunum using an EDTA incubation [27]. Briefly, the jejunum was incubated in 400 ml of preheated EDTA solution (10 mM EDTA, 0.9% NaCl) for 22 h at 40 °C. After incubation, the tissue was dipped in EDTA solution to remove the larvae. The solution was sieved through a sieve (mesh size: 20 µm) to collect the tissue larvae.

All recovered worms of both species from each hen were placed in Petri dishes for counting, sex differentiation and length measurements using a stereomicroscope. The total number of each of the *A. galli* and *H. gallinarum* worms present in the small intestine and caeca were recorded separately. Worm burden was recorded based on the identified morphological characteristics of worms such as sex (male or female) and by developmental stage (i.e., larvae, mature, immature) [25]. Briefly, identification of male worms was based on the presence of spicules. Worms of *H. gallinarum* were classified as adult females if eggs were present in the uterus. *Ascaridia galli* worms were classified using a predetermined cut-off (43.5 mm) to precisely separate ovigerous females (>43.5 mm) from immature females (<43.5 mm) [25]. Worm length was measured for both *A. galli* and *H. gallinarum* using a ruler with measurement precision of 1 mm. Length measurement was based on the worm classification (i.e., larvae, mature immature, males). Only intact worms for each classification (maximum of 10 per bird) were randomly selected and measured. The average of the selected worms was multiplied by the total number of worms in each classification [25]. The weight (mg) of *A. galli* was estimated from the length (mm) of female and male *A. galli* using the Le cren weight–length relationship model [28]. The average weight of *A. galli* was also calculated with respect to the total *A. galli* burden in each hen. To establish the Le cren weight–length relationship, we used a data set from a previous experiment (Additional file 1: Fig. S1), where both the weight and length of *A. galli* were precisely measured. For the measurement of *A. galli* weight, a precision (0.1 mg readability) analytical balance (Mettler Toledo GmbH, Gießen, Germany) was used. The precision of length measurements was the same as in the present study (i.e., 1 mm).

Faecal egg counts (FEC)

At each time point of necropsies (i.e., wpi 2–18), a random subsample (4 g) was obtained from thoroughly mixed daily faeces collected 1 day prior to hen necropsy and analysed with the McMaster egg counting technique [15]. A saturated NaCl solution (density = 1.2 g/ml) was used as the flotation fluid for the 4 g of faeces, which was then made up to 60 ml of the final suspension. The minimum detection level of the egg counting technique was set to 50 eggs per gram of faeces (EPG). Since eggs of *A. galli* and *H. gallinarum* cannot reliably be differentiated from each other [29], and regular faecal droppings cannot be precisely separated from the caecal droppings following a 24-h collection period, both regular and caecal droppings were mixed, and ascarid eggs were counted together.

Quantification of ascarid-specific antigens in faeces and ascarid-specific IgY in plasma and egg yolk

Homogenized faecal subsamples taken from the daily faeces of hens during the last 4 days of captivity (i.e., $n = 4$ samples/hen) were measured for worm antigen concentration according to the ELISA procedure described by Oladosu et al. [21]. Briefly, soluble antigens from *A. galli* were isolated from thawed intact worms by washing with phosphate-buffered saline (PBS) and 70% ethanol. Afterwards, the worms were homogenized in a mortar and extracted using basic buffer (35 mM Bis-Tris, 25 mM Tris). Extracted soluble antigen was used to immunize rabbits for the production of antibody. ELISA plates were coated overnight at 4 °C with 100 µl of the anti-ascarid polyclonal rabbit antibody to allow binding with soluble antigen in faecal samples. A total of 50 mg of daily faecal samples was weighed in sample buffer and mixed thoroughly with a vortex mixer. The supernatant was collected and pipetted into assay wells. An aliquot of 100 µl of soluble antigen with concentrations of 400, 200, 100, 50, 25 and 0 ng/ml were also added to the assay wells for standardization. The plates were then incubated and washed repeatedly before measurements. The antigen concentration in faeces was then measured as the amount of ascarid antigen per gram of faeces (APG, µg/g faeces) based on the standard curve for 400, 200, 100, 50, 25 and 0 ng soluble antigen/ml [21]. Anti-ascarid-specific IgY in plasma and egg yolk was quantified using another ELISA [13]. The microtiter plates were coated overnight at 4 °C with 100 µl of the isolated soluble *A. galli* antigen. Standard chicken serum was serially diluted and used as the standard curve in the assay. Samples were added to the coated plates and incubated for 2 h. After incubation, the plates were washed repeatedly followed by another 30 min incubation with enzyme conjugate, washing step and termination with hydrochloric acid. Antibody binding was expressed relative to the standard chicken serum with high antibody activity (1000 mU/ml per definition) using a four-parameter logistic (4-PL) [13].

Statistical analyses

Data were modelled based on the measurement of each variable, i.e., either single measurements or repeated measurements from a host over time. The relevant worm

burden parameters (worm counts, worm length, FEC, etc.) were measured at a single time point during necropsy, while antigen concentration was measured in each of the faecal samples collected on four consecutive days prior to necropsy (Fig. 1). APG, egg yolk IgY and plasma IgY data were analysed after log transformation [log(y+1)] to correct for the heterogeneity of variance and produce approximately normally distributed data. A description of all the variables measured is presented in Table 1.

Significant differences in the antigen and antibody concentrations between infected hens and non-infected control within wpi and their interactions were analysed with repeated-measures analysis of variance (ANOVA) using the PROC MIXED function of the SAS OnDemand for Academics cloud-based software (2021 SAS Institute Inc., Cary, NC, USA). The repeated statement was excluded for antibody concentration in plasma and egg yolk since only a single measurement at necropsy was done. The model for antigen and antibody concentrations included the fixed effects of infection, wpi and their interactions, while pen, host genotype and sampling day were considered as blocking effects in the analysis. The covariance structure was set to AR (autoregressive) (1) for the fitted model. Least-square means were computed for each fixed effect, and pairwise comparison was tested with the Tukey–Kramer corrections for multiple comparisons. Effects and differences were considered significant at $P<0.05$.

The intra-class correlation coefficient (ICC) between the repeatedly measured samples within each wpi was estimated to determine the repeatability of faecal antigen excretion. Faecal samples from both infection groups for quantifying antigen concentration were repeatedly collected from the same laying hens for each of four consecutive days ($n=4$ samples per hen) in each wpi ($n=4\times29$–34 samples per wpi). ICC estimates of these four repeated measurements and their 95% confidence intervals (CI) were calculated using the ICC function in the R psych package version 2.1.9 [30]. Estimates were based on the absolute agreement of measurements ($k=4$), two-way mixed-effects model [31]. Measurements with ICC values of less than 0.5 were defined as having poor reliability, values between 0.5 and 0.7 as moderate reliability, values between 0.75 and 0.9 as good reliability, and values greater than 0.9 as excellent reliability [31].

A receiver operating characteristic (ROC) analysis was performed to assess and compare the diagnostic accuracy of coproantigen ELISA with that of FEC, plasma, and egg yolk IgY ELISA using samples collected 1 day prior to slaughter. The pairwise comparison of the area under the curve (AUC) from all diagnostic tests was carried out using the DeLong post hoc test [32] because the faecal, egg yolk and blood samples used for the FEC, coproantigen and the IgY measurements were made on the same host. Test accuracy of the assays was interpreted based on the range of the AUC value and is classified as follows: low accuracy ($0.5<\text{AUC}\leq0.7$), moderate accuracy ($0.7<\text{AUC}\leq0.9$) or high accuracy ($\text{AUC}>0.90$) [33].

The data sets used for ROC comparison included APG, EPG, plasma, and egg yolk IgY values of experimentally infected laying hens with their corresponding controls obtained during necropsy. The analysis was performed for both data pooled across wpi and within wpi to determine differences between different time points, i.e., potential time-dependent differences in overall performance of different tests. In any case, the total number of observations ($n\geq28$) used for the analysis exceeded the minimum sample size required for an ROC analysis. The minimum sample size was calculated using MedCalc statistical software version 20.023 [34] with a preset significance level of 0.05, maximum AUC of 0.99 and group ratio of 1 [33]. All parameters of the ROC and DeLong comparison test were calculated using the pROC open-source package for R [35].

Pearson correlation coefficients were calculated to determine the interdependence among infection-related parameters including worm burden, worm length, worm weight, FECs and antigen concentration in faeces. The analysis was based on log-transformed data [log(y+1)]. To further assess the quality of each diagnostic method, Pearson correlations among all variables (i.e., ascarid-specific IgY in plasma and egg yolk, APG and EPG with worm burden parameters) were examined within each wpi. Pearson correlation analysis, descriptive statistics and visualization of data were performed in the R environment for statistical computing [36].

Table 1 Abbreviation, measurement unit, and short description of relevant variables

Abbreviation	Unit	Description
APG	µg/g faeces	Antigen per gram of faeces
Ag_Length	cm	*A. galli* length
Hg_Length	mm	*H. gallinarum* length
EPG	*n*/g faeces	Number of eggs per gram of faeces
Ag_Total	*n*/hen	Total number of *A. galli* in a hen
Hg_Total	*n*/hen	Total number of *H. gallinarum* in a hen
Ag_Weight	mg/hen	Average weight of all *A. galli* in a hen
Egg yolk_IgY	mU/mL	Ascarid-specific IgY in egg yolk
Plasma_IgY	mU/mL	Ascarid-specific IgY in plasma

Table 2 Effects of mixed-nematode infection on ascarid-specific IgY in plasma and egg yolk and the concentration of ascarid antigens in faeces of uninfected control or infected chickens

Variables	Infection status				Statistics								
	Control		Infected		Infection			WPI			Infection × WPI		
	LSM	SE	LSM	SE	*P*	*df*	*F*	*P*	*df*	*F*	*P*	*df*	*F*
Plasma IgY (mU/mL)	23.5	10.75	86.3	8.91	0.001	164	82.85	0.131	164	1.73	0.001	164	4.56
Egg yolk IgY (mU/mL)	3.77	7.81	41.94	6.53	0.001	151	95.38	0.009	151	3.19	0.008	151	3.26
APG (µg/g faeces)	0.13	0.04	0.65	0.03	0.001	164	211.05	0.001	164	18.6	0.001	164	15.85

Statistical analyses of the data are based on log-transformed data [log(y+1)]. whereas least-squares means (LSM) and their standard errors (SE) are based on untransformed data

APG was quantified from birds on four consecutive days prior to necropsy in each week. Thus, the number of observations used for the statistical analysis was $N = 179$ hens × 4 days = 716 APG measurements

The data used for the analysis of plasma and egg yolk IgY are based on single measurements at necropsy in each wpi ($n = 179$)

APG ascarid antigen concentration per gram of faeces, *IgY* ascarid-specific IgY in plasma or egg yolk, *P* *P*-value, *df* degrees of freedom, *F* *F*-value

Results

Ascarid antigen concentration in host faeces

The fixed effects of infection ($F_{(1,164)} = 211.05$, $P < 0.001$), wpi ($F_{(5,164)} = 18.60$, $P < 0.001$) and their interactions ($F_{(5,164)} = 15.85$, $P < 0.001$) on antigen concentration (APG) were significant (Table 2). Tukey–Kramer adjusted pairwise comparison for within-wpi effect revealed that antigen concentration was not significantly different between control and infected animals at wpi 2($t_{(164)}$, = 0.66, $P = 1.00$) and wpi 4 ($t_{(164)}$, = −3.09, $P = 0.094$) (Fig. 2). APG increased in infected laying hens by wpi 6 ($t_{(164)}$, = −6.74, $P < 0.001$), and significant differences ($P < 0.001$) were then observed between infected and uninfected laying hens until the end of the experiment at wpi 18. Control hens were not significantly different (post hoc, Tukey–Kramer adjusted $P > 0.05$) in

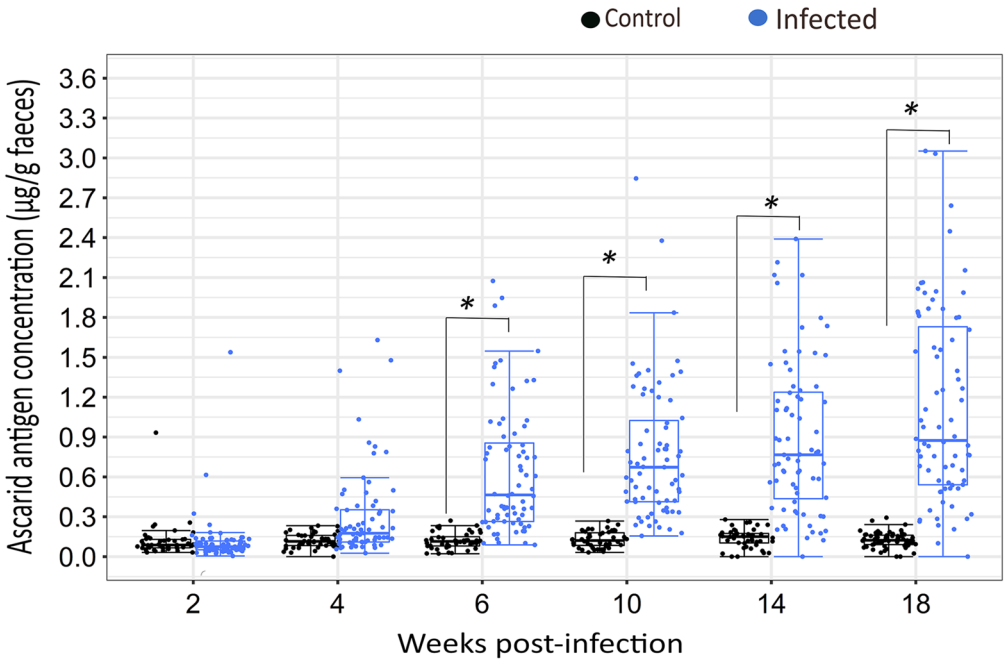

Fig. 2 Concentration of ascarid antigens in faeces (APG) of control (black circle) and infected (blue circle) laying hens. The effect of infection, wpi and their interaction were significant ($P < 0.001$). Statistical analyses are based on log-transformed data [log(y+1)] while visualization is based on untransformed data. * Indicates a significant difference between control and infected laying hens at a given time point (Tukey–Kramer, $P < 0.05$). Each dot on the plot represents an individual observation. Number of observations ($N = 716$) refers to 179 hens sampled for four consecutive days prior to slaughter. The vertical line inside the box plots shows the sample median, while the lower and upper ends of the box represent the 25th and 75th quantiles, respectively

their antigen concentration across different wpi throughout the experiment (Fig. 2), whereas there was a statistically significant increase ($P<0.05$) in the APG values of infected laying hens across the wpi. APG in the infected laying hens was significantly different ($t_{(164)}$, = −4.62, $P<0.001$) in the early stages of infection (between wpi 2 and 4), while at later phases (e.g., between wpi 6–18, [$t_{(164)}$, = −3.09, $P=0.094$]) there was no significant difference in the APG of infected laying hens.

Repeatability of worm antigen excretion through host faeces

The ICC estimates of the repeated measure of APG are given in Fig. 3. Overall, the analysis revealed a high repeatability estimate (ICC = 0.91; 95% CI = 0.89–0.93) for APG measurement from the same animal across four repeated measurements within a wpi. There were fluctuations, however, in the repeatability of APG across different weeks. The ICC estimates for the measurements in wpi 2 were low (ICC = 0.08; 95% CI = 0–0.47), but APG measurements throughout the remaining wpi showed moderate to high repeatability estimates (ICC 0.78–0.96). The highest repeatability estimate (ICC = 0.96; 95%CI = 0.94–0.98) was recorded in measurements obtained in wpi 6.

Ascarid-specific IgY in host plasma and egg yolks

A statistically significant difference was found in overall ascarid-specific IgY concentration in both plasma

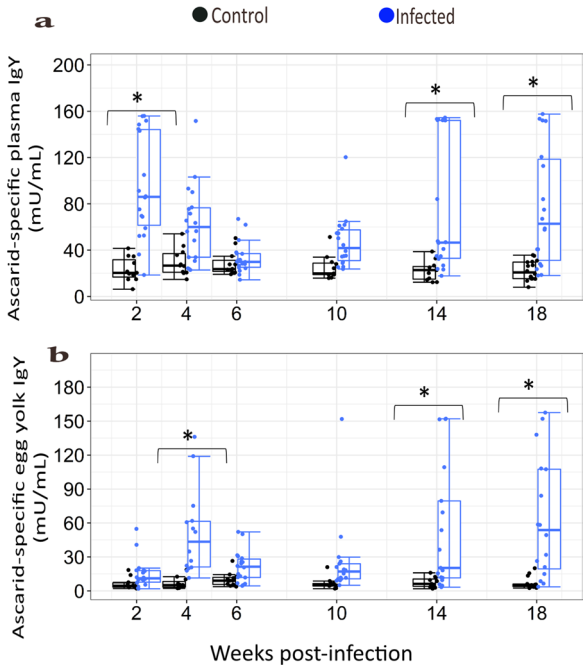

Fig. 4 Ascarid-specific IgY concentrations in **a** plasma and **b** egg yolks of control (black circle) and ascarid-infected laying hens (blue circle). The effect of infection and interaction with wpi were significant ($P<0.001$). Statistical analyses are based on log-transformed data [log(y+1)] while visualization is based on untransformed data ($n=179$ hens). * Indicates a significant difference between control and infected laying hens at a given time point (Tukey–Kramer, $P<0.05$). Each dot on the box plot represents an individual observation. The vertical line inside the box plots shows the sample median, while the lower and upper ends of the box represent the 25th and 75th quantiles, respectively

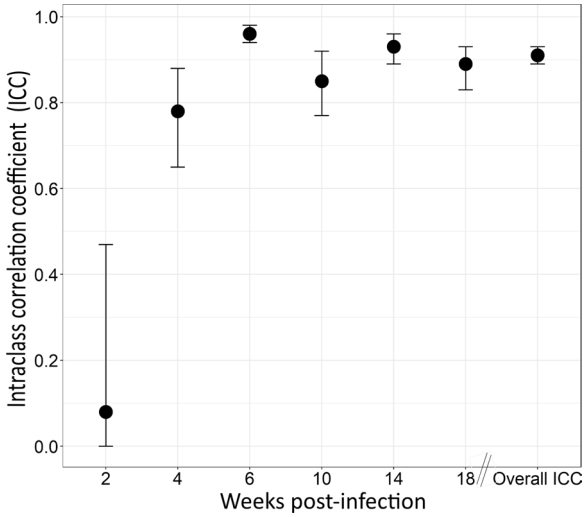

Fig. 3 Intra-class correlation coefficient (ICC) of repeated measurements of antigen concentration per gram of faeces within each week post-infection (wpi) and the overall ICC. Number of hens necropsied, $n=29$ in each wpi, and in wpi 18, $n=34$ (overall $N=179$ hens). Error bars represent 95% confidence intervals

($F_{(1,164)}=82.85$, $P<0.001$) and egg yolk ($F_{(1,151)}=95.38$, $P<0.001$) between infected and uninfected controls (Table 2). The overall average IgY concentration across all wpi in infected laying hens was higher than that in uninfected controls. However, the differences were time-dependent. The differences in the antibody response in plasma between the two groups were significant at wpi 2 ($t_{(164)}=-6.35$, $P<0.001$), wpi 14 ($t_{(164)}=-5.05$, $P<0.001$) and wpi 18 ($t_{(164)}=-5.37$, $P<0.001$) (Fig. 4a), while significant differences in egg yolk IgY concentrations were observed at wpi 4 ($t_{(151)}=-5.78$, $P<0.001$), wpi 14 ($t_{(151)}=-4.67$, $P=0.0004$) and wpi 18 ($t_{(151)}=-6.29$, $P<0.001$) (Fig. 4b).

Worm burden and FEC

A detailed presentation of worm burden in both host genotypes has been reported previously [24]. Figure 5 provides visual representations of the worm burden of the hens with *A. galli* and *H. gallinarum* as well as FEC resulting from both nematodes. The number of worms was highest at wpi 2 for both *A. galli* (Fig. 5a) and *H.*

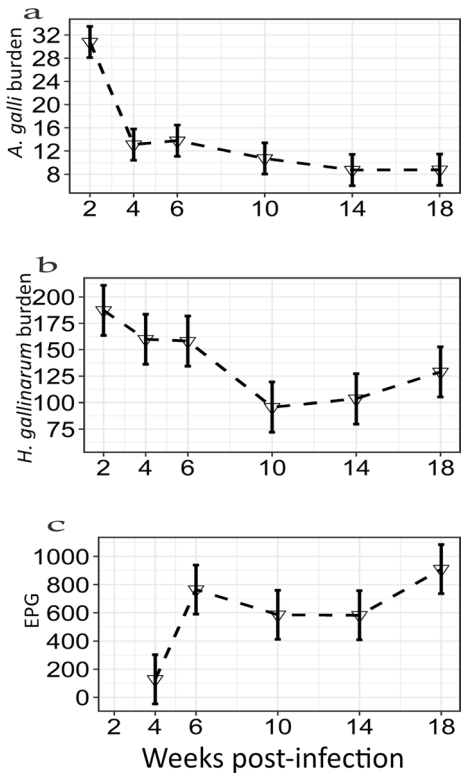

Fig. 5 Worm burden of laying hens experimentally infected with both *Ascaridia galli* (**a**) and *Heterakis gallinarum* (**b**), and the number of eggs per gram of faeces (EPG) (**c**) across different weeks post-infection ($n = 108$). Figures are LS means with their standard errors. The EPG was determined by wpi 4. Thus, the number of faecal samples for EPG was $n = 90$

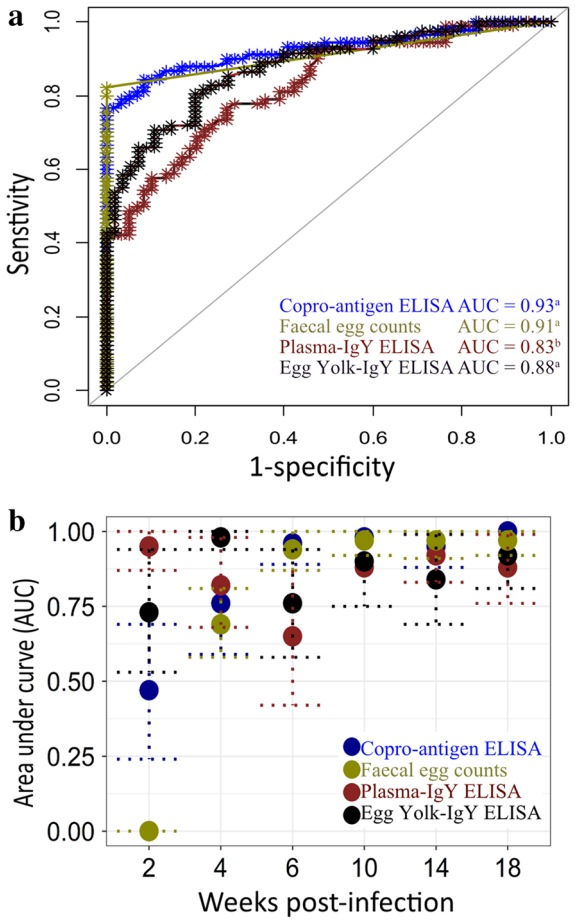

Fig. 6 Overall (**a**) and time-point-specific (**b**) diagnostic accuracy of the coproantigen ELISA in comparison with plasma and egg yolk IgY ELISA and faecal egg counts in chickens infected with *Ascaridia galli* and *Heterakis gallinarum*. The diagonal line corresponds to half of the maximum area under the curve (AUC = 1.0). The DeLong test was used to determine significant differences between the AUC of each diagnostic test. The significance level was preset at $P < 0.05$. AUC values bearing the same superscripts showed no significant difference. The farther the location of the ROC curve from the diagonal line, the higher the total test accuracy analyses. A summary of AUC values with their confidence intervals for all diagnostic methods within each wpi is shown in (**b**)

gallinarum (Fig. 5b). The number of *A. galli* worms decreased over time throughout the experimental period such that the lowest number of worms was recovered by wpi 18, whereas the lowest count of *H. gallinarum* was recovered in wpi 10. The number of *H. gallinarum* increased from wpi 14 through wpi 18 due to re-infections. EPG was not quantified until wpi 4 (Fig. 5c). The average EPG increased between wpi 4 and wpi 6 and then remained relatively constant until wpi 14, while the highest average EPG was observed at the last wpi. Worm eggs were not present in the faeces of control laying hens throughout the period of the experiment.

Diagnostic accuracy of coproantigen ELISA, antibody ELISA and FEC

ROC analysis was carried out to investigate the diagnostic accuracy of the coproantigen ELISA compared with FEC, egg yolk and plasma IgY ELISA using all measurements taken within each wpi during the experiment. The outcomes of the ROC analysis are summarized in Fig. 6, and detailed results with specific test performance parameters (e.g., AUC, sensitivity, specificity) are presented in Additional file 2: Table S1. The overall accuracy for coproantigen ELISA was high, with AUC = 0.93. Except for wpi 2 and 4, high accuracy (AUC > 0.90) was confirmed for this method within all wpi. Its diagnostic test accuracy was highest at wpi 18 (AUC = 1.00). Similarly, the specificity and sensitivity of the assay increased over time; by wpi 4, the assay yielded 100% specificity but with low sensitivity of 39%. To fully compare the accuracy of the coproantigen ELISA method (i.e., APG) with faecal egg counts (i.e., EPG) and plasma and egg yolk IgY ELISA, the available data for EPG and IgY assay

were used. The overall AUC for FEC was 0.91, while plasma and egg yolk IgY assay yielded overall accuracy of AUC = 0.83 and 0.88, respectively (Fig. 5a). The DeLong test showed no significant difference between coproantigen ELISA and FEC ($Z = 0.674$, $P = 0.501$) and egg yolk IgY ELISA ($Z = 1.645$, $P = 0.100$), whereas the overall accuracy of plasma IgY assay was significantly ($Z = 2.336$, $P = 0.019$) lower. Both FEC and coproantigen ELISA had 100% specificity, while specificity was lower for the plasma IgY assay (72.9%) and egg yolk IgY assay (80%). FEC demonstrated the highest sensitivity, at 82.2%, followed by egg yolk IgY with sensitivity of 80%, while both coproantigen and plasma IgY ELISA had sensitivity of 76.7%.

Correlations among infection-related parameters

We investigated the linear relationships among all infection-related parameters using Pearson correlation statistics with both pooled data across all the weeks (Fig. 7a) and within each wpi (Fig. 7b) by each diagnostic measurement. The parameters associated with the length and weight of the worms demonstrated higher positive correlation coefficients with APG than EPG and both IgY. The total average length of *A. galli* showed a significant positive correlation with APG ($r_{(105)} = 0.69$, $P < 0.001$) and EPG ($r_{(87)} = 0.65$, $P < 0.001$). In the case of *H. gallinarum*, the total worm length demonstrated significant positive correlations with APG ($r_{(102)} = 0.61$, $P < 0.001$) and EPG ($r_{(84)} = 0.38$, $P = 0.030$). However, a negative correlation was found between plasma IgY and the total length of both *A. galli* ($r_{(105)} = -0.31$, $P = 0.100$) and *H. gallinarum* ($r_{(102)} = -0.40$, $P = 0.010$). The weight of *A. galli* showed a significant correlation ($r_{(105)} = 0.71$, $P < 0.001$) with APG and EPG ($r_{(87)} = 0.68$, $P < 0.001$).

Only EPG exhibited a high correlation with the total worm counts of both *A. galli* ($r_{(87)} = 0.44$, $P < 0.001$) and *H. gallinarum* ($r_{(87)} = 0.32$, $P = 0.110$). However, when we examined the correlation with the number of worms by maturity, there was a significant positive correlation between APG and the total number of adult worms ($r_{(105)} = 0.6$, $P < 0.001$ for *A. galli* and $r_{(105)} = 0.52$, $P < 0.001$ for *H. gallinarum*), and a negative correlation with the total number of larvae ($r_{(105)} = -0.59$, $P < 0.001$ for *A. galli* and $r_{(105)} = -0.44$, $P < 0.001$ for *H. gallinarum*). EPG correlated negatively with the *A. galli* larvae ($r_{(87)} = -0.31$, $P = 0.160$) and positively with mature worms ($r_{(87)} = 0.69$, $P < 0.001$ with *A. galli* and $r_{(87)} = 0.33$, $P = 0.070$ with *H. gallinarum*). Plasma IgY demonstrated

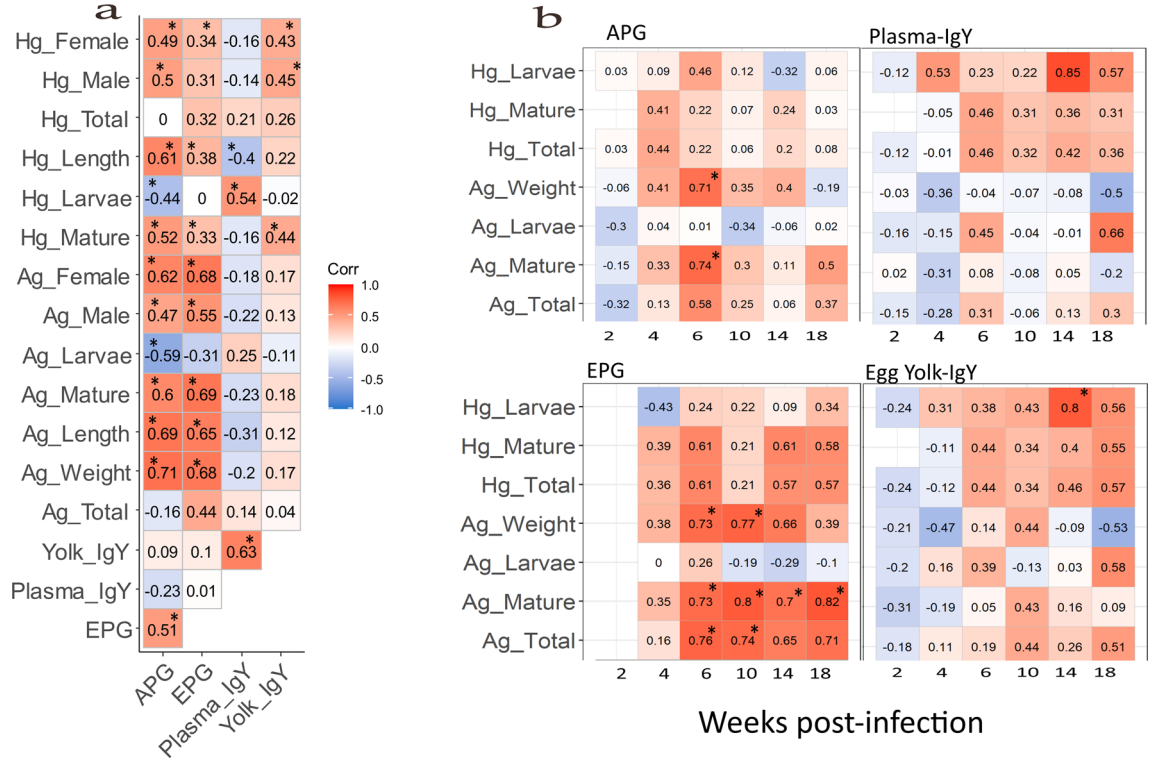

Fig. 7 Overall correlations with pooled data across wpi among different variables representing worm burden and diagnostic measurements (**a**), and Pearson correlation coefficients between each worm burden variable and APG, EPG and IgY within each wpi (**b**) ($n \geq 29$). The Pearson correlation coefficients (r) are presented in the squares. Significant ($P < 0.05$) correlations are indicated with an asterisk (*)

a positive correlation with *H. gallinarum* larvae ($r_{(105)}=0.54$, $P<0.001$) and *A. galli* larvae ($r_{(105)}=0.25$, $P=0.370$) but egg yolk IgY did not. We further investigated whether the correlations between worm burden and infection proxies are time-dependent. The result in Fig. 7b shows that the highest correlation occurred at different wpi for each of the four infection proxies. Correlations were lowest at wpi 2 for all methods. In general, EPG showed significant positive correlations with worm counts and size measurements in most wpi (Fig. 7b; Panel EPG).

Discussion

This study assessed the excretion pattern and repeatability of ascarid antigen in the faeces of laying hens over time and evaluated the performance of four different methods of diagnosing nematode infections, with an emphasis on alterations in test performance at different time points of infection. The methods included McMaster faecal egg counts, coproantigen ELISA, and plasma IgY and egg yolk IgY ELISA. It is evident from the results that soluble worm antigens are rather consistently excreted through the faeces of laying hens. Antigen concentrations increased over time in the faeces of infected hens, indicating the possibility that as worms mature, antigen concentration increases. In a time-dependent manner, increased antigen concentration may also be a reflection of higher worm burden and re-infection.

To confirm whether APG can be consistently measured on the same hen over time, we performed repeatability estimates of antigen excretion by measuring the antigen concentration from faecal samples of the same individual hens for four consecutive days within a given wpi. Repeatability is the measure that reflects the extent to which a set of measurements can reliably be replicated by measuring both the correlation and absolute agreement among the obtained values [37]. The overall repeatability (ICC = 0.91) confirms a considerable repetitive pattern in the excretion of antigens in faeces. From wpi 4, when antigen could be reliably quantified, the agreement among measurements across the four sampling days was high, suggesting that antigen excretion in faeces of the infected hens consistently occurred daily and is reliably measurable on the same animal.

Furthermore, the results also showed unique time-dependent differences in the test performance of different diagnostic methods, implying the need for specific selection of one or two tools to capture a more informative indication of nematode infections in chickens. Faecal egg counts are widely used for assessing *A. galli* and *H. gallinarum* infections, but due to challenges such as variability in worm fecundity, diurnal variation in excretion of eggs and uneven distribution of eggs in faeces [19, 38], new methods have been investigated. This includes measuring worm-specific antibody in host plasma and egg yolks [13, 20] and soluble worm antigens in host faeces [21], as well as PCR-based approaches [39]. Ascarid-specific plasma IgY was already significantly different between infected and non-infected hens at wpi 2, which is in agreement with all relevant past studies measuring immune response to nematode infection in chickens [22, 40–42]. We deduce that diagnosis with antibody ELISA can provide earlier detection of infection than faecal egg counting or coproantigen ELISA. *Heterakis gallinarum* larvae are carried to caeca nearly 9 h after the host's ingestion of the ova, where the larvae embed themselves into the superficial epithelium for a short period of time [43]. Similarly, the larvae of *A. galli* have a tissue-associated phase [44]. Thus, at wpi 2, the hosts' small intestine and caeca walls are mainly colonized by larvae, and almost no adult worms are present at this time, but by wpi 4, antibody response—as quantified by ELISA—decreases, coinciding with the presence of maturing worms in the lumen. This supports the idea that the larvae, which penetrate the intestinal wall of infected chickens, elicit a stronger humoral response than the adult worms that have migrated to the lumen [41]. Therefore, the migration of larvae from the intestinal walls to the lumen may have resulted in lower antibody production in subsequent weeks. However, at wpi 14, indicating the presence of the next generation of larvae due to re-infection, antibody response was again significantly higher in infected hens. The study by Marcos-Atxutegi et al. [41] established that soluble antigen from embryonated eggs stimulates a higher antibody concentration measured by ELISA than the adult worm antigen. Similarly, our results from the correlation between plasma IgY and worm stages further demonstrated that plasma IgY has a stronger positive relationship with the number of larvae than with adult worms. This result is also consistent with a previous report shown by Daş et al. [22]. This relationship was much stronger with *H. gallinarum* larvae than with *A. galli*, likely because of the higher re-infection with *H. gallinarum* than with *A. galli* [24].

As the chicken host develops cross-reactive antibodies against the two closely related nematode species [13], higher re-infection with *H. gallinarum* than with *A. galli* might explain higher correlations between ascarid-specific IgY and *H. gallinarum* larvae counts. Despite studying multiple time points, plasma IgY had no significant relationship with total worm burden, making it less suitable for quantitative diagnosis of nematode infection in chickens. However, in terms of qualitative diagnostic assessment, measuring ascarid-specific plasma IgY can be valuable considering its relatively high diagnostic accuracy, sensitivity and specificity obtained in this study.

The sensitivity values ranged from 66 to 88% across all wpi. This value is lower when compared with previous studies. Sharma et al. [20] reported diagnostic sensitivity of 96% for plasma IgY in detecting *A. galli* infection in chickens, which was similar to the result reported by Daş et al. [13], who also reported sensitivity of 94%. As indicated in this study, the time of sample collection influences the diagnostic performance of the method used. Here, the diagnostic performance of the antibody ELISA was assessed continuously at different wpi up to wpi 18, unlike in the aforementioned studies where evaluation was made only at 25 wpi and 28 wpi, respectively, and may be responsible for the differences in results. Nevertheless, plasma IgY measurements can be considered a reliable tool for early detection of nematode infection in chickens.

By contrast, FEC, although not quantifiable until wpi 4 due to the pre-patent period [45, 46], shows a consistent and considerably stronger relationship with total worm burden. It is no surprise that FEC could not be evaluated until wpi 4, because egg counting in faeces relies on the presence of female worms with reproductive maturity in the host intestine, and it takes up to 5–8 weeks for larvae to reach maturity and begin shedding eggs, depending on several factors [45]. It is well known that FEC is a good reflection of infection intensity, but as shown in this study, the relationship differs based on the time of measurement. At wpi 4, FEC showed a low correlation with adult worms, whereas from wpi 6, FEC demonstrated a moderately high correlation with adult and total worm burden (Fig. 7b). Feyera et al. [47] found a similar range of correlation at wpi 8 and 10. As the eggs of the two nematode species cannot be reliably differentiated [29], the present correlations between EPG and worm burden of each species might also have been underestimated.

The ROC analysis showed that at wpi 2, coproantigen ELISA could not accurately differentiate between infected and non-infected hens at this early phase. Similarly, there was no significant difference in the faecal antigen concentration between the two groups at this time point. This is likely because the coproantigen ELISA is not sensitive enough to detect the possibly low concentration of antigens released by small larvae. However, from wpi 4 until the end of the experiment, coproantigen exhibited diagnostic sensitivity and specificity in the range of 61–100% and 90–100%, respectively. Further studies are needed to confirm whether worm antigen excreted in faeces is dependent on or associated with egg shedding. Such a study could evaluate changes in faecal antigen concentration in the presence or absence of worm eggs spiked in faeces. In general, the ROC analysis demonstrated higher qualitative performance for coproantigen ELISA than for IgY ELISA and FEC except in wpi 2, when plasma-IgY ELISA was more sensitive. The correlation between APG and total burden of *H. gallinarum* and *A. galli* was highest at wpi 4 and wpi 6, respectively. APG correlated best with *A. galli* weight rather than with the number of *A. galli* worms, implying the excretion of more antigens from the larger worms; however, data for *H. gallinarum* weight is not available for comparison. Based on the available data, it may be a reasonable assumption that faecal antigen concentration is more reflective of worm size than the number of the worms.

Conclusion

We conclude that soluble worm antigens are consistently excreted through the faeces of an infected host and can be repeatedly measured on the same hen over time. In comparison with other methods, coproantigen ELISA provides the best qualitative diagnostic method. Plasma IgY assay is shown to be the most reliable tool for early diagnosis of nematode infection. Finally, in terms of infection intensity, FEC is superior, and remains a better indicator of total adult worm burden but only post-patency. We suggest that the combination of different tools rather than just one tool would give a better reflection of infections as a result of changes in developmental stage, worm size and fecundity over time. This suggests the necessity of complementary use of different tools for a more accurate diagnosis and quantification of infections.

Abbreviations

APG	Antigen per gram of faeces
AUC	Area under the curve
ELISA	Enzyme-linked immunosorbent assay
EPG	Eggs per gram of faeces
FEC	Faecal egg count
ROC	Receiver operating characteristics

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-023-05782-5.

Additional file 1: Figure S1. Weight–length relationship of *A galli* male (a) and female (b) worms. https://doi.org/10.5281/zenodo.7974367.

Additional file 2: Table S1. Qualitative test performance parameters of different diagnostic tests derived from the receiver operator characteristics (ROC) analysis.

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Author contributions

GD, CCM and MG conceived the study. MH and GD developed the coproantigen ELISA system. GD and MS induced the experimental infections and performed the experiments with chickens. OJO conducted the statistical analysis

of all the data. OJO and GD interpreted the data. OJO wrote the original draft of the manuscript. GD, CCM, MS, MG and MH reviewed the draft manuscript. GD, MG and CCM contributed to funding. All authors read and approved the final manuscript.

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Availability of data and materials
Data used in this study have been deposited in an open-source data repository (DOI: https://doi.org/10.5281/zenodo.7974367).

Declarations

Ethics approval and consent to participate
The Mecklenburg-Western Pomerania State Office for Agriculture, Food Safety and Fisheries, Germany gave approval for the experiment with the permission number AZ.: 7221.3-1-080/16. Animal handling and experimental procedures followed animal welfare rules.

Consent for publication
Not applicable.

Competing interests
Mark Hennies is working in a medical company network (TECOmedical Group) that develops and sells serological tests. The other authors declare no potential competing interests.

Author details
[1]Research Institute for Farm Animal Biology (FBN), Institute of Nutritional Physiology 'Oskar Kellner', Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany. [2]TECOmedical Group, Marie-Curie-Str. 1, 53359 Rheinbach, Germany. [3]Faculty of Science and Technology, Free University of Bozen-Bolzano, Universitätsplatz 5, 39100 Bolzano, Italy.

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4 Discussion

Intestinal nematode infections are of particular concern, especially in non-cage poultry production system. Keeping hens for example in a free-range or organic production system is becoming the most acceptable choice for egg production system, and this method will continue to be widely adopted globally due to its importance to animal welfare. On the contrary, even though non-cage farming may help improve the expression of natural bird behaviour, the system exposes chickens to nematode infections due to the ease of the spread of nematodes through the oral-faecal route. Without sustainable intervention, the spread of nematodes would also likely continue as non-cage farming becomes popular. As studies over the years have provided evidence for the risks that nematodes pose to chickens, including reduced feed intake, poor nutrient absorption, mortality in extreme cases, and the susceptibility of chickens to secondary infections, actions need to be taken.

A historical trajectory of research on ascarids reveals attempts to elucidate the impact of these nematodes on various dimensions of chicken health. A related example is the investigations of Stehr et al., (2019b) on the tolerance and resistance of chickens to nematodes. Their findings indicated a heightened sensitivity of high-performing genotypes to nematode infections which may be metabolically costly. This thesis, therefore, builds upon this preceding work and designs the first objective, which was to investigate the metabolic alterations in the plasma and liver of high-performing genotypes of chickens exposed to intestinal nematode infections.

In addition to understanding the physiological implications, the accurate diagnosis of nematode infections assumes paramount importance particularly in designing accurate intervention strategies. Prior efforts by researchers have explored the measurement of antibodies in plasma and egg yolk (Daş et al., 2017; Sharma et al., 2018b) as an alternative to classical FEC method of diagnosing nematode infections. While their methodologies reported commendable accuracy, the applicability of such method to all types of poultry birds (i.e. layers, broilers, breeders) remains limited. This thesis then contributes to the advancement of an alternative diagnostic approach and further conducts a comprehensive assessment of this new diagnostic method relative to the established methods, forming the last two objectives of this study.

Chapter 3 encloses the articles that delineate the experimental procedures undertaken to address the aforementioned objectives which already includes a detailed discussion of the obtained results. Nevertheless, within this chapter, an overarching significance, and limitations

of the results in relation to metabolic alterations and advancements in nematode diagnosis is presented. The conclusion of this chapter also proffers perspectives for future research.

4.1 Metabolic responses in chicken during mixed nematode infections

Selective breeding in chicken which has resulted in desirable productivity over the past 10 decades, might have also resulted in a weakness in the ability of high-performing chicken to balance growth, reproductive functions, and immunological challenges (Rauw et al., 1998; Stehr et al., 2019b). (See chapter 2). This impairment makes high-performing chickens to be especially vulnerable to the impact of helminth infections which induce physiological disturbances, leading to a reduction in feed intake, nutrient absorption, and utilization (Schwarz et al., 2011). Whereby, during nematode infections, the genotypes of chickens optimized for productivity, promptly allocate nutrients to sustain a metabolically costly immune reaction (Glazier, 2008; Most et al., 2011) likely at the expense of growth and performance.

To investigate the metabolic changes due to helminth infections, the liver and plasma of highperforming genotypes of laying hens (Lohmann Dual, LD), known for their heightened sensitivity to infection were profiled using a ^{1}H-NMR metabolomics method. The investigation spanned 18 weeks post-infection, with specific time points at 2-, 4-, 6-, 10-, 14-, and 18wpi, representing crucial moments in the progression of the nematode infection. Data were published in paper 1 (Chapter 3) (Oladosu et al., 2023a).

The findings from this experiment revealed several key pieces of new knowledge that actualized the first objective of this thesis. Firstly, a significant alteration in the plasma metabolites induced by the stage of infection which are more pronounced than those in the metabolically dynamic organ, the liver was observed (paper 1, figure 3 and 4). Perhaps the main significance of this alteration is that it occurred at the period when immune response is activated due to the penetration of larvae in hosts' mucosa (Luna-Olivares et al., 2015). As previously established, this immune response is notably resource-intensive in terms of metabolic utilization (Stehr et al., 2018) which may explain the increased plasma concentration of metabolites.

It could also be deduced from the results that some specific metabolites identified in this experiment could serve as proxies for intestinal infections in poultry. Some specific compounds elevated due to infection includes sarcosine, myo-inositol, isoleucine, 3-hydroxybutyrate, leucine, and creatine. Another significance is that the elevation in the concentration of these

compounds further reflect substantial metabolic changes, indicating an adaptation of the host to meet its physiological demands during gastrointestinal infection. For instance, myo-inositol, linked to immune barrier functions, has been associated with decreased intestinal immune functions (Li et al., 2018). Similarly, branched-chain amino acids is vital for the effectiveness of both the innate and adaptive immune systems, to ensure the integrity of intestinal mucosa (Kim et al., 2022). Since an effective worm expulsion process hinges on a vigorous adaptive immune activation (Dold and Holland, 2011), it could be proposed that significant changes observed in the branched-chain amino acids are possibly a metabolic mechanism for the activation of the adaptive immune system. Nevertheless, this assumption needs to be investigated in future research.

Further analysis was performed to particularly understand the impacts of the altered metabolites in the metabolic pathways. Thus, notable alterations in metabolic pathways were reported (Paper 1 Fig. 5), particularly in arginine proline metabolism and alanine aspartate and glutamate metabolism, in response to nematode infection. These pathways assume crucial roles in supporting the host's defence mechanisms and facilitating tissue repair amid intestinal inflammation. The availability of arginine in the host stimulates the regulation of immune system through the activation of T cells and macrophages. Proline and arginine contribute significantly to collagen synthesis, enhancement of the intestinal mucosa, and supporting tissue repair (Das et al., 2010; Zhu et al., 2013). Therefore, the observed upregulation in the arginine and proline metabolic pathway may also signify a metabolic response to the intestinal inflammation associated with gastrointestinal infections.

It is noteworthy to mention that the study revealed that liver metabolome was not significantly altered due to infection with the mixed nematodes. The lack of differences in the liver tissue of infected animals poses an unexpected outcome in that study, and explaining this phenomenon proves elusive. Despite being a metabolically active organ, the liver did not exhibit significant alterations in infected animals. While the liver is not the primary site of ascaris infection, known to primarily elicit a localized immune response in the gut, it was hypothesized that the liver metabolome could be altered during mixed intestinal parasite infection in high-performing chickens due to the possible involvement of *H. meleagridis* which may cause liver inflammation. Contrary to our hypothesis, the liver metabolome was not altered, although our study did not provide evidence of liver damage, even though signs of liver inflammation were observed as indicated by elevated acute-phase protein (alpha-1-acid glycoprotein, paper 1 Fig. 2). This result emphasizes the need for further investigation into the complex dynamics of infection involving both ascarids and the protozoon.

Nevertheless, it remained evident in the plasma metabolomics study that metabolic resources undergo alterations during mixed nematode infection, particularly during the period known for the activation of immune responses to infections. This suggests that the differential elevation of metabolites in the plasma of infected chickens of high-performing genotype serves as a signature metabolic response, likely indicating nutrient diversion for immune activities.

4.2 Advancement in the diagnosis of nematode infections in chickens

Investigations on the metabolic responses during mixed nematode infection contributes to our understanding of the dynamics of infection biology, nonetheless, further studies on infection dynamics would also depend largely on an accurate, easy diagnosis of helminth infections which is an important theme addressed in this thesis. This entails the development and assessment of a non-invasive diagnosis of intestinal nematodes. The correct identification of infected and non-infected animals is crucial for implementing effective intervention strategies and preventing the spread of infection within and between flocks. Early detection enables targeted flock treatment, potentially mitigating anthelminthic resistance development among parasites. Additionally, the soundest diagnostic tool should assess infection intensity by correlating its outcome of measurement with the definite worm burden of the infected animal.

Quantitative diagnosis is essential for evaluating anthelminthic efficacy, as reductions in FEC or worm counts through necropsy are currently relied upon. But reliability of FECs to assess nematode infection can be impaired by its dependence on the fecundity of worms and other challenges (Wongrak et al., 2015). An alternative method like ELISA measuring anti-ascarid antibodies in plasma and egg yolk were explored previously (Daş et al., 2017). Yet, these methods had drawbacks, such as the need for an authorized expert and invasive blood sample collection and non-application of egg yolk antibodies to male birds (Discussed in Chapter 2.5).

The new approach developed in this thesis utilized worm antigens in host faeces in what was we called coproantigen ELISA. This coproantigen diagnostics demonstrated an accurate differentiation of infected and non-infected chickens for both *A. galli* and *H. gallinarum* (Paper 2, Oladosu et al., 2022). It proved effective in capturing cross-reactive antibodies against both nematode species, allowing for the diagnosis of two different nematode infections with a single assay. In the specific context of coproantigen development, the process involved immunizing rabbits with soluble antigens extracted from *A. galli*. This immunization aimed to generate a polyclonal rabbit antibody with specificity towards ascarid antigens. Subsequently, the obtained rabbit antibody was utilized to coat an ELISA plate. Simultaneously, the soluble antigen extracted from *A. galli* served as standards in a sandwich ELISA procedure. The chosen methodology enabled the utilization of a single assay for both nematode species,

rendering it particularly advantageous in field conditions where co-infection is more prevalent than mono-infection. Notably, the diagnostic accuracy, sensitivity, and specificity exhibited robust performance for *A. galli*; however, marginally lower values were observed for *H. gallinarum*. Despite this discrepancy, the assay demonstrated the ability to correctly distinguish between infected and uninfected birds, with a comparable accuracy with similar assays reported for other species.

While the overall performance of the assay is high, further refinement is conceivable, particularly by directing focus towards specific worm proteins. This targeted approach has the potential to enhance sensitivity and specificity for each worm species, optimizing the diagnostic utility of the assay. Therefore, further efforts to fine-tune the methodology may yield improvements in its ability to discern between infections of *H. gallinarum* and *A. galli*.

In Paper 3 (Oladosu et al., 2023b), we conducted a comprehensive evaluation of the temporal dynamics of worm antigen excretion in faeces. Our findings revealed consistent patterns over time, with a notable correlation observed between antigen excretion and worm maturation. The repeatability analysis conducted further underscored the reliability of measurements within individual hens, affirming the robustness of coproantigen ELISA. A comparative analysis of four diagnostic methods (i.e. coproantigen ELISA, egg-yolk IgY ELISA, plasma IgY ELISA, and FEC) revealed distinct variations in the performance of all the diagnostic methods. This emphasized the critical importance of selecting specific tools for the optimal detection of nematode infections. Notably, despite a diminishing accuracy in diagnosis over time, plasma IgY, demonstrated early detection capability. In contrast, the coproantigen ELISA method outperformed other diagnostic techniques, showcasing its potential for accurate and stable diagnosis of nematode infections in chickens although not suitable for early detection in comparable to the antibody ELISAs.

4.3 Perspective for future research and general conclusions

While the thesis has achieved significant results in understanding how key metabolites, especially amino acids, are altered due to intestinal nematode infection, there is a considerable gap that is yet to be explained. The metabolic alteration was observed on plasma, but the metabolites measured in plasma could have originated from various organs in the chickens. Future research could explore the metabolites particularly in the intestinal tract as the site of ascarids infection in chickens, providing more insights into the site of infection and nutrient absorption. Additionally, studying metabolites in the kidney could enhance our understanding of metabolic processes and excretion during nematode infections.

Furthermore, it is crucial to acknowledge the limitations of the NMR-Metabolomics technique used in this study, which although known for its high accuracy may not capture a wide range of metabolites. The thesis recommends a complementary approach using techniques with broader range of compounds like GC-MS to obtain a more comprehensive understanding of metabolic exchange during nematode infections.

The study has advanced the diagnosis of intestinal parasite infections in chickens, introducing a newly developed copro-antigen ELISA that is highly specific and sensitive to ascarids. However, a clarification is needed regarding whether faecal antigen measurement depends on worm egg shedding. Future studies can address this limitation by designing experiments to detect larvae antigens recovered in faeces soon after infection starts. Despite this, the assay offers high diagnostic accuracy and has the potential for further development into a rapid dipstick test, enabling farmers to quickly self-identify parasite infections without the need for technical expertise or sophisticated laboratory equipment. The investigation into metabolite alterations also opens up possibilities for alternative methods of infection diagnosis. The study identified key metabolites, such as trimethylamine N-oxide (TMAO), associated with gut microbiota and diet. Further research on TMAO and other compounds as potential biomarkers for intestinal infection in poultry is recommended.

Finally, it is imperative to understand farmers' perceptions of the disease and its impact on poultry welfare. The continued adoption of free-range and organic production systems necessitates strategic intervention to address associated parasite infections. Further research is recommended on the effects of parasite infections on chicken health, particularly on welfare, as evidence is needed to challenge the notion that helminths pose no risk to poultry production systems. Further welfare indicators and additional evidence should be provided to support this argument.

In conclusion, this thesis reveals the alterations in the plasma metabolome of improved genotype of laying hens experimentally infected with *A. galli* and *H. gallinarum* concurrent *H. meleagridris*. The mixed infection stimulated an upregulation of key metabolic pathways, indicating a prioritized allocation of metabolic resources for an intensified immunological response. The identified key metabolites show potential as proxies for the identification of nematode infections. In addition, this work demonstrated the feasibility of achieving a noninvasive and accurate diagnosis of infection through a coproantigen ELISA. The results from the examination of multiple diagnosis methods emphasized the need for careful consideration when selecting diagnostic tools for assessing nematode infections in poultry.

5 Zusammenfassung

Metabolische Anpassungsreaktionen und diagnostische Neuerungen bei Hühnern mit gemischten Darmparasiteninfektionen

Diese Dissertation beschreibt die metabolischen Reaktionen auf intestinale Nematodeninfektionen bei Hochleistungshühnern und skizziert die Entwicklung eines effektiven nicht-invasiven diagnostischen Verfahrens für die detaillierte Charakterisierung von Infektionen mit weitreichenden Auswirkungen auf das Geflügelmanagement.

Hochleistungshühner des Genotyps Lohmann-Brown wurden experimentell mit *Ascaridia galli* und *Heterakis gallinarum* infiziert, was auch zu Histomonoseinfektionen führte. Die Tiere wurden zu spezifischen Zeitpunkten nach den Infektionen anhand von Leber- und Plasmaproben auf metabolische Anpassungsreaktionen auf die Infektionen untersucht. Die Studie liefert somit eine detaillierte und zeitabhängige Analyse der infektionsbedingten Stoffwechselveränderungen bei mit Nematoden infizierten Hühnern. Die Identifizierung von Schlüsselmetaboliten, die mit verschiedenen Infektionsstadien in Verbindung gebracht werden, einschließlich des Eindringens der Larven in die Schleimhaut, der Passage und der Reinfektion, ermöglicht ein tieferes Verständnis der dynamischen Wirt-Parasiten-Interaktion und erlaubt somit gehzielte Untersuchungen spezifischer Stoffwechselwege und ihrer Funktionen. Diese Erkenntnisse haben Auswirkungen auf Zuchtprogramme und Managementstrategien, die darauf abzielen, Tiergesundheit und Produktivität in der Geflügelzucht zu optimieren.

Die erhöhten Aminosäurekonzentrationen während der frühen Phase der Nematodeninfekion mit verstärkter Immunantwort deuten auf eine mögliche Umleitung metabolischer Ressourcen auf Kosten von Leistung und Produktivität hin. Die erhöhten Plasmaaminosäurekonzentrationen deuten auf ein mögliches Ungleichgewicht bei der Proteinsekretion und -rückresorption im Dünndarm hin. Die Studie identifizierte weiterhin veränderte Stoffwechselwege, einschließlich des Arginin-Prolin-Stoffwechsels, der für die Kollagensynthese und Gewebereparatur entscheidend ist. Dies unterstreicht die Bedeutung von Aminosäuren bei der Unterstützung der Abwehrmechanismen während der Nematodeninfektion. Darüber hinaus eröffnet die Identifizierung von Schlüsselmetaboliten wie Trimethylamin-N-oxid (TMAO), dessen hohe Konzentration auf das Vorhandensein von Nematodenmischinfektionen hinweisen könnte, eine interessante Perspektive für die Biomarkerforschung. Eine weitere Erforschung der Anwendbarkeit von TMAO in verschiedenen parasitären Fragestellungen sollte insbesondere in Bezug auf den

Mechanismus der TMAO-Produktion und seine mögliche Rolle bei der Unterscheidung des Status von Helmintheninfektionen bei Hühnern erfolgen.

Der Status der Nematodeninfektion wird derzeit durch Eizählungen in den Exkrementen ermittelt, die aufgrund von Variationen in der Eizahl der Würmer und weieren Gründen nicht immer zuverlässig sind. Eine alternative Methode besteht darin, Anti-Ascariden-Antikörper im Hühnerplasma zu messen. Diese Methode ist invasiv und muss die Tierschutznormen in der Geflügelhaltung entsprechen, die Entnahme erfolgt als veterinärmedizinische Maßnahme. Daher wurde im Rahmen dieser Dissertation ein neues, nicht-invasives Diagnoseverfahren entwickelt, das auf der Messung von Wurmantigenen in den Exkreta basiert. Die Entwicklung dieses Copro-Antigen-ELISA als nicht-invasive Diagnosemethode stellt eine Möglichkeit für ein verbessertes Parasitenmanagement dar. Dieser neuartige Ansatz unterscheidet bei beiden Nematodenarten zwischen infizierten und nicht-infizierten Hühnern und bietet eine vielversprechende nicht-invasive Alternative zur Eizählung. Während die Methode für *A. galli* eine hohe diagnostische Genauigkeit aufweist, muss die Genauigkeit und Empfindlichkeit gegenüber *H. gallinarum* weiter verfeinert werden. Zeiteffekte, die zwischen den verschiedenen Diagnosemethoden festzustellen waren, verdeutlichen die Notwendigkeit einer sorgfältigen Methodenauswahl. Der Copro-Antigen-ELISA zeigte bei der qualitativen Diagnostik eine hohe Genauigkeit und übertraf die anderen Methoden. Gleichzeitig zeigte der Antikörper-ELISA eine frühzeitige Erkennung einer Nematodeninfektion an, während die Eizählung sich als die Methode herausstellte, die am engsten mit der Wurmlast in den Tieren korrelierte und als zuverlässiges Maß für die Infektionsintensität diente.

Im Kontext der bisherigen Untersuchungen erweitert diese Studie unser Verständnis der potenziellen metabolischen Konsequenzen von Nematodeninfektionen bei Hochleistungshühnern. Die identifizierten Metaboliten und Stoffwechselwege tragen nicht nur zum grundlegenden Verständnis über die Wechselwirkungen zwischen Wirt und Parasiten bei, sondern bilden auch die Grundlage für gezielte Interventionen, um die negativen Auswirkungen einer Nematodeninfektionen auf die Produktivität des Geflügels zu mindern. Die Entwicklung eines innovativen, nicht-invasiven diagnostischen Mittels hat eine hohe Relevanz für die Anwendung und bietet Vorteile für Geflügelproduzenten, die mit den Herausforderungen von Nematodeninfektionen konfrontiert sind.

6 Summary

This thesis reveals the metabolic responses to intestinal nematode infections in highperforming chickens and delineates the development of an effective non-invasive diagnostic tool for the detailed characterisation of infections with significant implications for broader impacts on poultry management.

High-performing chickens of the Lohmann Brown genotype were experimentally infected with both *Ascaridia galli* and *Heterakis gallinarum,* which also led to concurrent histomonosis infections. The birds were necropsied at specific time points after infections, and the liver and plasma were investigated for the metabolic responses to the infections. Notably, this is the first study providing a detailed, time-dependent analysis of the infection-induced metabolic alteration in nematode infected chickens. The identification of key metabolites associated with different infection stages including larval penetration of the mucosa, patency, and reinfection provides a nuanced understanding of the dynamic host-parasite interplay, paving the way for targeted investigations into specific pathways and their functional consequences. This finding prompts a deeper investigation into the trade-offs between immune defence and overall performance, with implications for breeding programs and management strategies aimed at optimizing both health and productivity in poultry.

The elevated concentrations of amino acids during heightened immune responses at early phase of nematode infection indicated the possible diversion of metabolic resources towards immune response at the expense of performance and productivity. The increased plasma amino acids were attributed to a potential imbalance in protein secretion and re-absorption in the small intestine. The study further identified altered metabolic pathways, including arginine proline metabolism which are crucial for collagen synthesis and tissue repair, thus highlighting the importance of amino acids in supporting the defence mechanism during nematode infection. Additionally, the identification of key metabolites like trimethylamine Noxide (TMAO) whose high concentration may indicate the presence of mixed nematode infections, opens up a new perspective for biomarker research. Further exploration into the applicability of TMAO in diverse parasitic situations are encouraged particularly the mechanism of TMAO production and its role in discriminating the status of helminth infections in chicken.

The status of nematodes infection is currently achieved through faecal egg counts which may not always be reliable due to variations in worm fecundity amongst other reasons. An alternative method is to measure anti-ascarid antibodies in chicken plasma. However, this practice may not always meet current welfare standards in poultry production, as it involves an

invasive technique for blood collection that also requires licensed experts to perform. Therefore, this thesis further developed a new non-invasive nematode diagnosis based on the measure of worm antigens in chicken faeces.

The development of this coproantigen ELISA as a non-invasive diagnostic tool presents an opportunity for improved parasite management. This novel approach accurately differentiated infected and non-infected chickens for both nematode species, offering a promising non-invasive alternative to faecal egg counts. While showing high diagnostic accuracy for *A. galli,* further refinement is needed for accuracy and sensitivity to *H. gallinarum.* Temporal variations among different diagnostic methods highlighted the need for tool-specific selection. The coproantigen ELISA demonstrated superior performance in overall qualitative diagnostics, surpassing other methods. Concurrently, the antibody ELISA showcased an early detection capability, while faecal egg counting emerged as the method most closely correlated with worm burdens, serving as a reliable measure of infection intensity.

In the broader context of existing information, this study advances our understanding of the potential metabolic cost of defence against nematode infections in high-performing chickens. The identified metabolites and pathways not only contribute to the fundamental knowledge of host-parasite interactions but also lay the groundwork for targeted interventions to mitigate the impact of these infections on poultry productivity. The development of innovative noninvasive diagnostic tool adds an applied dimension to the research, providing tangible benefits for poultry producers grappling with the challenges of nematode infections.

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8 List of publications and oral presentations

Oladosu, O.J., Correia, B.S.B., Grafl, B. et al. ^{1}H-NMR based-metabolomics reveals alterations in the metabolite profiles of chickens infected with ascarids and concurrent histomonosis infection. Gut Pathog 15, 56 (2023). https://doi.org/10.1186/s13099-023-00584-7

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O. J. Oladosu, M. Hennies, M. Stehr, C. C. Metges, M. Gauly, G. Daş. Diagnostic performance of a copro-antigen ELISA to assess nematode infections in chickens. 73rd EAAP Annual Meeting, Porto, Portugal September 07, 2022. (**oral presentation)**

Oyekunle J. Oladosu, B. S.B. Correia, H. C. Bertram, C. C. Metges, G. Daş. ^{1}H-NMR metabolomics reveals alterations in the metabolism of ascarid-infected laying hens. 74th EAAP Annual Meeting, Lyon, France August 30th, 2023. (**oral presentation)**

O. J. Oladosu, B. S.B. Correia, H. C. Bertram, C. C. Metges, G. Daş. Plasma metabolomics profiling of nematode infected chickens 77. Jahrestagung der Gesellschaft für Ernährungsphysiologie 07.-09. März 2023 Georg-August-Universität Göttingen Oyekunle John Oladosu, Cornelia C. Metges, Gürbüz Daş Characterisation of metabolic responses in chicken exposed to nematode infections. Research Institute for Farm Animal Biology (FBN), 22nd Day of the Doctoral Student, 18 August 2022. (**oral presentation)**

Oyekunle J. Oladosu Cornelia C. Metges, Gürbüz Daş. Assessment of a new diagnostic tool, and the metabolic and transcriptional responses in chickens infected with mixed parasite species. Day of Doctoral Students Research Institute for Farm Animal Biology, Dummerstorf November 24, 2023. (**oral presentation)**

Oyekunle John Oladosu. Characterisation of metabolic responses in nematode infected chickens. Mono-Gut-Health project network meeting. Bydgoszcz, Poland, September 30, 2021. (**oral presentation)**

Oyekunle John Oladosu. A non-invasive tool for the characterization of nematode infections in chickens. MonoGutHealth project network meeting. Research Institute for Farm Animal Biology, Dummerstorf, Germany, May 20, 2022. (**oral presentation)**

Oyekunle John Oladosu. ^{1}H-NMR metabolomics reveals alterations in the metabolism of ascarid-infected laying hens. MonoGutHealth project network meeting. KU-Leuven, Belgium, April 25, 2023. (**oral presentation)**

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11 Conflict of interest

Mark Hennies who is a contributing author to published article 2 and 3 of this thesis is an employee of TECODevelopment GmbH. Other authors have no competing interest.

12. Declaration of independence

I hereby certify that I have prepared this thesis independently. I certify that I have used only the sources and aids indicated.

Berlin, 08.07.2024
Oyekunle John Oladosu

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