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Investigations on worm expulsion, resistance and tolerance to mixed-nematode infections in chicken genotypes with different performance directions

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Meiner Familie

TABLE OF CONTENT

SUMMARY

Since decades chicken genotypes have been selected to produce either high efficiently meat or eggs, based on the genetically determined antagonism between growth rate and reproduction. As a consequence the performance level of meat- and egg-type chickens has been increased steadily. We hypothesised that responses of host animals to their pathogens may be associated with their performance levels determined by their genetic makeup. Thus, the main objective of the present thesis was to investigate whether selection for divergent performance directions resulted in a different ability to cope with nematode infections. Therefore three divergent genotypes developed either for egg or meat production or for both purposes were used. Further on, the worm expulsion phenomenon, known to occur in mammalian host species, and the expulsion associated immune responses have been studied in the chicken host co-infected with *Ascaridia galli* and *Heterakis gallinarum*. The results obtained were used to discuss potential benefits of dual-purpose chickens compared to classical meat- and egg-type chickens. The studies covered in this thesis were performed at the Institute of Nutritional Physiology "Oskar Kellner" at the Leibniz-Institute for Farm Animal Biology, Dummerstorf, from 2015 and 2018. Therefore two experimental infection studies were performed separately.

In the first experiment male birds of three genotypes selected for either meat production (Ross-308, R) or egg production (Lohmann Brown Plus, LB) or for both purposes (Lohmann Dual, LD) were used. The birds were obtained as day-old chicks and were kept for 10 weeks in a floor husbandry system. All birds were fed on a diet developed for fattening of meat-type chickens. At one week of age approximately half of the birds were infected with a total of 500 infective eggs of *A. galli* and *H. gallinarum* (250 eggs per worm species) to produce a mixed nematode infection, whereas non-infected birds served as controls. Starting with 2 weeks post infection (weeks p.i.) a defined number of birds were slaughtered at weekly intervals to determine both worm burdens and selected host immune responses. Performance data (feed intake, body weight, etc.) were determined at weekly intervals. In the second experiment we compared hens of a classical layer-genotype (Lohmann Brown Plus, LB) with hens of a lower performing dual-purpose genotype (Lohmann Dual, LD). The hens were infected at 24 weeks of age with a total of 1000 eggs of *A. galli* and *H. gallinarum* (500 eggs per worm species). A comparable number of hens were used as uninfected controls. The study was run for 18 weeks, in which both genotypes exhibited their maximal performance level (i.e., laying peak). The hens were necropsied at defined time points (2, 4, 6, 10, 14 und 18 weeks p.i.), to determine worm burdens with both species. Performance data (i.e., laying rate

and egg weight) were determined daily. Feed intake, body weight as well as blood and egg yolk samples were taken weekly on a pen based level.

In both the male and the female experiments a strong decrease of worm burdens over time with both worm species was observed in all genotypes, independent of host sex and performance direction. Thus, we decided to further quantify both the extent and duration of worm expulsion with both worm species and investigated the accompanying humoral and cell-mediated host immune responses in association with population dynamics of the worms **(Chapter Two)**. We found that the chicken host is able to eliminate the vast majority of both *A. galli* and *H. gallinarum* in a short period of time. The worm expulsion occurred in three distinct phases. The first phase covers the first day of infection only, when the larvae must hatch and withstand the peristaltic movements of the gut. The second phase, the most efficient one, was probably mediated by local immune responses. Our data suggest that the increased expression of Th2 cytokines and mucin-regulating genes likely resulted in an increased gut motility that favours the expulsion of the worms. Worm expulsion in general was strongly associated with the developmental stages of the worms, where the elimination of juvenile stages was specifically targeted. Once escaped from the expulsion mechanism, a very small percentage of worms reached sexual maturity and induced reinfections (third phase of expulsion). Thus only a small number of worms were able to establish themselves successfully in the host.

In the male-bird study **(Chapter Three)** infections reduced the feed intake in all three host genotypes, whereas feed conversion was not affected despite low structural alterations of the intestinal wall. The infections reduced the growth performance in the high performing meat-type chickens (R) only, whereas the lower performing LB and LD genotypes tolerated the infections without any impairment in performance, indicating a performance-level dependent ability to tolerate nematode infections. The impaired tolerance in R birds was associated with a relative nutrient scarcity due to the infection-induced lower feed intake. Based on different nutrient and energy requirements between the genotypes the nutritious broiler diet supplied the slower growing LB and LD birds with surplus nutrients that most likely enhanced their tolerance against the nematodes. The *A. galli* burden following the experimental infection was highest in R than LB birds, whereas the burden of LD was not different from that of R and LB. The higher burden in R was associated with the intestinal environment of the genotype. The larger intestine, the higher amount of nutrients due to a higher feed intake as well as a higher quantity of lactic acid might have provided beneficial effects on worm establishment and survival. *Heterakis gallinarum* burden of the first

generation worms, however, was similar between the three genotypes, whereas susceptibility to re-infections with *H. gallinarum* was higher in LB than in LD. Re-infections in R birds were on a very low level. Differences in susceptibility to re-infections are probably associated with host behaviour.

In the female study **(Chapter Four)** the feed intake and body weight were not affected by infections in both genotypes, whereas feed conversion rate was worse in infected hens. Laying rate was reduced by infections in both genotypes. Infected LB hens reduced their laying performance already in the early phase of infection (i.e., by 3 weeks p.i.), whereas LD hens laid less number of eggs much later (i.e., by 14 weeks p.i.). The reduction in laying performance was associated with the genotype specific production peak, which was delayed in LD hens compared to LB. Nevertheless, the results clearly demonstrated that host tolerance against nematodes is performance-level dependent. In contrast to the male study, worm burdens with both species originating from the experimental infections were not different between the two genotypes. However, susceptibility to re-infections was higher in LB than LD hens as well, which was likely associated with behavioural differences between two genotypes. The infections caused a shift in egg size classes, leading to a smaller frequency of larger eggs in both genotypes. In addition, the infections reduced the egg and yolk weight, altered yolk colour and reduced the fat content in the egg yolks. The proportion of polyunsaturated fatty acids (PUFA) especially that of n-6-PUFA, was also lower in the egg yolks of the infected hens. Thus, infections negatively affected market-relevant egg quality traits and altered yolk nutrients.

It is concluded that tolerance to nematode infections is dependent on both growth rate and laying performance level in male and female chickens, respectively. The time dependent impairment in laying performance was likely due to differences in genetic programming for production peak and persistency of the two genotypes. Differences found between male birds may be associated with a mismatch between the genotype-specific nutrient requirements and the nutrient supply during infectious challenges. Thus, both the host individual performance level as well as the nutrient supply appears to play a key role in tolerance against nematode infections in chickens. Resistance to infections, however, is dependent on the type of infection (i.e., experimentally vs. naturally re-infection). While overall resistance against infections likely depend on the host immune status and the intestinal conditions of the respective genotype, susceptibility to re-infections, which finally determines the risk for an infection, may be associated with host behaviour.

CHAPTER ONE

General Introduction

1 Foreword

The poultry production has been the most rapidly growing livestock sector worldwide in the last decades (Scanes, 2007; FAO, 2017) and the forecasts predict it will continue this way in future (Mottet and Tempio, 2017), for both egg production (Preisinger, 2018) and the meat producing sector (FAO, 2006; Alexandratos and Bruinsma, 2012). The increasing demand for food of animal origin is not only due to an expected increase in the *per capita* consumption (FAO, 2006; Alexandratos and Bruinsma, 2012) but is also a logical consequence of the growing world population (Dunwell, 2013; Alexandratos and Bruinsma, 2012; FAO, 2017). To ensure food security for the human species, food production needs to be doubled by 2050 (Dunwell, 2013), and poultry meat is considered to account in large part for protein source then (Alexandratos and Bruinsma, 2012), due to the high nutritional quality, high efficiency, lower meat cost and the absence of cultural or religious barriers (Magdelaine et al., 2008). However, this trend will be much more pronounced for developing countries than for industrial nations (Alexandratos and Bruinsma, 2012; Preisinger, 2018).

In Germany the continuous growth in poultry production has been and is still being accompanied by challenges through legislations predominantly concerning the layer industry, driven by consumer concerns and the pressure of NGO´s. Challenges that have to be managed by farmers, scientists and the industry. One of the most challenging tasks in the current decade is to find an approach to avoid the world-wide practiced culling of day-old male chicks in the layer hatchery, a consequence of a breeding strategy to achieve specialized and highly efficient chicken genotypes, not only practiced in conventional but also in organic poultry farming. Modern genotypes are therefore either selected to produce large amounts of meat in a short time (Schmidt et al., 2009; Zuidhof et al., 2014) or to lay a large number of eggs (Preisinger, 2018). Based on the genetically determined antagonism between growth and reproduction (Damme, 2015), both production traits cannot be improved at the same time in the same animal. Despite a slight sex-dependent dimorphism for growth, both male and female broilers are used for meat production (Awad et al., 2018). Contrarily, as only female birds lay eggs, the poor and inefficient growth potential results in the culling of the male layer chicks. Responsible therefore is the low profitability of the male birds of the layer genotypes (Koenig et al., 2012; Kaufmann and Andersson, 2013) and the pressure for low prices of consumers and the food retailing. Although no statistics are available for the exact number of killed male layer chicks, the number of hatched layer females provides reliable information, as at hatch sex ratio is expected to be 1:1 for males and females (Feng et al., 2006). Therefore number of killed male layer chicks is estimated to be about 46 million in Germany (Destatis, 2017), 397 million in the EU (Anonymous, 2018a) and 7.5 billion worldwide (FAO, 2016). However, as ethical concerns raised in the last years (Bruijnis et al., 2015) the research for suitable alternatives is necessary and is therefore funded by the German government (BMEL, 2018). The two strategies, the in-ovo sex determination, to determine the sex before hatch (Weissmann et al., 2013; Galli et al., 2017; Krautwald-Junghanns et al., 2018), and the use of dual purpose genotypes are currently the most considered approaches to avoid the culling of day-old male chicks.

The replacement of conventional cage system for laying hens with the so-called alternative housing systems (i.e. floor husbandry and free range systems) in Germany in 2010, EU-wide since 2012 (CEC, 1999), benefited the expression of natural behaviours of the hens but has apparently negatively affected the general health status of the birds (Lay et al., 2011; Grafl et al., 2017). Infectious diseases with multiple parasites, e.g., nematode species, as a fact, have drastically re-emerged since then (Kaufmann et al., 2011a; Sherwin et al., 2013; Wongrak et al., 2014; Thapa et al., 2015; Wuthijaree et al., 2017) in laying hen farms, and occur in both outdoor and indoor systems (Permin et al., 1999; Jansson et al., 2010; Kaufmann et al., 2011a; Bestman and Wagenaar, 2014; Grafl et al., 2017). Nematode infections, however, constitute not only a health risk for laying hens but also concern broiler chickens. This applies for backyard chickens in developing countries (Abdelqader et al., 2008; Katoch et al., 2012; Ferdushy et al., 2016) and might thus also account for broiler chickens in industrialised countries kept under organic farming conditions with a prolonged fattening time, increasing the risk for parasite infections (Kijlstra and Eijck, 2006). Studies in the USA reported nematode infections of commercial broiler chickens in indoor systems, too (Wilson et al. 1994; Sarathi et al., 2004; Jansson et al., 2010; Yazwinski et al., 2013). Comparative studies in the response to nematode infections, however, have mainly been performed for layer genotypes (Permin and Ranvig, 2001; Gauly et al., 2002; Schou et al., 2003; Kaufmann et al., 2011b; Wongrak et al., 2015a). Nevertheless, there is evidence that breeding chickens for divergent performances has caused physiological differences between layers and broilers in many cases (Leshchinsky and Klasing et al., 2001; Lilburn and Loeffler, 2015; Schokker et al., 2015; Walugembe et al., 2015), implying the possibility that chicken genotypes selected for different performances may respond differently to nematode infections. Thus, the main objective of the present thesis was to investigate whether selection for divergent performance directions in chickens has resulted in a different ability to cope with nematode infections. Therefore three divergent chicken genotypes developed either for egg or meat production or for both purposes were used.

In the following sections, we will give a literature overview about nematode infections in chickens, host-parasite interactions and genetically determined differences between meat- and layer-type chickens to introduce the following investigations performed in this thesis.

1.1 Nematodes and their life-cycle

Among other worm species, the nematodes *Ascaridia galli* and *Heterakis gallinarum* are most common in chickens globally (Kaufmann et al., 2011a; Katoch et al., 2012; Sherwin et al., 2013; Wongrak et al., 2014; Thapa et al., 2015; Wuthijaree et al., 2017). In Germany about 88% of the laying hens kept in organic productions systems were infected with the large roundworm *A. galli*, whereas almost all birds (98%) were infected with the small roundworm *H. gallinarum* (Kaufmann et al., 2011a). The high prevalence's of the worms are related to the direct lifecycle and the high effective faecal-oral transmission route (Ramadan and Abou Znada, 1991; Daş et al., 2014). As the birds are frequently in contact with their own faeces the ingestion of infectious nematode eggs from the surrounding environment, that are periodically released by the worms within a day (Wongrak et al., 2015b; Daş et al., 2019), ensures the completion of their life cycle in a short time period. Wongrak et al. (2014) demonstrated that primary infection of birds in free range is only a matter of time, probably a consequence of the low biosecurity and the transmission of nematode eggs from wild birds. However, there exists a 50% higher risk of the birds to get infected in the summer than winter, implying a seasonal dependency in the presence of infections (Kaufmann et al., 2011a). Even if ascarid eggs remain viable for year's outdoors (Farr, 1961; Velichkin and Merkulov, 1970), the percentage of eggs remaining infective in the environment is low (Thapa et al., 2017).

The two ascarid species *A. galli* and *H. gallinarum* are genetically closely related species (Nadler et al., 2007; Wang et al., 2016a), which inhabit different areas in the gastrointestinal tract (small intestine and caecum, respectively), but share an almost similar direct life-cycle (Ramadan and Abou Znada, 1991; Daş et al., 2014) with two fundamental exceptions. First, the prepatent period of *A. galli* is longer (5 to 8 weeks; Ramadan and Abou Znada, 1991) than that of *H. gallinarum* (approximately 4 weeks; Daş et al., 2014), while time for embryonation of eggs in the extra-intestinal environment is similar for both species (i.e., 2 weeks; Püllen et al., 2008). Second, *A. galli* larvae undergo a tissue phase (Luna-Olivares et al., 2012; Ferdushy et al., 2013), which is dose dependent and can last up to about 54 days (Herd and McNaught, 1975). Although there exist many studies in the past investigating the tissue phase of *A. galli* (Ackert, 1923, 1931; Todd and Crowdus, 1952; Tugwell and Ackert, 1952; Herd and McNaught, 1975), this phase has recently been described to be more a superficial mucosal phase rather than a true histotrophic phase, at least in the early phase of infection, as larvae were predominantly recovered from the epithelium and the lumen of the crypts than from the lamina propria (Luna-Olivares et al., 2012). However, larvae have been found to migrate accidentally in the abdominal cavity, too (Borji and Razmyar, 2012). Whether larval stages of *H. gallinarum* undergo a similar tissue phase as described for *A. galli* is not fully clarified yet, since observations made are contradictory. Some studies reported kind of a tissue phase for *H. gallinarum* in the early stage of life (Roberts, 1937; Hsu and Li, 1940; van Grembergen, 1954), whereas Baker (1933) found that only the anterior part of the larvae was borrowed into the glandular crypts of the mucosa. Others, however, showed that the larvae developed only in the caecal lumen (Dorman, 1928; Clapham, 1933). Vatne and Hansen (1965) found that *H. gallinarum* larvae only stay short tissue-associated (up to 12 days), but do not penetrate into the tissue wall *per se*.

Heterakis gallinarum, additionally, plays a key role in the transmission of the protozoan hyper-parasite *Histomonas meleagridis*, the causative agent of the so-called blackhead disease (McDougald, 2005), and is high frequent in layer flocks (Grafl, et al., 2011). While turkeys can further get infected laterally via the “cloacal-drinking” phenomenon (Hu et al., 2004), due to the retrograde peristalsis into the caeca (Hu et al., 2004), the transmission of *H. meleagridis* in chickens is restricted to the ingestion of the caecal worm (McDougald, 2005). Worms of both sexes serve as natural carriers of the protozoan parasite (Swales, 1948; Gibbs, 1962; Lund, 1968; Springer et al., 1969). Lund and Burtner (1957) reported that one of every 200 *Heterakis* eggs is contaminated with the protozoan parasite, whereas Fine (1975) found almost every fourth egg contains Histomonas.

1.2 Adverse effects of nematode infections on host performance

Infections with nematodes impair the performance and productivity of the host animal through several direct and indirect effects. Direct effects on host performance, i.e., growth and laying activity (Daş et al., 2010, 2011a, 2012; Sharma et al., 2018a) mainly arise from a reduced availability of dietary nutrients, a result of a nematode induced impairment in the nutrient absorption (Hurwitz et al., 1972; Walker and Farell, 1976; Schwarz et al., 2011b), feed intake and feed conversion (Daş et al., 2010, 2011b, 2012). In addition nematode induced immune responses against the worms (Schwarz et al., 2011a,b; Luna-Olivares et al., 2015; Daş et al., 2017, 2018) are metabolic costly (Colditz et al., 2008; van der Most, 2011). Tissue injury to the intestinal wall caused by *A. galli* larvae (Luna-Olivares et al, 2012) as well as alterations in the intestinal morphology (Marcos-Atxutegi et al., 2009; Dänicke et al., 2009, 2013; Luna-Olivares et al, 2015) may contribute to the loss of nutrients available. Hurwitz et al. (1972) reported an impaired utilization of protein sources in *A. galli* infected chickens through a reduced proteolytic enzyme activity in the jejunum. Further on the energy metabolism and nitrogen retention (Walker and Farell, 1976) and the transport of alanine and glucose (Schwarz et al., 2011) were reduced by an *A. galli* infection. Indirect effects emerging from the infection are based on vectoring roles (i.e., *H. meleagridis* (McDougald, 2005)*; Salmonella enterica* (Chadfield et al., 2001)) and effects of concurrent infections with other pathogens. Infections with *H. meleagridis* caused a drop in egg production and increased mortality rates in layer flocks (Esquenet et al., 2003; Liebhart et al., 2013). Co-infections of *A. galli* with the bacteria *Escherichia coli* (Permin et al., 2006), *Salmonella enteritidis* (Eigaard et al., 2006) and *Pasteurella multocida* (Dahl et al., 2002) increased the severity of infection**.** Mortality in free-range layers is associated with nematode infections (Hinrichsen et al., 2016), although *A. galli* is also known to cause mortality by obstruction of the small intestine directly (Ramadan and Abou Znada, 1991). In two studies the development of immunity after new castle disease vaccinations was influenced if concurrently infected with *A. galli* (Hørning et al., 2003; Pleidrup et al., 2014). Further on *A. galli* promotes the absorption of feed mycotoxins, as shown in higher DON values in blood plasma of infected birds (Dänicke et al., 2013), that impair the performance and health of chickens (Awad et al., 2013). Behavioural changes have been reported for *A. galli* infected laying hens (Gauly et al., 2007).

A meta-analysis investigating the effects of endo-parasites on the performance of pigs demonstrated a 5 % lower feed intake of infected pigs, accompanied by a 31% lower weight gain and worse (17 %) feed conversion (Kipper et al., 2011). The lower weight gain of infected pigs was in large part (59%) explainable due to the reduction in feed intake and was only less affected (6%) due to the parasites directly (Kipper et al., 2011). In chickens such a meta-analysis has not been performed to our knowledge.

1.3. Factors affecting worm burdens

The outcome of intestinal nematode infections is multi-factorial (Dold and Holland, 2011). Host sex (Todd and Hollingsworth, 1952), hormones (Gauly et al., 2005), diet (Abdelqader et al., 2012; Daş et al., 2011a,b, 2012), chemical properties of the gastrointestinal environment (Springer et al., 1970; Johnson and Reid, 1973) and host immunity (Dold and Holland, 2011) are known to influence the infection procedure. The chicken’s age, if at all, plays only a minor role (Idi et al., 2004; Gauly et al., 2005). Current research in mammals (e.g. Reynolds et al., 2014; Jiang et al., 2016) and chickens (e.g. Daş et al., 2011a,b, 2012) report an interaction between the worms and the intestinal environment, too. However, host immunity is the major mechanism that directly and specifically keeps fighting against the worms (Dold and Holland, 2011).

Even if the main mechanisms or interactions between various factors contributing to resistance against the intestinal nematodes are not fully understood yet, the above-mentioned factors must contribute to the variations in worm burdens found between and within genotypes (Permin and Ranvig, 2001; Gauly et al., 2002; Schou et al., 2003; Kaufmann et al., 2011b; Wongrak et al., 2015a). The genetic background of the host is largely responsible for variations in worm burdens, confirmed by high heritability estimates for both *A. galli* and *H. gallinarum* burdens in chickens (Kaufmann et al., 2011b; Wongrak et al., 2014). Both worm species are likely controlled by similar genetically determined mechanisms in the host animal (Wongrak et al., 2014).

1.3.1 Immunity against nematodes and worm expulsion

Previous studies mainly have focused on immunity against *A. galli* rather than *H. gallinarum*. For *A. galli* increased abundances of mast cells and eosinophils locally in the jejunum have been documented already in the early phase of infection (Darmawi et al., 2013; Luna-Olivares et al., 2015). Both *A. galli* and *H. gallinarum* induce specific humoral and cell-mediated immune responses. The production of ascarid specific IgY-antibodies is well-known (Marcos-Atxutegi et al., 2009; Schwarz et al., 2011b; Norup et al., 2013; Daş et al., 2017, 2018) and is triggered mainly by larval stages (Marcos-Atxutegi et al., 2009; Daş et al., 2018), probably due to the close contact to the intestinal tissue, as already proved for *Ascaris suum* infections in pigs (Miquel et al., 2005). However, a protective effect of worm specific IgY has not been proven (Andersen et al. 2013; Daş et al., 2018). The interferon gamma gene has been shown to be associated with variations in *A. galli* worm burdens and was suggested as a candidate gene for nematode resistance (Lühken et al., 2011). The nematode induced cell-mediated immunity is strongly dominated by a Th2 cell polarization (Degen et al., 2005). In both spleen and intestinal tissue increasing abundances of the Th2 cytokines IL-4 and IL-13 in *A. galli* and *H. gallinarum* infected chickens were found (Degen et al., 2005; Schwarz et al., 2011a,b; Pleidrup et al., 2014; Dalgaard et al., 2015). In mammals, similar Th2 polarizations during parasitic challenges are known (Lawrence, 2003; Dawson et al., 2005; Scales et al., 2007) and there is evidence that expulsion of the worms heavily depends on the Th2 pathway (Urban et al., 1998; Ishiwata et al., 2002; Lawrence, 2003; Scales et al., 2007; Horsnell et al., 2007; Oeser et al., 2015). Additionally, goblet cell derived products, e.g. mucins (D’Elia et al., 2009; Hasnain et al., 2010, 2011; Turner et al, 2013; Forman et al., 2012; Oeser et al., 2015), the gut peristaltic (Vallance et al., 1997, 1999; Masure et al., 2013) and an increased influx of fluids in the intestinal lumen (Zaph et al., 2014) are thought to be involved in worm expulsion in mammals. Luna-Olivares et al. (2015) reported that goblet cell counts were not affected by *A. galli* infections. However, mechanisms and the pattern of worm expulsion have only rarely been described in avian species (Tompkins and Hudson, 1999; Ferdushy et al., 2013). Although ascarid-specific IgY are transferred to egg yolks (Daş et al., 2017; Sharma et al., 2018b) there is currently no evidence for a maternal protection against *A. galli* (Rahimian et al., 2017).

1.3.2 Interactions between helminths and the intestinal environment

Several factors affect the intestinal environment by shaping the intestinal microbiota as kind of an interface (Kers et al., 2018). There is evidence that also intestinal parasites interact with the bacterial community as shown in humans, mammalians (Midha et al., 2017) and in the avian species (Hauck, 2017). Studies in mammalian have shown that helminth species not only affect (Walk et al., 2010; Li et al., 2012; Wu et al., 2012; Rausch et al., 2013; Reynolds et al., 2014; Holm et al., 2015; Houlden et al., 2015; Biswal et al., 2016) but also are affected by the intestinal conditions (i.e., the microbiota), implying the existence of two-way interactions. Studies in the chicken host investigating the interaction between intestinal bacteria and *A. galli* (Johnson and Reid, 1973) and *H. gallinarum* (Springer et al., 1970) pointed out a more successful worm establishment and survival in chickens harbouring a ‘normal’ intestinal flora compared to germ-free or gnotobiotic conditions. Similar observations were made in host-parasite systems of mice (Wescott, 1968; Chang and Wescott, 1972). Antibiotic treatments further affect worm burdens negatively (Todd, 1951; Greuel et al., 1972; Hayes et al., 2010; Biswal et al., 2016), indicating that a well-balanced intestinal microbiota is necessary for a successful infection. If *H. gallinarum* larvae were reciprocally transferred between pheasants and Japanese quail the survival ability in the reciprocal host was low, probably a matter of different intestinal environments (Lund and Chute, 1974). In chickens *A. galli* burdens were lower if worms co-existed with higher levels of the pathogenic bacteria *E. coli* (Permin et al., 2006) and *S. enteritidis* (Eigaard et al., 2006). Application of probiotics, based on L*actobacillus* strains, enhanced the mucosal immunity against coccidian infections in chickens (Dalloul et al., 2003). Contrarily, in mammalian species specific *Lactobacilli* have had beneficial effects on worm burdens (Walk et al., 2010; Reynolds et al., 2014; Jiang et al., 2016). Several studies reported interactions between the worms and the host diet (Petkevicius et al., 2003, 2004; Daş et al., 2010, 2011a,b, 2012; Pan and Yu, 2014; Wang et al., 2016b). For chickens Daş et al. (2011a,b, 2012) demonstrated that diets containing increased amounts of non-starch polysaccharides (NSP) increased the establishment and survival of both *A. galli* and *H. gallinarum*, probably a consequence of NSP induced changes in the intestinal environment. However, mechanisms being responsible for the environmental impact on the worms are probably multi-factorial, and are thought to act directly through the microbiota or *via* changes in the functional metabolite profile. In some cases it has been reported that a physiological contact between the larva and specific bacteria is necessary to induce egg-hatching, although a worm species dependency exists (Hayes et al., 2010; Koyama et al., 2013; Vejzagić et al., 2015). Bacteria also serve as a nutritional source for parasites, e.g. for *H. gallinarum* (Bilgrami and Gaugler, 2006). Walk et al. (2010) suggested that specific bacteria have anti-inflammatory and immune suppressive effects that thereby can favour the worm's survival. Alterations in the profile of microbial metabolites, e.g. short-chain fatty acids (SCFA) and lactate that derive from fermentation processes (Biswal et al., 2016) can affect the intestinal worms. The metabolites undoubtedly drive the intestinal luminal pH (Li et al., 2012, 2016) and thereby change the conditions for the worms, as pH optima for parasites are known (Lowther et al., 2009; Morin-Adeline et al., 2015). Petkevicius et al. (2004) reported a negative influence of SCFA and lactate on *Oesophagostomum dentatum* worm burdens in pigs. However, the other way round, the worms also induce alterations in the intestinal environment (Walk et al., 2010; Li et al., 2012; Rausch et al., 2013; Reynolds et al., 2014; Holm et al., 2015; Houlden et al., 2015; Biswal et al., 2016). Okulewicz and Złotorzycka (1985) found lower abundance of bacteria in digesta of *A. galli* infected hens compared to controls and suggested an antibacterial property of the worms. The mechanisms responsible for the changes are not fully understood yet. Nevertheless, a direct modulation of the helminths *via* secretory and excretory molecules (Abner et al., 2001; Berrilli et al., 2012) is discussed on one hand. Biswal (2016) discussed a so called "traffic jam" concept describing a slower food passage through the gut as a consequence of a parasite induced barrier function, allowing a higher fermentation of dietary ingredient. Consequently microbial metabolites may increase as described in a helminth-murine model (Zaiss et al., 2015). Parasite induced immune responses potentially alter the intestinal environment as well (Zaiss and Harris, 2016).

If both *H. meleagridis* and *H. gallinarum* concurrently reside in the caeca, lower establishment rates and lower survival ability have been described for *H. gallinarum*, probably due to environmental changes in the caeca (Daş et al., 2011a). It is therefore known that *H. meleagridis* induces heavy destructions in the caeca (e.g. mucosal bleedings, enlarged caeca, thickened caecal wall and fibrinous content; Hess et al., 2006). A concurrent infection with the caecal coccidian *Eimeria tenella* and *A. galli*, however, had no effects on worm burdens (Riedel, 1950).

1.4 Alternatives to culling of day-old-male chicks of layer genotypes

The research for alternatives to avoid the culling of male layer chicks is kind of an “old story” that is debated since decades (Krautwald-Junghanns et al., 2018). Through a higher progress in breeding and technical possibilities in the recent years the practical application of certain alternatives to avoid the culling comes apparently closer. The fattening of male layer chicks, the in-ovo sex determination and the use of dual-purpose genotypes are therefore under investigation. Several other methods / approaches to avoid the culling procedure have failed (Krautwald-Junghanns et al., 2018). The idea to influence the sex ratio through environmental factors, i.e. incubation temperature, known for brush turkeys and quails (Göth and Booth, 2005; Yilmaz et al., 2011), but do not account for chickens (Collins et al., 2013). However, even if possible, reducing the percentage of male chicks does not solve the culling problem. Further on, in contrast to mammalian species (Vazquez et al., 2009; Seidel, 2014), sperm sexing in avian species is not possible as the sex is determined by female chromosomes (Burt, 2001).

1.4.1 Fattening of male layer hybrids

The utilization or rather the viability of male layer type chickens has often been studied in the past years (Damme and Ristic, 2003; Gerken et. al., 2003; Murawska and Bochno, 2007; Koenig et al., 2012; Kaufmann and Andersson, 2013). In Germany there are several projects (e.g., Bruderhahn Inititative, Spitz und Bube, Haehnlein, etc.) that allow the male layer chicks to grow up on a manageable number of farms (Anonymous, 2016, 2018b, c). Even if the meat quality is comparable to that of meat-type chickens (Lichovníková et al., 2009; Choo et al., 2014), the demand for the higher priced premium products of such chickens increases only slowly (Anonymous, 2016), and is still negligible when compared with the demand for the cheaper broiler products.

1.4.2 In-ovo sex determination

Sexing layer-type chicks before hatching is currently under heavy investigation. Among other approaches to determine the sex before hatch (Göhler et al., 2017; Krautwald-Junghanns et al., 2018) mainly two techniques have been studied, which take advantage of the differences in the allantoic fluid hormones (Weissmann et al., 2013, 2014) or the fluorescence spectra of blood cells (Galli et al., 2016, 2017, 2018). The use of an endocrine based method to evaluate the level of embryonic sex hormones (i.e., the concentration of estrone sulfate), released into the allantoic fluid, allows sex determination at day 9 of incubation (Weissmann et al., 2013, 2014). For both brown and white layers the sexing accuracy is high (98.7% and 100%, respectively), whereas hatchability following the invasive sampling of allantoic fluid was much better for the brown than white layers (90.1 and 81.3%, respectively) compared to the untreated controls (Weissmann et al., 2014). Even if no negative effects on animal performance were observed a practical use is currently not yet in sight, mainly because of the low hatching rates and costs for analyses (Weissmann et al., 2014). The second method, the near infrared (NIR) fluorescence and Raman spectroscopy (Galli et al., 2016, 2017, 2018), uses sex related differences in the optical spectra (fluorescence signals) of the embryonic blood cells, caused due to the higher DNA content (2% difference) in the erythrocytes of males than females (Steiner et al., 2011). Sex determination, with accuracy about 93%, is already possible following 3.5 days of incubation, when the extraembryonic blood circulation system has been developed (Galli et al., 2016, 2017, 2018). During the first steps of development an invasive method windowing the egg shell was used, which reduced the hatching rate of the chicks by 10% (Galli et al., 2016). However, due to recent advances sex identification is hence possible contact-less and non-invasive through the egg shell membrane (Galli et al., 2018). Hatching rate, post-hatch behaviour and chick’s development are thus no longer negatively affected (Galli et al., 2018).

1.4.3 Dual-purpose genotypes

Dual purpose breeds combine the performance traits of high performing genotypes in one breed, with the compromise of a reduced growth rate (Fig. 1) and laying activity compared to the high specialized meat- and layer-type chickens (LTZ, 2013; Icken et al., 2013). The use of dual-purpose breeds was a common practice before breeding companies started to select only for one performance direction in the 1960th. The hitherto used pure-bred chicken (e.g., Sulmtaler, Vorwerk, Sundheimer, Mechelner, Rhode Island Red; Lambertz et al., 2018) can thus be called “traditional” dual purpose genotypes.

In the past there have already been considerations to use again traditional dual purpose chickens for meat and egg production (Lange, 1995; Hahn et al., 1995). However, the efficiency of the traditional dual purpose breeds is very poor (Lange, 1995; Hahn et al., 1995;

Damme, 2015; Mueller et al., 2018), thus their use was not further pursued. However, since the debate on culling chicks received increasing attention, new dual-purpose genotypes (e.g., Lohmann Dual, Les Bleues, Walesby Special and Novogen Dual) have been bred by commercial breeding companies (Damme, 2015; Schmidt and Damme, 2017). Comparing several dual-purpose genotypes the Lohmann Dual chicken have been considered to be one of the most promising one, concerning performance and efficiency traits (LTZ, 2013; Icken et al., 2013; Damme, 2015; Schmidt and Damme, 2017; Mueller et al., 2018; Lambertz et al., 2018). However, based on the genetically determined antagonism (Damme, 2015), female birds of this genotype lay about 50 eggs less compared to conventionally used laying hens (LTZ, 2013), whereas male birds need the double time to reach the same slaughter weight as conventional meat-type chickens (Damme, 2015; Mueller et al., 2018). Considering the performance level the dual-purpose genotype Lohmann Dual is comparable with the slower growing broiler chickens (Mueller et al., 2018).

1.5 Performance improvement through selective breeding

Based on the genetically determined antagonism between growth and reproduction (Damme, 2015), commercial breeding organizations have selected chickens highly specialized either for meat or egg production. Modern chicken genotypes are thus characterized by a high productivity and efficiency. Comparing broiler chickens of 1957 and 2005, the body weight has increased about 400 % accompanied by a 50% reduction in feed conversion (Zuidhof et al., 2014). In the same time period laying hens increased their productivity about 230 % (LTZ, 2015). At 1960 birds could lay 140 eggs per year, whereas modern laying hens have the potential to lay about 320 eggs (LTZ, 2015). Havenstein et al. (1994) reported that 85-90 % of the improvement in body weight development for the broiler chickens is based on genetics. Progress in the development of breeding tools (Siegel et al., 2006; Preisinger, 2011) has contributed their parts in this development. However, it is not negligible, that modern broiler chickens exploit their growth potential only when fed on modern diets with high energy and nutrient contents (Havenstein et al., 1994, 2003a, b; Cheema et al., 2003), indicating a strong diet dependency (Applegate and Angel, 2014). The different growth potential between broiler and layer genotypes is accompanied by a much higher (2 to 3 fold) feed intake for broilers than layers, a consequence of different satiety mechanisms and partly through a different sensitivity to specific satiety hormones (Cassy et al., 2004; Saneyasu et al., 2011; Buzala et al., 2015). In addition, the faster growth of broiler chickens compared to that of laying hens

(Fig. 1) is associated with the development of their intestines (Smith et al., 1990; more details in chapter 1.6.3).

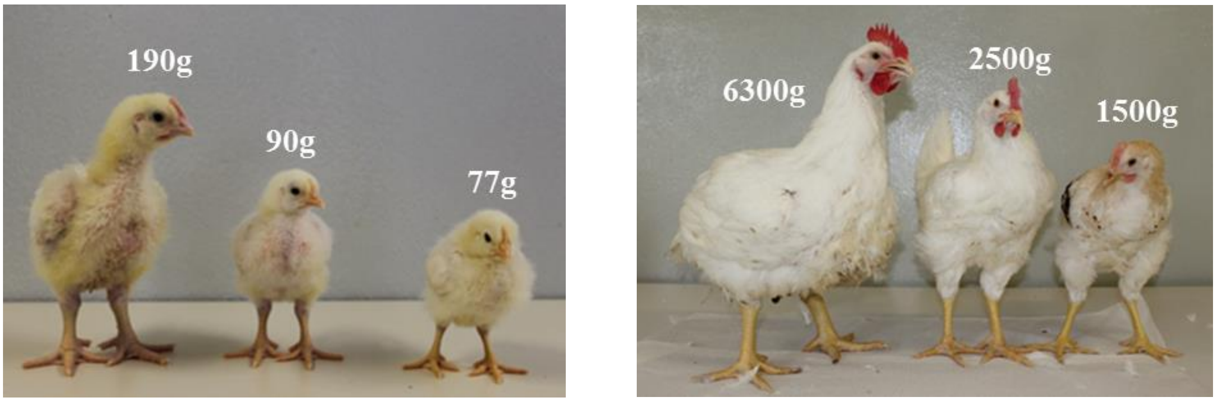

Fig.1. Growth performance of a conventional meat-type chicken (Ross-308; left), a dual purpose chicken (Lohmann Dual; middle) and a male layer-type chicken (Lohmann Brown Plus; right) at one (left panel) and ten weeks (right panel) of age.

1.6 Differences between broilers and layers

Selection for divergent performance traits either for meat or egg production consequently resulted in a large number of behavioral, physiological and immunological differences between broilers and laying hens (Rauw et al., 2012; Buzala et al., 2015; Buzala and Janicki, 2016; Malchow et al., 2018), already manifested during the embryonic development (Ohta et al., 2004; Druyan, 2010; Ho et al., 2011; Nangsuay et al., 2015; Buzala et al., 2015).

1.6.1 Fitness prospects of broilers and layers

The literature review clearly shows that selection for high performances in chickens have had risky side effects on physiological traits and the immunological status of the host (Rauw et al., 1998, 2007, 2009). The long-term selection for high performance is associated with several metabolic disorders and reduced the fitness prospects of the birds; a major problem in poultry production (Julian, 2005). However, such problems not only account for the poultry sector, but are also present in other farmed animal species. For instance, in dairy cattle, sheep and goats milk yield and mastitis incidence are positively correlated (Rupp and Boichard, 2003; Raynal-Ljutovac et al., 2007; Veerkamp et al., 2009). In broiler chickens problems like the sudden death syndrome due cardiovascular failures, ascites, malabsorption syndrome, foot-pad dermatitis, skeletal disorders accompanied by a low mobility are consequences of the selection for rapid growth rates (Decuypere et al., 2000; Julian, 2005; Rebel et al., 2006; Olkowski et al., 2007; Knowles et al., 2008). In laying hens the pressure to perform high has caused problems that concern e.g., the skeletal system (keel bone deformations and breakage) partly due to osteoporosis (Rodenburg et al., 2008; Jahja et al., 2013; Habig and Distl, 2013; Harlander-Matauschek et al., 2015; Candelotto et al., 2017), the oviduct (salpingitis; Jordan et al., 2005; Grafl et al., 2017) and the liver health (e.g., fatty liver syndrome; Dimitrov et al., 1980). Although there is only little evidence host responses to infectious diseases seem to be associated with the traits they have been selected for (Shapiro et al., 1998; Calenge et al., 2014; Han et al., 2016).

1.6.2 Consequence of selection for high performance on the immune system

In the last decades several studies aimed to investigate the impact of the long term artificial selection for divergent production traits on immune functions. Since then there is increasing evidence that broiler and layer genotypes differ in their immune competence. Koenen et al. (2002) reported different kinetics of the innate humoral immune response, with the layer-type chickens mounting a high, long-term humoral IgG response, whereas broiler chickens respond with strong but short IgM responses following a LPS challenge. The cell-mediated immune response was lower in broilers than in layers in this study (Koenen et al., 2002). Although birds were not in a challenging situation Simon et al. (2014) found lower ileal cytokine expression in broilers than in layers, whereas ileal mRNA expression levels of IgA, IgM, and IgY were higher in broiler chickens compared with layers in response to early feeding. Leshchinsky and Klasing (2001) found lower pro-inflammatory responses of broiler chickens, although there was a trend that anti-inflammatory agents were increased instead. However, results of Parmentier et al. (2010) did not confirm the previously reported differences between broiler and layer genotypes. Willson et al. (2017) compared three chicken genotypes selected for different growth rates (broiler, layer and layer x broiler cross) and found that the innate immune system, the heterophils: lymphocyte ratio and most organelle stress indicators were not different between the genotypes. Even if observations were not consistent among the studies, the differences found are likely a consequence of different growth rates. A meta-analysis by van der Most et al. (2011) demonstrated a trade-off between growth and immune functions, indicating that selection for increased growth rates have had negative effects on the immunological status of the birds. Lower body weights and smaller eggs were found if birds were selected for resistance to Marek´s disease (Warner et al., 1987). The negative association does not only account for broiler chickens (Miller et al., 1992; Qureshi and Havenstein, 1994; Parmentier et al., 1998; Cheema et al., 2003) but has also been documented for layer-type chickens (Siegel and Gross, 1980) and turkeys (Bayyari et al., 1997). Nevertheless, selection for egg production has less affected the humoral immune response (Rauw, 2012). However, the negative association between growth rate and immune status of the host has also been found to account *vice versa*, which means that selection for improved immunity negatively affects growth performance of the host. Selection for increased antibody responses in layer type chickens resulted in reduced body weights (Gross et al., 2002; Lamont et al., 2003), and in sheep studies selection for lower faecal egg counts were associated with lower weight gains of lambs (Morris et al., 2000; Bisset et al., 2001).

1.6.3 Consequence of selection on the intestinal physiology

Selection for high growth rates increased the absolute length and weight of the small intestine in broilers and resulted in a faster maturation of the intestines if compared with chickens of a layer line (Nir et al., 1992; Zavarize et al., 2012) and unselected heritage lines (Mitchell and Smith, 1991; Schmidt et al., 2009; Lumpkins et al., 2010; Mabelebele et al., 2014, 2017). Schmidt et al. (2009) reported that the small intestine of a broiler genotype was 20% longer than that of an unselected genotype. However, the relative weight and length of the small intestine were reduced due to selection (Mitchell and Smith, 1991). A similar pattern was found in chickens selected for divergent digestive efficiencies (de Verdal et al., 2010). First results demonstrated smaller intestines in a dual-purpose genotype than in classical broiler genotypes (Masri et al., 2016). Concerning histo-morphological traits, long-term selection for faster growth rates increased the villus size (i.e., villus height and width) and villus surface area (Smith et al., 1990; Zulkifli et al., 2009; Lumpkins et al., 2010; Kadhim et al., 2012), whereas the villus number was reduced during the same selection period (Kadhim et al., 2012). The larger mucosal surface probably permits an enhanced absorption of available nutrients and may be additionally responsible for the faster growth of the broiler chickens (Smith et al., 1990; Yamauchi and Isshiki, 1991; Uni et al., 1996). Selection for different growth rates has further affected the gene expression of the intestinal nutrient transporters (Mott et al., 2008).

Among several factors (e.g., diet, sex, age, housing, medication; Kers et al., 2018) affecting the intestinal microbiota the host genotype is a key factor that determines the intestinal microbiome. Research of the last years has described host genetic effects for humans (Khachatryan et al., 2008), pigs (Camarinha-Silva et al., 2017), mice (Snijders et al., 2016) and ruminants (Paz et al., 2016) as well as for avian species (Kers et al., 2018). Several studies investigated genotype differences of the intestinal microbiota between chicken genotypes designed for different growth performances and production efficiencies (Lumpkins et al., 2010; Stanley et al., 2012; Zhao et al., 2013; Singh et al., 2014; Schokker et al., 2015;

Mignon-Grasteau et al., 2015; Liu et al., 2015), whereas comparative studies with broiler and layer-genotypes are rather rare (Videnska et al., 2014; Walugembe et al., 2015; Han et al., 2016). The production of SCFA in the caecum has been shown to be higher in broiler than in laying hen chicks (Walugembe et al., 2015). Zulkifli et al. (2009) found a 10 fold higher abundance of lactic acid producing bacteria (i.e. *Lactobacillus sp.*) in the ileum and caeca of a conventional broiler genotype compared with an unselected line, the red jungle fowl. Comparing a broiler and a multi-purpose genotype no differences in the microbial load were found (Lumpkins et al., 2010). As the intestinal transit time is known to interact with the gut microbiota in mammalian species (Kashyap et al., 2013; Tottey et al., 2017) and chickens (Siegerstetter et al., 2017) differences in the microbiota between broilers and layers may therefore be a consequence of the digesta time spent in the different parts of the gastro-intestinal tract and the amount of fermentable substrates available (Shires et al., 1987).

1.7 Outline of this thesis

Due to the several known physiological differences between chicken genotypes of divergent performances directions the main objective of the present thesis was to investigate whether chicken genotypes selected either for meat or egg production or for both purposes differ in their ability to cope with nematode infections. The secondary aim was to determine whether the worm expulsion phenomenon that is known to occur in mammalian hosts occurs in the chicken host too.

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CHAPTER TWO

Co-expulsion of *Ascaridia galli* and *Heterakis gallinarum* by chickens

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Abstract

Worm expulsion is known to occur in mammalian hosts exposed to mono-species helminth infections, whilst this phenomenon is poorly described in avian hosts. Mono-species infections, however, are rather rare under natural circumstances. Therefore, we quantified the extent and duration of worm expulsion by chickens experimentally infected with both *Ascaridia galli* and *Heterakis gallinarum*, and investigated the accompanying humoral and cell-mediated host immune responses in association with population dynamics of the worms. Results demonstrated the strong co-expulsion of the two ascarid species in three phases. The expulsion patterns were characterized by non-linear alterations separated by species-specific time thresholds. *Ascaridia galli* burden decreased at a daily expulsion rate (e) of 4.3 worms up to a threshold of 30.5 days p.i., followed by a much lower second expulsion rate (e = 0.46), which resulted in almost, but not entirely, complete expulsion. *Heterakis gallinarum* was able to induce reinfection within the experimental period (9 weeks). First generation *H. gallinarum* worms were expelled at a daily rate of e = 0.8 worms until 36.4 days p.i., and thereafter almost no expulsion occurred. Data on both humoral and tissue-specific cellular immune responses collectively indicated that antibody production in chickens with multispecies ascarid infections is triggered by Th2 polarisation. Local Th2 immune responses and mucin-regulating genes are associated with the regulation of worm expulsion. In conclusion, the chicken host is able to eliminate the vast majority of both *A. galli* and *H. gallinarum* in three distinct phases. Worm expulsion was strongly associated with the developmental stages of the worms, where the elimination of juvenile stages was specifically targeted. A very small percentage of worms was nevertheless able to survive, reach maturity and induce reinfection if given sufficient time to complete their life cycle. Both humoral and local immune responses were associated with worm expulsion.

Keywords: Ascarids, Avian host, Immunoglobulin Y, Modelling expulsion, Multispecies infections, Th2 immunity

Introduction

Helminthic parasites of chickens have received increasing attention since their re-emergence in layer farms, partly due to an European Union (EU)-wide ban on conventional cage systems. Since the ban in 2012, laying hens have been kept in so-called alternative housing systems with or without outdoor access, where co-infections with *Ascaridia galli* and *Heterakis gallinarum* are highly prevalent (Kaufmann et al., 2011a; Wongrak et al., 2014; Thapa et al., 2015; Grafl et al., 2017). *Ascaridia galli* and *H. gallinarum* have different predilection sites in the gastrointestinal tract of chickens, with *A. galli* targeting the small intestine whilst *H. gallinarum* invades the caeca. Both nematodes have a similar direct life cycle, but *A. galli* has a longer prepatent period than *H. gallinarum* (Ramadan and Abou Znada, 1992; Daş et al., 2014). Additionally, whilst *A. galli* larvae are known to undergo a mucosal/ histotrophic phase (Luna-Olivares et al., 2012; Ferdushy et al., 2013), no true histotrophic phase has been described for *H. gallinarum*, with only a short tissue-associated phase taking place (Vatne and Hansen, 1965).

Worm expulsion is the first effective immune-regulatory mechanism of the host animal to address helminth infections (Dold and Holland, 2011). It is known to occur for several helminth species residing in mammalian hosts (Vallance et al., 1999; Dawson et al., 2005; Miquel et al., 2005; Masure et al., 2013; Kringel et al., 2015; Oeser et al., 2015; Sun et al., 2016). Although it has not been specifically investigated, there is evidence that worm expulsion may also occur in avian species exposed to monospecies infections (Tompkins and Hudson, 1999; Permin and Ranvig, 2001; Ferdushy et al., 2013). Low nematode establishment rates (Marcos-Atxutegi et al., 2009; Daş et al., 2010, 2012; Schwarz et al., 2011a,b) and even inability to induce patent infections (Ferdushy et al., 2013) following mono-species experimental infections of chickens provide further evidence that the chicken host may also be able to induce effective immune responses that lead to worm expulsion.

Although chicken immunity against nematodes has received increasing attention, focused on achieving a better understanding of host-parasite interactions, the mechanisms responsible for worm expulsion are not fully understood yet. Both *A. galli* and *H. gallinarum* induce strong humoral immune responses (Martín-Pacho et al., 2005; Marcos-Atxutegi et al., 2009; Schwarz et al., 2011a; Norup et al., 2013; Ferdushy et al., 2014; Dalgaard et al., 2015) by stimulating the production of antibodies, which are likely cross-reactive (Daş et al., 2017). Although immunoglobulins play a role in worm expulsion by mammalian hosts (Miquel et al., 2005; McCoy et al., 2008; Harris and Gause, 2011), there is no evidence of a protective effect of worm-specific antibodies against avian ascarids (Andersen et al., 2013; Norup et al., 2013), whereas local immune responses are thought to play a major role. For mammalian hosts, it was shown that expulsion of gastrointestinal helminths is associated with Th2 immune responses (Dawson et al., 2005; Masure et al., 2013; Oeser et al., 2015), whereas Th1 immunity deals primarily with chronic infections (Cortés et al., 2017). Nevertheless, Degen et al. (2005) reported a similar Th2 polarisation taking place in avian hosts, which was later confirmed in mono-infections of chickens with either *A. galli* or *H. gallinarum* (Schwarz et al., 2011a,b; Pleidrup et al., 2014; Dalgaard et al., 2015).

With a few exceptions (e.g., Jenkins and Behnke, 1977; Petersen et al., 2014; Andreasen et al., 2015, 2016), the vast body of research on worm expulsion in mammalian hosts has been carried out systematically with mono-species infection models. However, monospecies infections under natural circumstances occur rarely, if at all, whereas multiple infections are almost unexceptional (Carstensen et al., 2002; Eijck and Borgsteede, 2005; Kaufmann et al., 2011a; Domke et al., 2013; Wongrak et al., 2014; Thapa et al., 2015; Raue et al., 2017). Because different parasite species may vary in their pathogenicity, reside in different predilection sites or compete for the same host resources within the same predilection site, hosts are predicted to produce interactive, nonadditive immune responses when exposed to multiple species co-infections instead of mono-infections (Eswarappa et al., 2012). This implies the need for a better understanding of co-expulsion of different parasite species. However, worm expulsion after multiple species exposure has never been systematically quantified in avian species. Since *A. galli* and *H. gallinarum* globally coexist in naturally infected chickens (Kaufmann et al., 2011a; Wongrak et al., 2014; Thapa et al., 2015), the host animal may have considerably different overall responses to mono-species infections than to mixed infections. Therefore, the aim of this study was to quantify the extent and duration of worm expulsion in association with humoral and cell-mediated immune responses, as well as with alterations in the population dynamics of the worms, in chickens experimentally infected with both *A. galli* and *H. gallinarum*.

2. Materials and methods

2.1. Experimental design and ethics

In an experiment with both temporal and spatial replications, 1 day-old male chicks (n = 192) of a brown layer genotype (Lohmann Brown Plus) were reared in a floor husbandry system with wood shavings as litter material. A total of 124 birds were orally infected at the age of 7 days to induce mixed infections with both *A. galli* and *H. gallinarum*. Sixty-eight birds were left as uninfected controls. The experiment was conducted twice in succession (temporal replication), and in each iteration there were two infected and two uninfected groups of birds (spatial replication) that were kept in separate pens. Pens containing infected and uninfected animals were located in two separate rooms in a poultry research facility to avoid cross-contamination between the infected and uninfected groups. The climatic conditions in the rooms were fully controlled by an automatic system, ensuring uniform temperature, light and aeration conditions across the pens within and between rooms. Infected and uninfected birds were necropsied at weekly intervals from 2 to 9 weeks p.i, to quantify infection intensities and collect blood and intestinal tissues. The number of infected birds necropsied at each week p.i. ranged from 12 to 16 across temporal and spatial iterations. The corresponding numbers of uninfected control birds ranged from eight to 12 per week p.i. Ethical approval was obtained from a state ethics committee for animal experimentation (Mecklenburg-Western Pomerania State Office for Consumer Protection and Food Safety, Germany; permission no.: AZ.: 7221.3-1-066/15). Animal care and handling, stunning, necropsies and all sampling procedures were performed by trained and authorised staff and followed animal welfare rules. Experimental infection procedures were in line with the relevant guidelines of the World Association for the Advancement of Veterinary Parasitology for poultry (Yazwinski et al., 2003).

2.2. Infectious material

Infectious material was collected from mature female worms isolated from the field-sampled intestines of naturally infected chickens (i.e., free-range chickens). The intestines were collected from several slaughterhouses and farms across northern Germany. The small intestine and caeca were separated and opened to collect mature worms by rinsing the intestinal contents through sieves (500 µm for *A. galli*, 250 µm for *H. gallinarum*). The collected worms were sexed, and sexually mature females were isolated and rinsed several times with tap water. *Ascaridia galli* females were cut with scissors and then squeezed gently by rubbing a pestle on a plastic tea strainer in a mortar to release the eggs into a petri dish. The body tissues were first coarsely removed through sieving with the tea strainer, and the eggs were then rinsed with tap water through a series of sieves with mesh aperture sizes of 100 and 75 µm. Finally, the eggs were collected in a 36-µm sieve as previously described (Rahimian et al., 2016). Freshly collected *A. galli* eggs were then incubated in 0.1% potassium dichromate ($K_2Cr_2O_7$) for approximately 4 weeks at room temperature. The egg density in the incubation media was <25 eggs/µl, as suggested by Eriksen (1990). A small quantity of the incubation medium was added occasionally to maintain a constant volume, and the egg cultures were aerated several times each week. Adult *H. gallinarum* females were kept in a formalin (0.5%) medium. On the day of infection, the eggs of *H. gallinarum* were released and isolated in the same way as described for *A. galli*. Eggs of each species were maintained in their specific media and never exposed to low temperatures (i.e., not stored in a refrigerator). On the day of infection, the ages of the eggs (relative to worm isolation from the host) of both species ranged from 5 to 11 weeks for different batches and collection dates.

2.3. Experimental infection

On the day of infection, the eggs of *A. galli* and *H. gallinarum* were separately rinsed in a 36-mm sieve and collected in a saline solution (NaCl, 0.9%) at room temperature. The eggs were assessed several times to determine the percentage of embryonated eggs by morphological classification, as described by Rahimian et al. (2016). Only fully embryonated eggs were considered infectious. A fixed number of infectious eggs of each species per bird (250 *A. galli* eggs (S.D. = 23.7) + 250 *H. gallinarum* eggs (S.D. = 12.4)) were then combined into a final inoculum and adjusted to a dosage of 0.2 ml/bird, containing a total of 500 eggs, to be given orally by using a 5 cm oesophageal cannula as described previously (Daş et al., 2010). Uninfected control birds received a sham oral treatment with the same amount (0.2 ml) of NaCl (0.9%).

2.4. Worm harvest and larval recovery from the intestinal wall

From 2 weeks p.i. onwards, infected (n = 12–16/weeks p.i.) and uninfected control birds (n = 8–12/weeks p.i.) were necropsied at weekly intervals until 9 weeks p.i. to quantify the burdens of both species of worms. To ensure standardised emptying of the gastrointestinal digesta, birds were fasted for 3 h before necropsy. Immediately post mortem, the gastrointestinal tract was removed and the small intestine and caeca were separated. The jejunum and ileum were divided at the Meckel's diverticulum, a unique and frequently used morphological reference point (Svihus, 2014). The jejunum and ileum were opened longitudinally, and the intestinal contents, separated by section, were washed through sieves with a mesh size of 36 µm and 100 µm at 2–5 weeks p.i. and 6–9 weeks p.i., respectively. The quantification of tissue-associated *A. galli* larvae was performed in the jejunal section, the main predilection site for *A. galli* larvae (Ferdushy et al., 2013). For recovery of larvae, individual jejunums were processed through an EDTA incubation method (Kringel et al., 2002; Katakam et al., 2010; Ferdushy et al., 2012), in which EDTA binds Ca^{2+} in the exposed tissue and causes disruption of tight junctions between the epithelial cells, thereby releasing the larvae from the intestinal tissue (Kringel et al., 2002). For *A. galli* larva recovery, EDTA incubation is as efficient as other common methods (Ferdushy et al., 2012). Immediately after the luminal contents were removed for wet sieving, the jejunal wall tissue to be used for EDTA incubation was prepared as follows. The tissue was rinsed in a sieve under running lukewarm tap water to remove any attached lumen worms. At the same time, the tissue was gently squeezed through a pair of fine pencil-pincers to remove luminal worms that were accidentally attached to the intestinal wall and thereby ensure a precise differentiation between luminal and tissue-embedded larvae. Within 15 min post mortem, the washed jejunum was hung in 400 ml of a preheated EDTA solution (10 mM EDTA, 0.9% NaCl) for an incubation time of at least 22 h at 40 °C. After incubation, the jejunal tissue was dipped in the EDTA solution several times to remove any attached larvae. Finally, the EDTA solution was passed through a 20 µm sieve to collect the tissue larvae. *Heterakis gallinarum* were harvested only from the lumen contents as described for *A. galli*, but the worms were collected on finer mesh sieves (20–36 µm). All worms of both species in each bird were then placed in petri dishes for counting, sex differentiation and length measurements using a stereomicroscope. Uninfected control birds were also examined for the presence of worms in the small intestine (tissue and lumen) and caeca to exclude accidental infection with either nematode.

2.5. Worm population structure

The harvested worms were first classified into larvae, females and males based on morphological characteristics. Male worms were identified based on the presence of spicules and were not classified further on the basis of sexual maturation. In contrast to males, female worms were further classified as adults or immatures based on the presence of eggs in the uterus. Whilst the presence of eggs in worm uteri can be easily confirmed in *H. gallinarum*, the larger and thicker body of *A. galli* makes it difficult to distinguish between eggs and egg-like structures in the uterus. Instead of checking for the presence of eggs in the uterus of each *A. galli*, which is extremely time consuming (particularly when worm burdens are high), we determined a cut-off (43.5 mm) for worm length, which allowed the precise separation of ovigerous females (>43.5 mm) from sexually differentiable but not egg-bearing ones (immature, 43.5 mm). A detailed description of the determination of the length cut-off as a threshold for maturity is provided in Supplementary Fig. S1. All Heterakis worms until 4 weeks p.i. were considered to originate from the experimental infection and were thus defined as the first generation worms. By 5 weeks p.i., the number of first-generation worms was calculated as the total worm burden minus the numbers of larvae and immature worms, as these juvenile worms must have originated from reinfections.

2.6. Faecal egg counts

By 3 weeks p.i., faecal samples (n = 110) were collected from the birds to quantify excretion of nematode eggs. Individual faecal samples were collected from the birds 1 day prior to necropsy during captivity in individual cages (W 40 x L 45 x H 50 cm) designed for quantitative daily faeces collection. The daily total faeces were thoroughly mixed, and a random subsample (4 g) was analysed with a modified McMaster egg counting technique (MAFF, 1986) using saturated NaCl as the flotation liquid (density $\geq$ 1.2 g/ml) to quantify egg concentration in faeces (eggs per gram faeces, EPG). The minimum detection level was 50 EPG. The number of eggs excreted within 24 h (eggs per day, EPD) was then estimated by multiplying the amount of daily faeces by EPG. Eggs of *A. galli* and *H. gallinarum* were not differentiated and were counted together because they cannot be reliably differentiated.

2.7. Ascarid-specific antibodies

At each necropsy (2–9 weeks p.i.), blood samples were taken immediately after cervical dislocation of stunned birds. Plasma was separated by centrifugation at 2500 g for 20 min and stored at 20°C in an anticoagulant (EDTA) until analysis. Ascarid-specific IgY antibodies were quantified in plasma samples (n = 190) with an ELISA as described earlier (Daş et al., 2017). The laboratory-specific intra- and inter-assay coefficients of variability for the assay were 5.0% and 8.4%, respectively.

2.8. Cellular immunity

Tissue samples from the mid-jejunum and caecum, representing the predilection sites of *A. galli* and *H. gallinarum*, respectively, were taken from a total of 36 chickens (six controls and six infected at weeks p.i. 2, 5 and 9). As all the jejunal tissue was used for larval recovery, tissue samples for quantification of cellular immunity were obtained from counterpart birds used in the same study for another purpose (digesta collection). All the control and infected birds used in that study, including those sampled for tissue, were identically treated/infected, housed, fed and necropsied on the same day to exclude any potential confounding effect. Immediately post mortem, intestines were removed, and pieces of tissue (2–3 cm each) from the mid-jejunum and left caecum were taken. The intestinal tissue samples were first flushed with 0.9% NaCl by using a syringe with a cannula to remove the intestinal content and were placed separately on ice afterwards. The tissues were opened longitudinally, flushed again with 0.9% NaCl and then cut into small pieces. The tissue pieces were then snap frozen in liquid nitrogen, transferred into cryogenic vials and stored at 80 °C for quantitative real-time PCR analysis.

2.9. Quantitative real-time PCR

2.9.1. Biochemical indices

Jejunal and caecal tissues from each chicken were homogenised separately in peqGOLD TriFast reagent (PEQLAB GmbH, Munich, Germany), and total RNA and DNA were extracted according to the manufacturer's protocol. Nucleic acids were quantified using a spectrophotometer and then used to determine biochemical indices (Supplementary Table S1) measuring protein synthesis capacity (RNA:DNA ratio), cell count (DNA) and translational capacity (RNA).

2.9.2. RNA extraction and cDNA synthesis

RNeasy Mini Kits (Qiagen, San Diego, CA, USA) were used to purify total RNA extracted for biochemical measurements. Purified RNA was quantified using a spectrophotometer, and RNA quality assessed by running 1 µg on a 1% non-denaturing agarose gel stained with EvaGreen® Fluorescent Gel Stain (Jen Biosciences, Dortmund, Germany). Total RNA (2 µg) was reverse transcribed for 120 min at 42°C to produce cDNA using the SuperScript® VILO™ cDNA Synthesis Kit according to a modified version of the manufacturer's protocol (Thermofischer Scientific, Darmstadt, Germany).

2.9.3. Primer design

Primers were sourced from published studies and, where applicable, designed for this study using the GenScript Real-time PCR (TaqMan) Primer Design Tool (https://www.genscript.com/tools/real-time-pcr-tagman-primer-design-tool) against Gallus gallus mRNA sequences from publicly available from the U.S. National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.-gov). Primers were made by Metabion (Munich, Germany) and purified using desalting to remove short truncated products and small organic contaminants. The primer sequences are presented in Table 1.

Table 1. Primers and probes used for quantitative real-time PCR targeting immune system-related genes in chickens.

Gene Name	Symbol	Source	Hybridisation	Primers (5′ to 3′)
Reference genes				
Jejunum				
Hypoxanthine phosphorribosyltransferase 1	HPRT	Yang et al., 2013	Forward	ACTGGCTGCTTCTTGTG
			Reverse	GGTTGGGTTGTGCTGTT
Ribosomal protein L30	RPL30	Yang et al., 2013	Forward	GAGTCACCTGGGTCAATAA
			Reverse	CCAACAACTGTCCTGCTTT
Caeca				
18S ribosomal RNA	18S	Yin et al., 2011	Forward	GGCGGCTTTGGTGACTCTAG
			Reverse	ATCGAACCCTGATTCCCCGT
Beta-actin	ACTB	Yang et al., 2013	Forward	CTGTGCCCATCTATGAAGGCTA
			Reverse	ATTTCTCTCTCGGCTGTGGTG
Target Genes				
Beta-2-microglobulin	B2M	Yang et al., 2013	Forward	CGTCCTCAACTGCTTCGTG
			Reverse	TCTCGTGCTCCACCTTGC
Claudin 1	CLND1	Shao et al., 2013	Forward	TGGAGGATGACCAGGTGAAGA
			Reverse	CGAGCCACTCTGTTGCCATA
Interleukin 13	IL-13	Dalgaard et al., 2015	Forward	AAGGACTACGAGCCCCTCAT
			Reverse	GCAAGAAGTTCCGCAGGTAG
Interleukin 22	IL-22	Crhanova et al., 2011	Forward	CAGGAATCGCACCTACACCT
			Reverse	TCATGTAGCAGCGGTTGTTC
Interleukin 4 Receptor - Alpha Chain	IL-4Rα	This study, 2018	Forward	CTGCGGGAGTTGCGAAGACG
			Reverse	CAGGGTATGCAGCGCAGGAA
Inducible Nitric Oxide	iNOS	Kano et al., 2009	Forward	TACTGCGTGTCCTTTCAACG
			Reverse	CCCATTCTTCTTCCAACCTC
Mucin 2	MUC2	Forder et al., 2012	Forward	ATGCGATGTTAACACAGGACTC
			Reverse	GTGGAGCACAGCAGACTTTG
Mucin 5 (transcript variants A and C)	MUC5	Forder et al., 2012	Forward	TGTGGTTGCTATGAGAATGGA
			Reverse	TTGCCATGGTTTGTGCAT
Signal transducer and activator of transcription 6	STAT6	This study, 2018	Forward	GCCTGGAACTTGGTCTGCGT
			Reverse	GGCGCATTTCTCCTCCGTCA

2.9.4. Quantitative PCR (qPCR) assay

Quantitative real-time PCR was performed using the SSOFAST EVA Low ROX Kit (Bio-Rad, Munich, Germany) on a Roche (Mannheim, Germany) LightCycler® 96 using the manufacturer's recommended volumes. The quantitative PCR conditions were set at 95 °C for 30 s, followed by 40 cycles of 5 s at 95 °C and 20 s at 60 °C (two step amplification). The specificity of each qPCR was assessed by analysing the melting curves of the products and by verifying the sizes of the amplicons. Each PCR included two negative controls, one with no template and one with no enzyme, and unknown samples that were run in duplicate. The PCR efficiency and quantification cycle (Cq) values were obtained for each sample using LinRegPCR (Ramakers et al., 2003). Two reference genes each for jejunal (Hypoxanthine phosphoribosyltransferase 1 (HPRT), Ribosomal protein L30 (RPL30)) and caecal (18S rRNA, (18S), Beta-actin, (ACTB)) samples were identified using the analysis software qBase+ (Biogazelle). Reactions were performed in triplicate, with all data entered into LinRegPCR 2017.1 (Ruijter et al., 2014), and the fold change in abundance ratios between the means of two treatment groups was determined. Abundance levels are expressed as normalised relative quantity (NRQ). All qPCR data were reported as specified by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE (Bustin et al., 2009)) guidelines.

2.10. Data analysis

Uninfected control animals were excluded from the analysis of worm burden and faecal egg count data. Variables describing infection intensity (worm burden, faecal egg counts) and antibody data were log transformed [ln (y + 1)] and subjected to analysis of variance by using the MIXED procedure in the software SAS/STAT (Version 9.4), part of the SAS System for Windows (SAS OnlineDoc® Version 9.4. Cary, NC, USA: SAS Institute Inc.). The statistical model for worm burden parameters included fixed effects of weeks p.i. and run (temporal replication) and their interaction, plus pen effects. The experimental unit was an individual chicken. For worm location analyses (jejunum versus ileum or lumen versus tissue), a random animal effect was additionally included in the mixed model to account for the individual effect of host animal with two locations. As anti-ascarid IgY antibodies were also measured in uninfected control birds, an extended model including effects of infection and weeks p.i. and their interaction, plus run and pen effects, was used. Pearson's correlation analysis was performed to investigate relationships between the burdens of the two worm species. The qPCR data were analysed with a model including effects of infection, weeks p.i. and their interaction, plus a run effect. Pairwise multiple comparisons were performed with the Tukey-Kramer test with a significance level at $P < 0.05$. Pooled worm burden data across spatial and temporal iterations ($n = 124$) were further modelled using a non-linear segmented regression analysis (Kaps and Lamberson, 2004) to quantify the magnitude and time dependency of expulsion for each nematode species. The analyses were based on the following model with two conditional equations:

(I) $y_i = a + bx_i$ for $x_i \leq x_0$

(II) $y_i = (a - cx_0) + (b + c) x_i$ for $x_i > x_0$

which meet at the breakpoint x_0 E (y_i | x_0). X_0 served as a threshold time point that identified different worm expulsion patterns taking place below and above this point. The parameters a, b and c were estimated, with (*a*) being a constant, (*b*) the slope of the first and (*c*) of the second regression lines. By using the outcomes of the equations based on the estimated parameters, daily worm expulsion rates (e, number of worms expelled from a host per day) were then calculated for each time period before and after the threshold. The model was implemented in Proc NLIN of SAS. The pseudo-goodness of fit (pseudo-R^2) was calculated as suggested by Schabenberger and Pierce (2002) for assessing the adequacy of non-linear models. Graphical representations of pooled data were created in Microsoft Excel 2010 or in JMP 12 (JMP®, Version 12. SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Reproducibility of experimental infections and main outcomes

Repetition of the experiment over runs ($n = 2$) and pens ($n = 2$/run) did not significantly influence total burdens of either *A. galli* or *H. gallinarum* and did not affect their combined egg excretion ($P > 0.05$). There was also no significant interaction between run and weeks p.i. on the total burden of either nematode ($P > 0.05$). Except for two genes out of 16 (eight genes in each of two tissues), transcript abundance was also not influenced by run effects ($P > 0.05$).

3.2. Changes in worm burden by time and location

Total counts of *A. galli* and *H. gallinarum* did not correlate significantly at any week p.i. ($P > 0.05$). Correlation analysis for pooled worm count data (2–9 weeks p.i.) of both nematodes also did not indicate a significant relationship between the two species ($P = 0.192$). From 2 to 4 weeks p.i., all experimentally infected birds harboured *A. galli*. In the following weeks (5–9 weeks p.i.), the prevalence of *A. galli* among birds ranged from 81.3% to 100%. At 2 weeks p.i., all experimentally infected birds harboured *H. gallinarum*, whilst the percentage of birds harbouring this nematode varied from 56.3% to 93.8% in the following weeks (i.e., 3–9 weeks p.i.). The average *A. galli* burden per host decreased significantly ($P < 0.001$) from approximately 97 worms at 2 weeks p.i. to two worms at 9 weeks p.i. (Fig. 1A). *Ascaridia galli* burden did not significantly differ between 2 and 3 weeks p.i. ($P < 0.05$), whereas significant decreases were quantified from 2-4 weeks p.i., 4–5 weeks p.i. and 5–9 weeks p.i. ($P < 0.05$). A similar decreasing pattern was also observed for *A. galli* larva counts ($P < 0.001$; Fig. 1B). Overall average *A. galli* worm counts were significantly ($P < 0.001$) higher in the jejunum (14.93 ± 2.82, means ± S.E.) than in the ileum (6.73 ± 1.11) (Fig. 1C). Approximately 69% of all *A. galli* worms were isolated from the jejunum. Although total *A. galli* counts decreased in both locations across experimental weeks ($P < 0.001$), there was a significant interaction between worm location and week p.i. ($P < 0.05$), indicating elevated ($P < 0.05$) worm counts at 2, 4, 5, 7 weeks p.i. and a strong tendency ($P = 0.051$) at 8 weeks p.i. in favour of the jejunal section. With the exceptions of 3 and 6 weeks p.i., the proportion of *A. galli* worms was also higher ($P < 0.05$) or tended ($P < 0.10$) to be higher in the jejunum than in the ileum (% data not shown). The number of larvae was significantly higher in the lumen of the jejunum than in its tissue ($P < 0.001$), with 87.5% of larvae being isolated from the lumen. The numbers of both luminal and tissue-embedded larvae decreased significantly ($P < 0.001$) over time, and very few tissue larvae could be found by 6 weeks p.i. (data not shown).

Total *H. gallinarum* burdens changed significantly across weeks of infection ($P = 0.002$; Fig. 2A) with a concave pattern, represented by decreasing worm counts until 7 weeks p.i., followed by an increase in the next 2 weeks. Similarly, the number of larvae differed significantly among the experimental weeks ($P < 0.001$). As shown in Fig. 2B, larval counts decreased from 2 to 4 weeks p.i. ($P < 0.05$), followed by a low larva phase up to 7 weeks p.i.; thereafter, increasing numbers of larvae were quantified by 8 weeks p.i. Despite the significant changes in both total *H. gallinarum* burden and larval counts throughout the experimental period ($P < 0.002$), no significant differences ($P > 0.05$) were quantified between 2 and 9 weeks p.i. for either worm burden or larval counts.

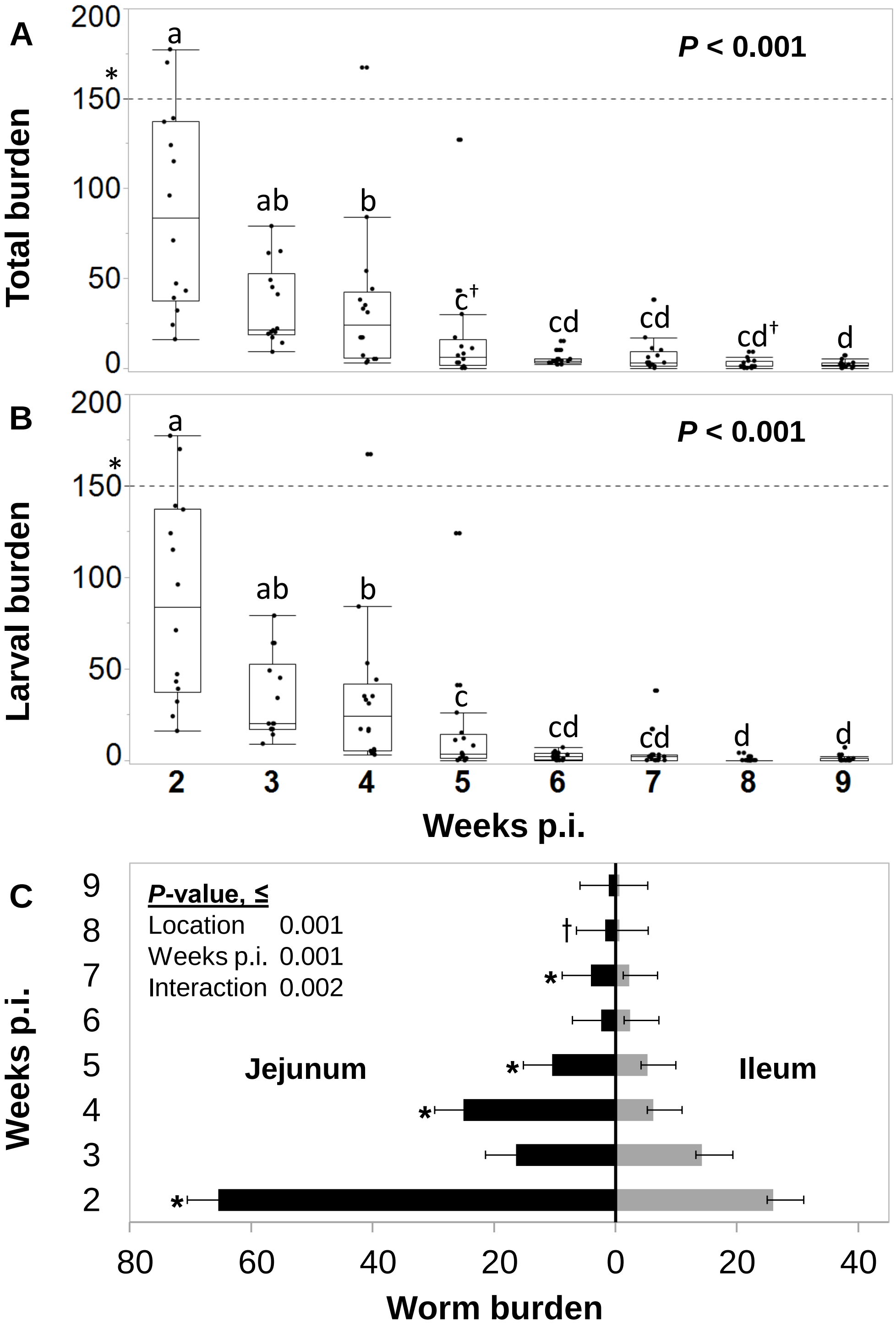

Fig. 1. Time-dependent alterations in total *Ascaridia galli* burden (**A**), larval *A. galli* burden (**B**), and distribution of worms in the jejunum and ileum (**C**) of infected chickens. The total number of birds necropsied at each time point ranged from 12 to 16 weeks p.i. (wpi). Pooled data across spatial and temporal replications (sample size, n = 124) are represented by outlier box plots, where the horizontal line within the box represents the median while lower and upper ends of the box represents the 25th and 75th quantiles, respectively. Each dot represents the worm count of an individual host. Outliers above the dashed line (*>150) are shown on an extended reduced scale (reduced x-axis = (y * 0.01 + 150)) to provide a focused picture of the whole dataset.

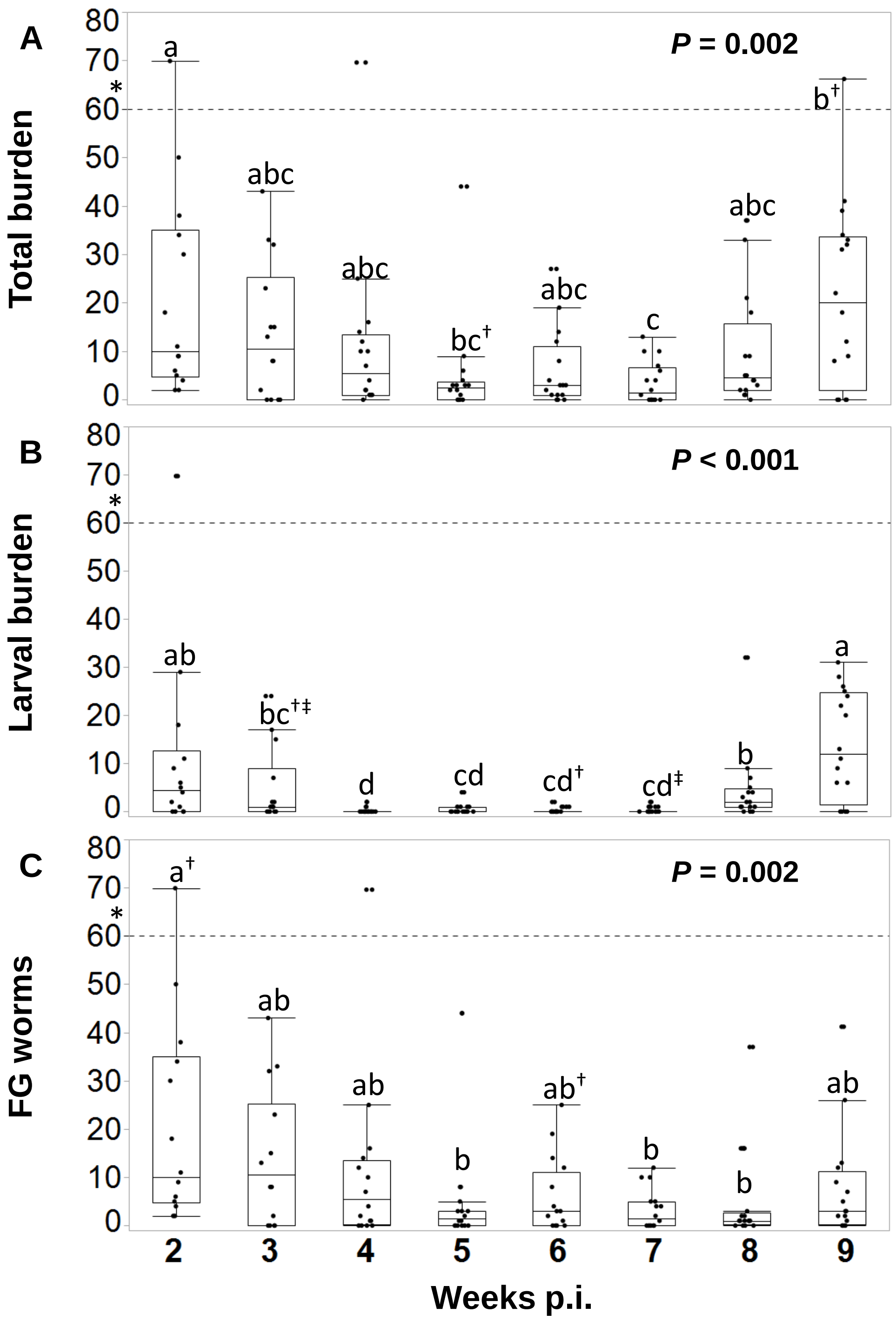

Fig. 2. Time-dependent alterations in total *Heterakis gallinarum* worm burden (**A**), larval *H. gallinarum* burden (**B**) and number of first-generation worms (**C**) in infected chickens. Outliers above the dashed line (*>60) are shown on an extended reduced scale (reduced x-axis = (y * 0.01 + 60)) to provide a focused picture of the whole dataset. Pooled data across spatial and temporal replications (sample size, n = 124) are represented by outlier box plots, where the horizontal line within each box represents the median while lower and upper ends of the box represent the 25th and 75th quantiles, respectively. Each dot represents the worm count of an individual host. The total number of birds necropsied at each time point ranged from 12 to 16 weeks p.i. The number of first generation worms was calculated as the total worm burden minus the numbers of larvae and immature worms by 35 days p.i.

3.3. Quantification of worm expulsion

The segmented regression models fit the worm burden data significantly ($P < 0.001$) for both species, indicating the existence of non-linear relationships of worm burden in response to time after infection. The goodness of fit of the model was higher for *A. galli* (Pseudo-R^2 = 0.42–0.43) than for *H. gallinarum* (Pseudo-R^2 = 0.13–0.19). Estimated parameters describing the exact shape of the expulsion curves – with a constant (*a*), two slopes (*b, c*), a breakpoint (X_0) and expulsion rates (*e*) below and above the threshold for each of the examined variables – are summarised in Table 2.

Table 2. Non-linear model parameters describing characteristics of prediction lines (*a, b, c,* X_0), daily expulsion rates (*e*) below and above the threshold (X_0), and the model adequacy parameters for worm burdens fitted to days p.i. (dpi) by segmented regression analysis. Model parameters refer to estimated initial worm burden (constant, *a*), slopes of the first (*b*) and second (*c*) regression lines, and a time threshold (X_0 in dpi) separating changing relationships between time and worm burden before and after X_0.

	Parameter estimates				**SE of estimates**				***e***		**Model fit parameters**		
Item	***a***	***b***	***c***	X_0	***a***	***b***	***c***	X_0	$\leq X_0$	$>X_0$	$P \leq$	**F**	**P-R^2**
Ascaridia galli													
Total burden	146.3	-4.296	3.832	30.52	19.142	0.867	0.944	3.200	4.30	0.46	0.0001	28.58	0.42
Larva	146.3	-4.320	3.873	30.88	19.164	0.868	0.945	3.203	4.32	0.45	0.0001	29.66	0.43
Heterakis gallinarum													
Total burden	32.43	-0.772	1.509	40.42	6.964	0.266	0.370	3.918	0.77	-0.74	0.0009	5.90	0.13
Larva	19.28	-0.597	1.238	38.98	4.454	0.170	0.237	3.029	0.60	-0.64	0.0001	9.17	0.19
1st gen. worms	32.19	-0.779	0.842	36.07	6.430	0.245	0.341	6.452	0.78	-0.06	0.0007	6.06	0.13

e, daily expulsion rate, number of worms expelled from a host in a day (-*e* indicates an increase due to reinfection); F, F-value; P-R^2, pseudo-R^2, N, Number of observations = 123–124 for all variables; DF, degrees of freedom for model: DF = 3 and for corrected total: DF = 122–123.

As shown in Fig. 3A, *A. galli* worms (total burden) were eliminated from the host with an expulsion rate of 4.3 worms/day up to 30.5 days p.i. Following this time threshold, the slope was more horizontal and represented an expulsion rate of 0.46 worms/day, which resulted in the almost complete elimination of *A. galli* by the end of experiment (estimated worm burden at 63 days p.i. = 0.1 worms/bird). Almost the same pattern and parameters observed for total worm burden were estimated for *A. galli* larvae (Table 2). *Heterakis gallinarum* (total burden) was eliminated from the host at an expulsion rate of 0.77 worms/day until a threshold of 40.4 days p.i., but thereafter, the worm burden increased at a daily rate of 0.74 worms/bird until the end of the experiment (Fig. 3B). A similar pattern was also quantified for *H. gallinarum* larvae (Table 2). When only the worms originating from the experimental infection were considered (first generation worms; Fig. 2C), a very similar expulsion rate (0.78 worms/day) was estimated for the time period before the threshold (36.4 days p.i.), but thereafter, the regression line was almost fully horizontal (Fig. 3C) and indicated an increase of 0.06 worms/day. This represented 5.8 worms/bird (2.3% of the infection dose) originating from the first generation and surviving until the end of the experiment (63 days p.i.).

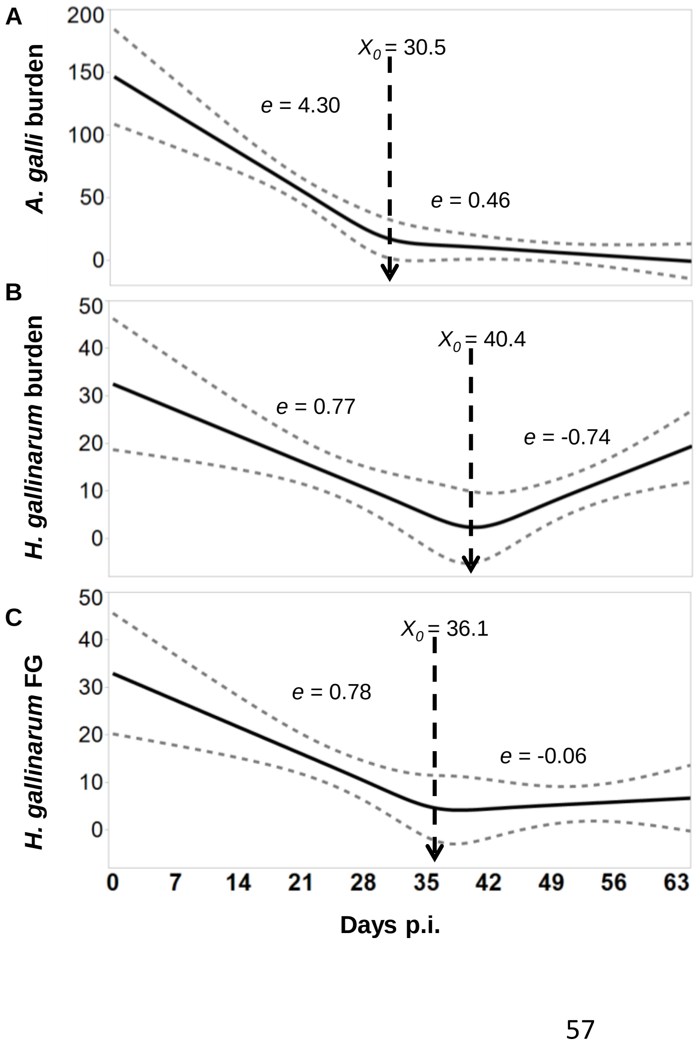

Fig. 3. The extent and duration of worm expulsion by chickens co-infected with *Ascaridia galli* and *Heterakis gallinarum*.
The expulsion curves for *A. galli* burden (**A**), *H. gallinarum* burden (**B**) and first-generation (FG) *H. gallinarum* worms (**C**) are based on outcomes of segmented regression. The solid line (-) represents the predicted expulsion curve, and the dashed lines (---) indicate the upper and lower 95% confidence intervals for the mean prediction; e indicates the daily worm expulsion rate before or after the time threshold (X_0). Analyses are based on pooled data across spatial and temporal replications (sample size, n = 124).

3.4. Worm growth and development

The vast majority (>98%) of *A. galli* worms were larvae until 5 weeks p.i. (Fig. 4A). Thereafter, the proportion of larvae in the total worm burden decreased to 45% at 6 weeks p.i. and varied from 20% to 69% in the following weeks. As shown in Fig. 4B, the proportions of different developmental stages in the *H. gallinarum* population corresponded well to the changes in the worm counts. At 2 weeks p.i., approximately 58% of the worms were larvae, the rest being classified as either immature female (23%) or male worms (19%). Thereafter, the proportion of larvae decreased up to 1.5% by 4 weeks p.i. and remained at low levels (i.e., <7%) until 7 weeks p.i. At the end of the experiment, the proportion of larvae was 65%, and immature females (3%) were encountered again.

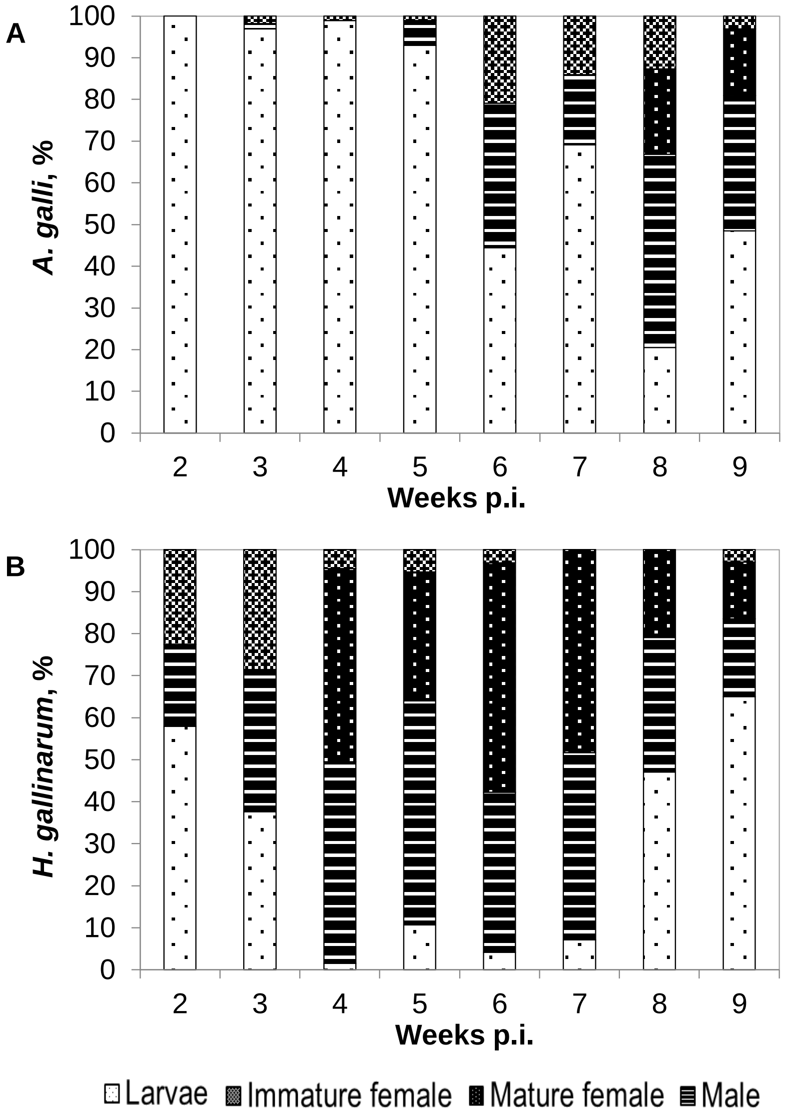

Fig. 4. Proportion of *Ascaridia galli* (**A**) and *Heterakis gallinarum* (**B**) worm burdens in relation to developmental stages and sexes.

Worm growth in relation to developmental stages/maturation and sex for both species are summarised in Fig. 5. By 3 weeks p.i., sexually differentiable male and female *A. galli* worms were identified for the first time, and female worms reached sexual maturity (i.e. harboured eggs in the uterus) by 8 weeks p.i. (Fig. 5A). The cut-off for maturity of *A. galli* females was set at a length of >43.5 mm (see Supplementary Fig. S1). Female worm length at 9 weeks p.i. was 56.8 ± 5.17 mm, whilst males were shorter (35.9 ± 3.65 mm). Jejunal lumen larvae were significantly longer than tissue-associated larvae (P < 0.001). Overall, larva length was significantly greater in the jejunum than in the ileum (P < 0.001). For *H. gallinarum*, sexually distinguishable worms were first encountered by 2 weeks p.i. (Fig. 5B). Mature female worms were first identified at 4 weeks p.i., when egg shedding also started (see below for faecal egg counts). At this age (4 weeks), mature females were on average 8.8 ± 0.22 mm, and the mean length increased significantly (P < 0.001) until 9 weeks p.i. (10.7 ± 0.31 mm).

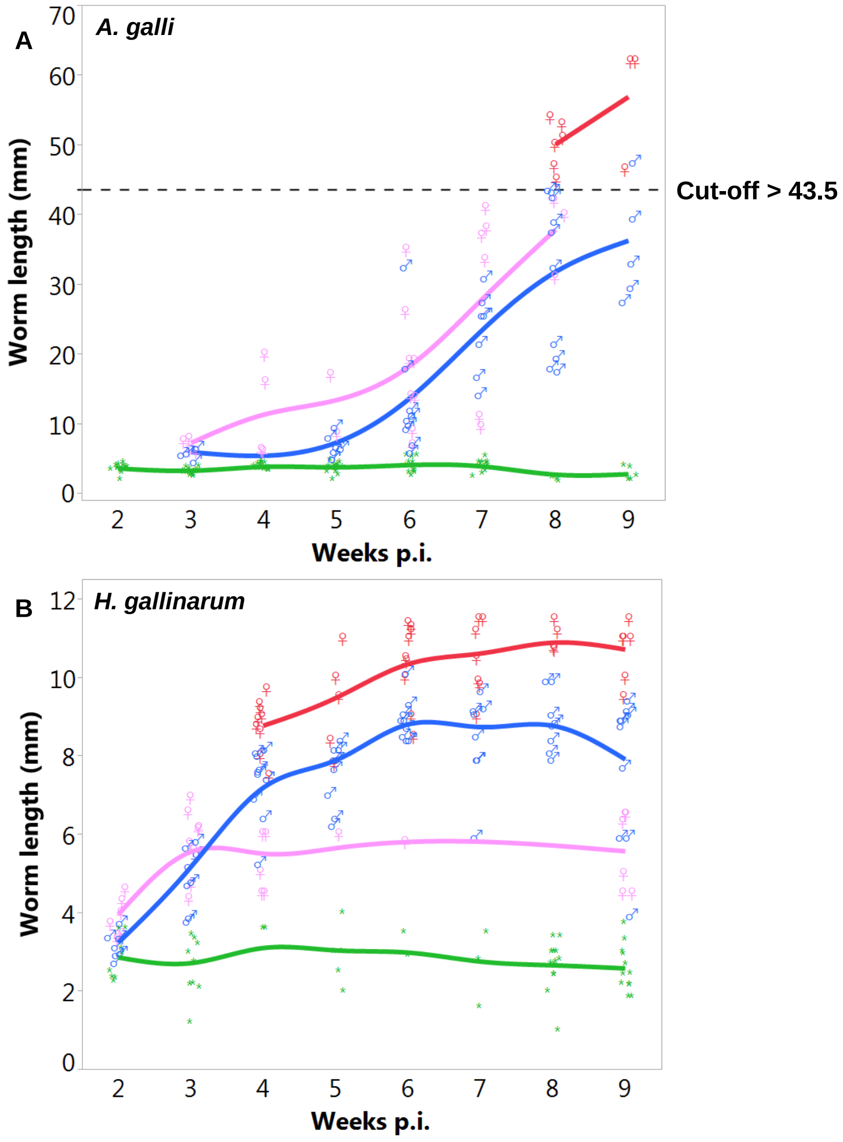

Fig. 5. Worm growth in relation to developmental stage/maturation and sex for *Ascaridia galli* (**A**) and *Heterakis gallinarum* (**B**). Each sign indicates the average length of species-specific larva (⋆), male (♂), immature female (♀) and mature female (♀) worms in a host animal.

3.5. Faecal egg counts

Faecal egg counts of individual chickens were quantified from 3 to 9 weeks p.i. in terms of both EPG and EPD. At 3 weeks p.i., all faeces samples were negative. As shown in Fig. 6, there was a slight, non-significant (P = 0.139) increase in EPD from 4 weeks p.i. to 9 weeks p.i.

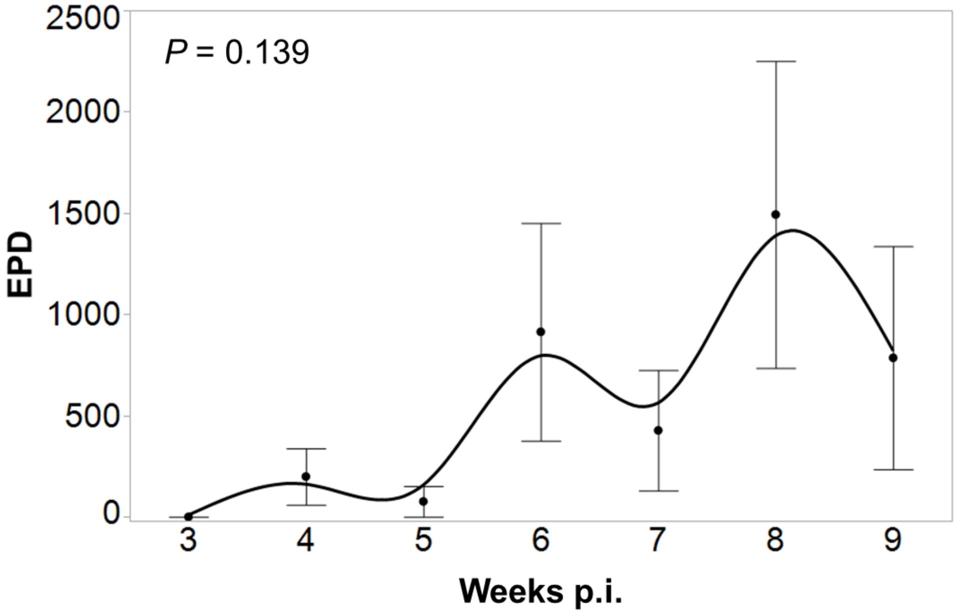

Fig. 6. Faecal egg counts in chickens co-infected with *Ascaridia galli* and *Heterakis gallinarum*. Data represents average number of eggs per day (EPD). Means and S.E. represent untransformed data; P values are based on the transformed data. Sample size, n = 110 faeces samples collected from 3 to 9 weeks p.i.

3.6. Ascarid-specific IgY

Overall ascarid-specific plasma antibody levels were higher in infected birds than in controls (P < 0.001). As indicated by a significant interaction (P < 0.001) between infection and time (weeks p.i.), development of ascarid-specific IgY in the infected birds took approximately 5 weeks after the challenge. As shown in Fig. 7, infection groups did not differ significantly from 2-4 weeks p.i. (P > 0.05). At 5 weeks p.i., infected birds had a strong tendency (P = 0.051) towards elevated levels of IgY compared with those of uninfected controls. From 6 to 7 weeks p.i., infected animals had higher plasma IgY concentrations than their uninfected counterparts (P < 0.05). At 8 weeks p.i., there was a slight decrease in the IgY levels of infected birds, which nevertheless still tended to be higher than the IgY levels of the controls (P = 0.080). At 9 weeks p.i., the IgY levels of infected birds reached a maximum. The IgY levels of uninfected control birds were relatively constant and did not differ significantly from

2 weeks p.i. to 9 weeks p.i. (P > 0.05). IgY levels at 2 weeks p.i. were significantly correlated with the burden of *H. gallinarum* larvae (r = 0.67; P = 0.009) but not *A. galli* larvae (P = 0.157). At 5 weeks p.i., when antibody levels tended to be elevated among infected birds (Fig. 7), IgY levels strongly correlated with *A. galli* larvae (r = 0.81; P < 0.001), whereas no correlation with *H. gallinarum* larvae was detected (P > 0.05). No further significant correlations between worm burdens and IgY levels were detected in other weeks.

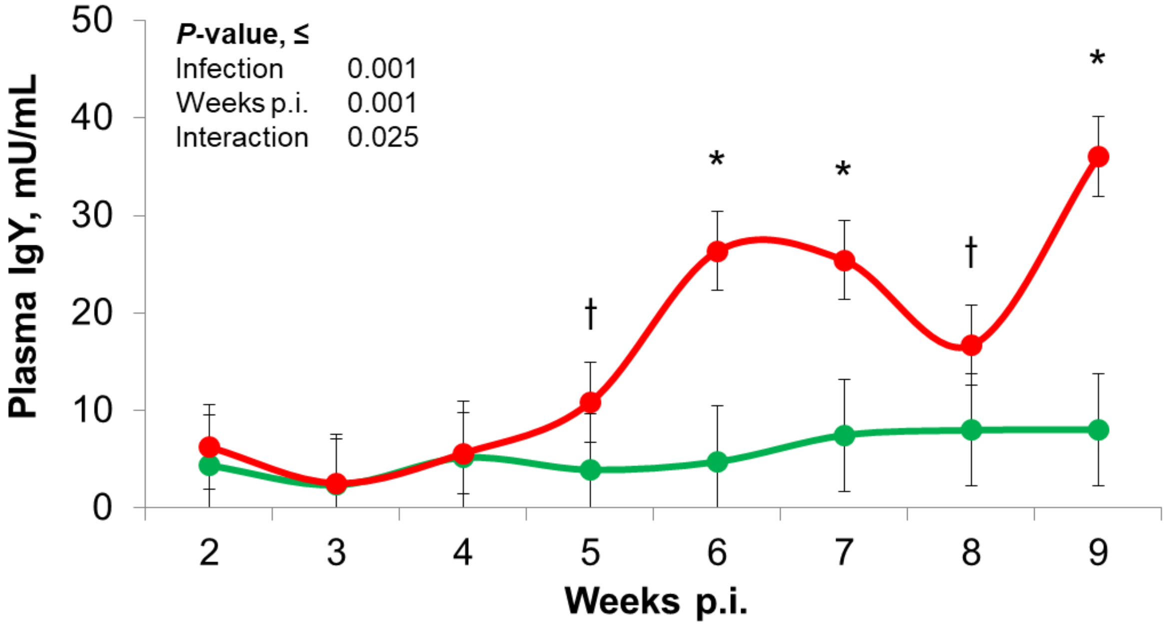

Fig. 7. Time-dependent alterations in plasma IgY antibodies against both ascarids in infected (●) and uninfected control (●) birds. An asterisk (*) indicates significant difference at the given time point weeks p.i. (wpi); (t) indicates statistical tendency to differ. Least-squares means and S.E. represent untransformed data; P values are based on the transformed data. Sample size, n = 190 plasma samples (n = 23–24/wpi).

3.7. Cell-mediated immune responses at predilection sites

To determine whether worm expulsion was related to immunological alterations in the tissues of worm-specific predilection sites, we performed a qPCR analysis. For both jejunal and caecal tissues, infected animals showed the highest overall abundance of the Th2-cytokine IL-13 (P < 0.001; Fig. 8). In the jejunum, IL-13 abundance reflected an infection group x time interaction (P = 0.002), with an elevated abundance of IL-13 in the infected birds only at 2 weeks p. i. (Fig. 8). Infection increased the expression of IL-13 in caecal tissue at 2 and 9 weeks p.i. (P < 0.05) and tended to increase expression at 5 weeks p.i. (P = 0.098; Fig. 8). The abundance of IL-4Ra tended to be decreased in the jejunal tissue of infected animals (P = 0.093; Table 3), whilst this receptor was upregulated by the infection (P = 0.049) in the caecal tissue. STAT6 (Signal transducer and activator of transcription 6) abundance in the jejunum was downregulated at 2 weeks p.i. ($P < 0.05$), although it remained unaffected by infection in the caecum ($P > 0.05$; Table 3). Infection did not influence the abundance of IL-22 in the jejunal tissue ($P < 0.05$), whereas the caecal abundance of IL-22 was significantly elevated ($P < 0.05$) or tended to be elevated ($P < 0.10$) at 2 and 9 weeks p.i., respectively.

Table 3. Effects of multiple parasite co-infections on expression of immune genes in the tissue[a] of parasite-specific predilection sites in the chicken host.[b]

	Infection			***P* values, ≤**			
Item	**Control**	**Infected**	**S.E.**	**Infection**	**wpi**	**Inf.*wpi**	**Run**
Jejunum							
IL-13	-0.375	0.284	0.12	0.0006	0.1521	0.0015	0.5731
IL-22	-0.015	-0.054	0.09	0.7671	0.1510	0.2759	0.4531
IL-4Rα	0.021	-0.022	0.02	0.0925	0.0291	0.1573	0.7578
STAT6	0.028	-0.022	0.02	0.1307	0.3843	0.0016	0.4821
MUC2	0.051	-0.124	0.04	0.0048	0.1112	0.0211	0.2159
MUC5ac	-0.094	0.091	0.03	0.0001	0.0113	0.0069	0.0426
iNOS	-0.020	-0.085	0.03	0.1723	0.2458	0.0188	0.4182
CLDN1	0.017	-0.050	0.02	0.0430	0.7118	0.0151	0.5897
Caecum							
IL-13	-0.404	0.342	0.08	0.0001	0.0042	0.0411	0.6009
IL-22	-0.080	0.104	0.08	0.1185	0.2039	0.0108	0.2492
IL-4Rα	-0.020	0.052	0.02	0.0489	0.2041	0.0868	0.4993
STAT6	0.014	0.018	0.02	0.8860	0.6287	0.2145	0.8310
MUC2	-0.320	0.186	0.08	0.0002	0.1567	0.2180	0.8390
MUC5ac	-0.308	0.288	0.05	0.0001	0.8599	0.0089	0.7258
iNOS	-0.169	0.197	0.07	0.0014	0.0557	0.1528	0.0117
CLDN1	-0.032	0.064	0.04	0.0859	0.0371	0.0232	0.7749

Inf, infection; STAT6, signal transducer and activator of transcription 6; MUC2, mucin 2; MUC5ac, Mucin 5 (transcript variants A and C); iNOS, inducible Nitric Oxide; CLDN1, Claudin 1.

[a] Tissue samples were collected from 12 birds (six infected, six uninfected controls) at each sampling time point (weeks p.i. (wpi)).

[b] Sample size: n = 72 (12 birds *x* three time points *x* two tissues).

Infection affected the expression of both mucin-regulating genes (MUC2 and MUC5 transcript variants A and C (MUC5ac)) in both tissues ($P < 0.05$). Overall MUC2 abundance was decreased in the jejunal tissue of infected animals ($P = 0.005$), with significant differences at 2 and 5 weeks p.i. (Fig. 8). Contrarily, infected animals had an elevated abundance of MUC2 in the caecal tissue ($P < 0.001$), and the abundance was constant over time as indicated by a non-significant interaction (P = 0.218). Infection strongly elevated the abundance of MUC5ac in both the jejunum and the caecum (P < 0.001). MUC5ac abundance was elevated in the jejunum at 2 and 9 weeks p.i. (P < 0.05) and in the caecal tissue at all investigated time points (P < 0.05). Reduced abundance of inducible Nitric Oxide (iNOS) in the jejunum was detected at 2 weeks p.i. in the infected animals (P = 0.001; Table 3). In the caecal tissue, overall iNOS abundance was higher (P = 0.001) in infected animals than in their uninfected counterparts, with particularly higher abundance levels at 2 and 9 weeks p.i. (P < 0.05). Whilst the abundance of Claudin 1 (CLND1) was significantly (P < 0.05) decreased in the jejunum of infected birds, its abundance in the caecum was upregulated by the infection (Table 3). For both locations, the changes occurred only at 2 weeks p.i. (P < 0.05; data not shown).

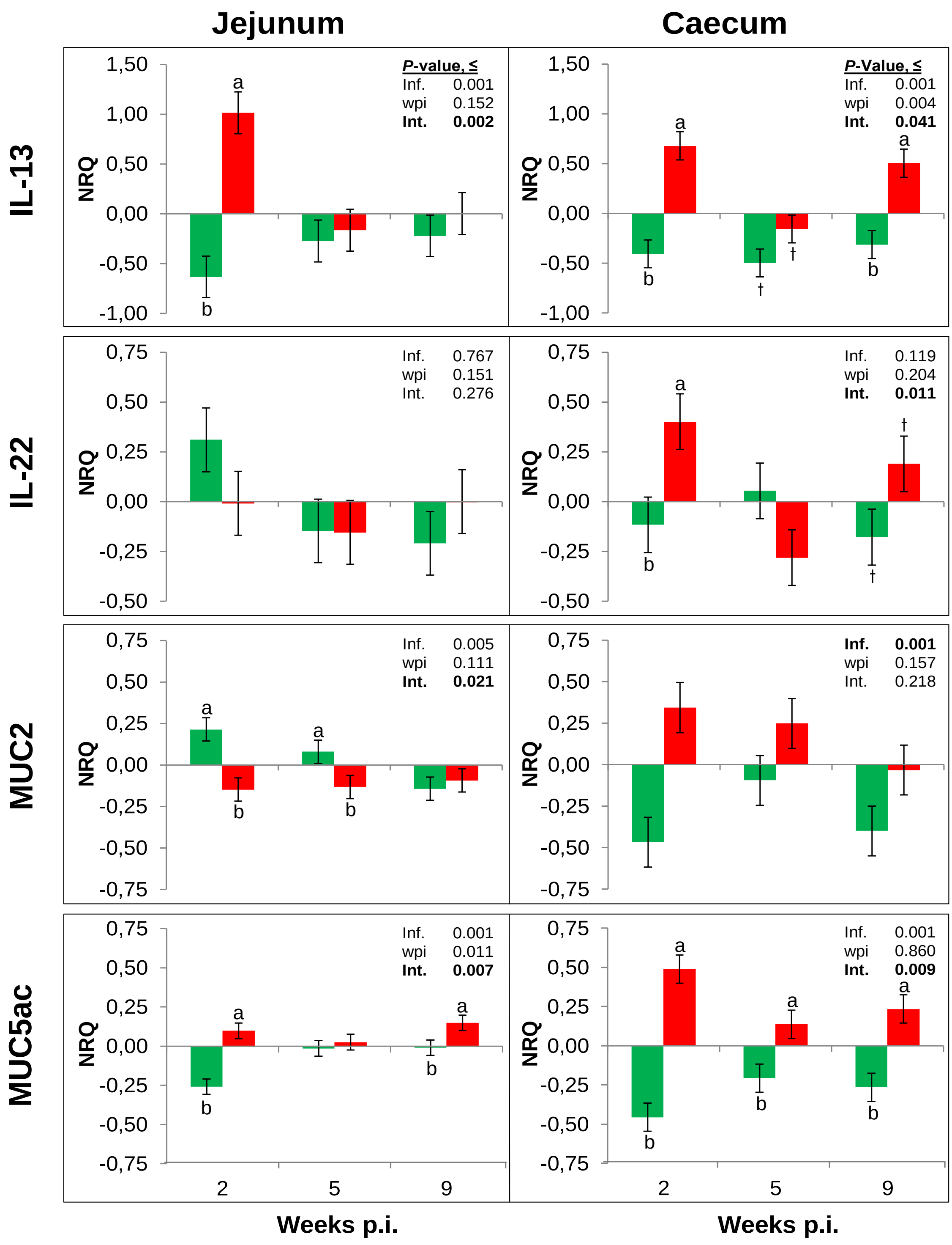

Fig. 8. Predilection site-dependent cellular immune responses in nematode-infected (●) and uninfected control (●) birds at 2, 5 and 9 weeks p.i. Expression levels are presented as normalised relative quantity (NRQ). Values without a common letter at the same time point indicate significant ($P < 0.05$) differences between infected and uninfected control birds. Values sharing the sign (t) tend to differ ($P < 0.10$). LSMEANS and S.E. represent untransformed data; P values for infection (Inf.) weeks p.i. (Wpi) and the interaction (Int.) effects are based on the transformed data. Tissue samples were collected from 12 birds (six infected, six uninfected controls) at each sampling point (wpi). Sample size, n = 72 (12 birds *x* three time points *x* two tissues).

4. Discussion

We investigated whether and how efficiently worm expulsion occurs in chickens experimentally infected with both *A. galli* and *H. gallinarum*, and we related worm expulsion to the population dynamics of the worms and the immune responses of their hosts. To mimic the outcome of naturally occurring nematode infections in an optimal way, we used a multiple species co-infection model. The infectious material was collected from several geographical locations to ensure high genetic diversity in both worm species, thereby potentially increasing worm pathogenicity (Johnson and Hoverman, 2012). Derived from an infection experiment with both temporal and spatial replications, our results clearly demonstrate that the chicken host is able to eliminate the vast majority of both *A. galli* and *H. gallinarum*. Here, we report for the first known time the rates and timeframes representing the extent and phases of expulsion of those nematodes from the chicken host. Worm expulsion was strongly associated with the developmental stage of the worms, where the elimination of juvenile stages was especially high. A very small percentage of the worms were nevertheless able to survive, reach maturity and induce reinfection if given sufficient time to complete their life cycle. Both humoral and local immune responses were associated with worm expulsion, with the latter likely having causative effects.

The numbers of first generation *A. galli* and *H. gallinarum* worms, defined as those originating from the experimental infections, decreased over the study period. This clearly indicates strong expulsion of both worm species from their shared host. Worm expulsion with either nematode species is nevertheless a rather non-linearly functioning process, with different expulsion rates before and after species-specific time thresholds. The majority of the first-generation worms were expelled by 31 and 36 days p.i. for *A. galli* and *H. gallinarum*, respectively. Beyond these time thresholds, worm expulsion was extremely reduced for *A. galli* and close to zero for *H. gallinarum*. The time threshold and slopes quantified for expulsion of *H. gallinarum* conform to the data shown for *Heterakis* mono-infection in the pheasant host, where the number of *H. gallinarum* decreased until 30 days p.i. and remained constant afterwards (Tompkins and Hudson, 1999). For *A. galli* mono-infections, Ferdushy et al. (2013) demonstrated a linear decrease in the larval stages with extremely arrested growth until 6 weeks p.i., and patency was not observed. Inducing patent *A. galli* infections following inoculation of chickens with embryonated eggs has indeed been challenging for many research groups, and as a consequence, new approaches for infection models are continuously being suggested (e.g., Sharma et al., 2017). Because most of the worms were shown to be expelled from the chicken host, our data may partially explain the extremely low worm burdens resulting from experimental infections with a single necropsy performed several weeks after infection, even if worm-specific antibodies could be quantified in plasma and egg yolks as early as 2 weeks p.i. (Marcos-Atxutegi et al., 2009; Daş et al., 2010, 2012; Schwarz et al., 2011a). In long-term studies, where sufficient time for reinfection was ensured, higher worm burdens that are comparable to those of naturally infected chickens were quantified (Daş et al., 2017).

Based on the presented data, we propose that worm expulsion occurs in three distinct phases and that juvenile worms are specifically targeted by phase-specific host immune responses, leading to the hypothesis that the earlier the worms are expelled, the lower the overall metabolic and pathophysiological costs of infection are for the host. The first phase covers the first day of infection only, when the larvae must hatch and either remain in the intestinal lumen or embed themselves in the adjacent superficial tissues. Excluding parasite-specific factors (e.g., larval hatchability), the only factor effectively controlling worm expulsion during the first phase is the host digesta transit time. Establishment of larvae during the first day of infection is probably the most challenging part of their lives. Not all the embryonated eggs given to the host animal are able to hatch and remain in the intestine, where they are passively transported together with digesta through peristalsis. By using a standard indigestible marker technique (TiO_2), Rougiere and Carre (2010) demonstrated that the mean retention time of digesta through the entire gastrointestinal tract of 9 days old chicks, approximately the same age as in our experiment, was only 5 h, with less than 50% of the time spent in the intestine, thus outlining the limits of available time for larvae to hatch and become established in their particular habitats. The segmented regression analysis estimated that 59% of *A. galli* larvae were able to survive the first day of infection, whilst Ferdushy et al. (2013) recovered 20% of the administered *A. galli* at 3 days p.i., potentially indicating a greatly elevated expulsion rate during the first days of infection. For *H. gallinarum,* the establishment rate on the first day was estimated to be much lower at 13%, which is likely an underestimate due to the potential involvement of *Histomonas meleagridis*, known to impair the establishment and survival ability of *H. gallinarum* due to pathological alterations occurring later in the caecal tissue and lumen (McDougald, 2005; Daş et al., 2011).

The second phase of worm expulsion is the most efficient one. It is partly species-specific and operates on both tissue-associated larvae and juvenile stages residing in the lumen, through activation of both humoral and cell-mediated immunity. The estimated breakpoints corresponded approximately to the end of this phase, when worm maturity was already achieved in the case of *H. gallinarum,* or larvae were mostly immature but no longer in the tissue phase in the case of *A. galli*. Whilst *A. galli* larvae experience a mucosal/histotrophic phase (Luna-Olivares et al., 2012; Ferdushy et al., 2013), which can last up to approximately 8 weeks depending on the infection dose (Herd and McNaught, 1975), only a short (maximum 12 days) superficial tissue-associated phase is known to occur for *H. gallinarum* (Vatne and Hansen, 1965). Most *A. galli* larvae were found in the lumen (87%), and tissue-associated larvae had almost disappeared by 6 weeks p.i., indicating completion of the tissue phase. Since tissue-associated larvae return to the lumen, it cannot be inferred whether these larvae were particularly exposed to expulsion. Nevertheless, the expulsion of the first generation worms of each species was accompanied by a selective compositional change in the worm populations. The population structures of both species changed from larval stages to a small population consisting of only a few mature worms, indicating that juvenile stages were specifically targeted. Although most *A. galli* larvae were recovered from the jejunum rather than the ileum, we found smaller larvae in the ileum than in the jejunum, suggesting a more posterior localization for those larvae unable to counter the expulsion and more likely to be expelled soon. This indicates targeted expulsion of slow growing larvae and further confirms the data from *Ascaris suum* (Morimoto et al., 2003) showing that only actively developing larvae with high metabolic activity go on to parasitize the host animal.

Although strong expulsion of both nematodes was observed over relatively similar periods of time, there was no significant correlation between worm burdens of *A. galli* and *H. gallinarum*. The lack of a correlation at any examined time point may indicate the existence of independent expulsion mechanisms for these two species with different predilection sites. The two nematodes, however, are genetically closely related species (Nadler et al., 2007; Wang et al., 2016) and are additionally under strong genetic control by the host animal (Kaufmann et al., 2011b; Wongrak et al., 2015). Furthermore, host antibodies developed against one species are similar to those developed against the other, indicating the similar antigenic properties of the two species (Daş et al., 2017). Despite high genetic correlations ($r_G = 0.37–0.88$) (Kaufmann et al., 2011b; Wongrak et al., 2015), only weak phenotypic correlations ($r_P = 0.20–0.25$) have been shown to exist between worm burdens of *A. galli* and *H. gallinarum* in naturally infected chickens.

In the third phase of expulsion, there were only a few *A. galli* left, whereas a substantial number of second generation *H. gallinarum* resulting from reinfections were present in this phase. For both species, a very small number of first generation worms were nevertheless able to survive and reach maturity. This not only indicates the exceptional ability of the chicken host to eliminate the vast majority of the nematodes but also points to the survival ability of the very few worms that were able to withstand host immune responses and counter expulsion. The surviving worms were nevertheless able to induce reinfection when given sufficient time for completion of their life cycle. As shown by the slopes of the segmented regression after the thresholds, almost all the remaining worms were able to survive once maturity was achieved. Notably, it is thus implied that there is substantial variation in the survival ability of worms. This is supported by mammalian studies showing that worm establishment depends on the genetic background of the worms (Johnston et al., 2005; D’Elia et al., 2009; Nejsum et al., 2009; Lippens et al., 2017). Given that the few surviving worms are able to pass their genetic material to the next generation worm populations, these results collectively provide insight into the magnitude of the host-parasite battle, with implications for the selection history of the nematodes. The fact that the reinfections occurred for *H. gallinarum* but not for *A. galli* is fully attributable to the shorter prepatent period of *H. gallinarum* (Ramadan and Abou Znada, 1992; Daş et al., 2014; Daş and Gauly, 2014). The occurrence of reinfections with *H. gallinarum* by 7 weeks p.i. was in the range of expected time for life cycle completion of this nematode, with the start of egg excretion occurring by 4 weeks p.i. at the latest, and the additional time (i.e., 2 weeks) needed for the embryonation of the eggs (Püllen et al., 2008). Since *A. galli* females reached maturity by 8 weeks p.i., as confirmed by the presence of eggs in the uteri of female worms and a slight increase in EPD at 9 weeks p.i., this nematode was less likely than *H. gallinarum* to cause reinfection within the 9 week study period.

The production of nematode-specific antibodies (IgY) has already been reported in previous mono-infection studies in chicken (Martín-Pacho et al., 2005; Marcos-Atxutegi et al., 2009; Schwarz et al., 2011a; Norup et al., 2013; Ferdushy et al., 2014; Dalgaard et al., 2015). These antibodies are probably cross-reactive against both nematodes (Daş et al., 2017). The onset of the humoral immune response occurs within approximately 2 weeks in *A. galli* mono-infections (Schwarz et al., 2011a; Ferdushy et al., 2014), whereas no information is yet available for *H. gallinarum*. Although *H. gallinarum* larval counts were positively correlated with the IgY level at 2 weeks p.i. ($r = 0.67$), IgY levels in the infected animals did not increase compared with those in the controls until 5 weeks p.i., at which time a significant correlation with *A. galli* larvae ($r = 0.81$) was recorded. Although the IgY levels of infected birds increased in a delayed manner, the IgY levels were generally lower than those reported in previous research (Marcos-Atxutegi et al., 2009; Daş et al., 2017). The delayed onset of antibody production and the lower IgY levels must be due in large part to the age of the host animal (Ulmer-Franco et al., 2012), as the birds used in this study were much younger than those used in previous studies. Although infected birds had elevated levels of IgY, reinfections with *H. gallinarum* were not prevented. This is in line with previous data on *A. galli* (Norup et al., 2013; Daş et al., 2018). Only very low IgY levels, not different from those of control birds, were quantified during the first weeks of infection, when most of the worms were expelled. In a pig study, specific antibodies against *Trichuris suis* were correlated weakly with worm burdens when expulsion of the whipworm occurred (Kringel et al., 2015). Whether the IgY antibodies contributed to the generation of a protective effect could not be directly clarified. Nevertheless, production of worm-specific IgY is triggered by the polarisation of naïve T helper cells to Th2 cells, which promote B cell class switching (Harris and Gause, 2011). Although a protective function is evident in murine models (Harris and Gause, 2011), nematode-specific IgY in chickens is expected to be part of a general immune response rather than providing a direct protective effect.

In the mammalian host, expulsion of gastrointestinal helminths depends mainly on parasite-induced Th2 immunity (Turner et al., 2003; Dawson et al., 2005; Masure et al., 2013; Oeser et al., 2015; Sun et al., 2016). Although chickens display equal Th2 polarisation following mono-infections with *A. galli* or *H. gallinarum* (Degen et al., 2005; Schwarz et al., 2011a,b; Pleidrup et al., 2014; Dalgaard et al., 2015), worm expulsion has not been systematically quantified in relation to local host immune responses. In agreement with previous mono-species nematode infections (Schwarz et al., 2011a,b; Pleidrup et al., 2014) the Th2 cytokine IL-13 was strongly upregulated in both jejunal and caecal tissues. IL-13 cytokines activate the STAT6 pathway through the receptor IL-4Rα. This pathway has been demonstrated to play a crucial role in worm expulsion (Urban et al., 1998; Ishiwata et al., 2002; Scales et al., 2007; Horsnell et al., 2007; Oeser et al., 2015). Although IL-13 abundance was elevated in both jejunal and caecal tissue in response to infection, IL-4Rα was upregulated only in the caecum and downregulated in the jejunum. Similarly, STAT6 abundance was reduced in the jejunum and remained unaffected in the caecum. Our data demonstrated the involvement of IL-22 in avian host-parasite interactions for the first known time, even if infection altered its abundance only in the caeca and not in the jejunum. The increased abundance of IL-22 in the caecum at 2 and 9 weeks p.i. might be associated with the presence of larvae, as these time points represent host immune responses to experimentally and naturally occurring reinfections, respectively. Both IL-13 and IL-22 are known to regulate worm expulsion, acting by inducing goblet cell hyperplasia and stimulating mucin production (Turner et al., 2013; Oeser et al., 2015). The mucin-regulating genes MUC2 and MUC5ac are essential for mucus protection and are important for worm expulsion in mice (Hasnain et al., 2010, 2011). Infection affected the effector molecules in both tissues, although the course was not the same for the two molecules. Whilst MUC5ac was upregulated in both the jejunum and the caeca, MUC2 was upregulated only in the caeca and downregulated in the jejunal tissue.

Th2 cytokines promote smooth muscle contractility (Zhao et al., 2003; Dawson et al., 2005), thereby decreasing intestinal transit time (Vallance et al., 1997, 1999; Masure et al., 2013). Thus, it is possible that increased gut motility accompanied by the secretion of mucins, particularly in the early stages of infection, might have provided unfavourable conditions for the nematodes. As a result, larvae that were not able to counterbalance the host expulsion mechanism might have lost the ability to parasitize the host successfully and might have been flushed out. Although local immune responses were not determined at weekly intervals, immunity to both ascarids appears to be temporarily induced mainly in the larval stages. Jejunal IL-13 was upregulated only in the early stage of infection, when larval stages were present. Contrarily, caecal IL-13 was significantly affected at 2 and 9 weeks p.i., the time points at which *Heterakis* larvae existed from experimental and natural reinfection, respectively. IL-22 showed a similar pattern in the caecum. Therefore, the temporary upregulation of the cytokines, as confirmed by Schwarz et al. (2011a,b), might be connected with the short tissue-associated phase of the nematode life cycle in the early stage of infection, implying the necessity of close contact between the worm and the host tissue in order for local immune responses to occur. This is fully consistent with the larva-dependent antibody production (Daş et al., 2018) being triggered by Th2 immune components (Harris and Gause, 2011). Data on both humoral and tissue-specific cellular immune responses may thus collectively indicate that antibody production in chickens with multi-species parasite co-infections is also triggered by Th2 polarisation. Despite the infection-induced changes in the local Th2 responses and mucin-regulating genes, which were clearly associated with worm expulsion, a direct proof of the local immune responses as the causative effects leading to worm expulsion needs to be demonstrated in future studies.

In conclusion, the chicken host is able to eliminate the vast majority of both *A. galli* and *H. gallinarum* in three distinct phases. Worm expulsion was strongly associated with the developmental stages of the worms, where the elimination of juvenile stages was specifically targeted. A very small percentage of the worms nevertheless survived, reached maturity and induced reinfections as long as they had sufficient time to complete their life cycles. Both humoral and local immune responses were associated with worm expulsion.

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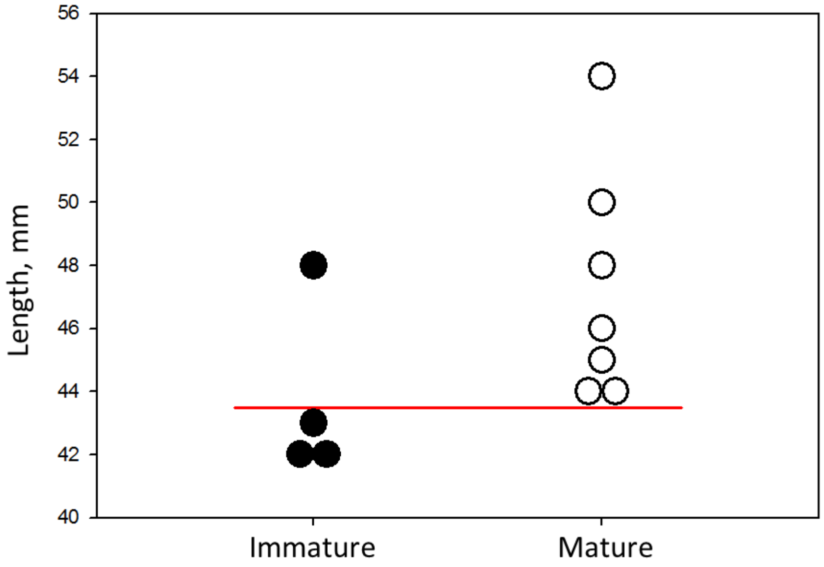
Supplementary Data



Supplementary Fig. S1. A length cut-off to differentiate between mature and immature female Ascaridia galli. Our preliminarily observations indicated that female worms longer than 55 mm have eggs in their uteri, and these eggs can relatively easily be visually inspected under a stereo microscope. Below this length, however, female worms need to be carefully examined for the presence of eggs in the uteri. We examined 11 female worms, ranging 40 to 55 mm in length, dubitable for the presence of eggs in the uteri. Individual worms were first measured for length, and thereafter dissected with a pair of fine-tweezers to check directly for the presence of eggs. A length-threshold (43.5 mm) which separated dubitable worms with or without eggs in the uterus, was then estimated with a receiver-operating- characteristics (ROC) analysis (Swets, 1988) using Sigmaplot Version-11 (Systat Software, Inc., San Jose California USA).

Supplementary Table S1. Biochemical indices derived from quantitative real-time PCR analyses.

		Jejunum[a]					Caecum[a]				
		Control		Infected			Control		Infected		
Biochemical measure	wpi	NRQ	S.E.	NRQ	S.E.	*P* value	NRQ	S.E.	NRQ	S.E.	*P* value
DNA[b]	2	5.104	0.56	5.291	0.62	0.8249	2.398	0.35	3.385	0.35	0.0608
	5	4.530	0.56	5.080	0.62	0.5175	3.536	0.35	3.505	0.40	0.9535
	9	4.724	0.56	5.556	0.56	0.3065	2.447	0.45	2.628	0.45	0.7781
RNA[b]	2	5.583	0.35	4.439	0.35	0.0264	3.750	0.34	4.317	0.34	0.2408
	5	4.637	0.35	3.991	0.35	0.1965	4.549	0.34	4.000	0.34	0.2559
	9	4.644	0.35	4.538	0.35	0.8309	3.471	0.34	2.903	0.34	0.2407
RNA DNA	2	1.097	0.09	0.855	0.10	0.0921	1.601	0.11	1.360	0.11	0.1510
	5	1.075	0.09	0.879	0.10	0.1681	1.386	0.11	1.112	0.13	0.1245
	9	0.987	0.09	0.877	0.09	0.4104	1.367	0.15	0.953	0.15	0.0598

[a] Tissue samples were collected from 12 birds (six infected, six uninfected controls) at each sampling time point (weeks p.i. (wpi)).

Sample size: *n* = 72 (12 birds *x* three time points *x* two tissues).

[b] µg / mg tissue

NRQ, normalized relative quantity.

A total of 36 control and infected birds were used for these analyses. Infected birds were inoculated with a sum of 500 eggs of *Ascaridia galli* and *Heterakis gallinarum* in equal proportion. Tissue samples were taken from the mid-jejunum and caecum of each bird at 2, 5 and 9 weeks p.i. (wpi).

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CHAPTER THREE

Resistance and tolerance to mixed nematode infections in chicken genotypes with extremely different growth performances

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Abstract

Fast growing broilers are less able to cope with fitness related challenges. As the allocation of metabolic resources may be traded off between performance and defence functions in parasitized hosts, we hypothesized that fast growing broilers are more sensitive to mixed nematode infections compared with slower growing genotypes under the same environmental conditions. Therefore, we compared male birds of genotypes selected for either meat production (Ross-308, R) or egg production (Lohmann Brown Plus, LB) or for both purposes (Lohmann Dual, LD), to assess their resistance and tolerance to mixed nematode infections with *Ascaridia galli* and *Heterakis gallinarum*. While infections reduced feed intake in all three genotypes, feed conversion efficiency was not affected. Infections impaired growth performance only in R birds, indicating lower tolerance in the fast growing genotype compared with slower growing LB and LD genotypes. Impaired tolerance in R birds was associated with a relative nutrient scarcity due to an infection-induced lower feed intake. Resistance to experimentally induced infections depended on host genotype as well as on the worm species involved. Overall, the *A. galli* burden was higher in R than LB, whereas the burden of LD was not different from that of R and LB. In contrast, the *H. gallinarum* burden of first generation worms was similar in the three genotypes. Susceptibility to re-infection with *H. gallinarum* was higher in LB than in LD, whereas very low levels of re-infection were observed in R birds. Our data collectively suggest that resistance and tolerance to mixed nematode infections are sensitive to growth rate in chickens. These differences amongst genotypes may partly be associated with a mismatch between the actual nutrient supply and genotype-specific nutrient requirements.

Keywords: Ascarids, Avian host, Growth rate, Host performance, Multi-species infection, Resistance, Susceptibility, Tolerance

1. Introduction

Infections with multiple nematode species are common in layer chickens (Kaufmann et al., 2011a; Thapa et al., 2015; Wuthijaree et al., 2017), and they occur not only in outdoor (Permin et al., 1999) but also in indoor systems (Grafl et al., 2017). Although nematode infections are rarely observed in broilers kept in intensive systems (Wilson et al., 1994; Kumar et al., 2015), slow growing broilers kept in organic systems may particularly be at risk due to the obligatory outdoor access and a prolonged fattening period that increases exposure to the infections. In line with the longer life expectancy, nematode infections can be highly prevalent (i.e. 89.9%) even in broiler breeders (Yazwinski et al., 2013). *Ascaridia galli* and *Heterakis gallinarum* are, globally, the two most prevalent nematode species parasitizing the chicken host (Abdelqader et al., 2008; Thapa et al., 2015; Wuthijaree et al., 2017). Infections with both ascarids are known to impair host performance (Dänicke et al., 2009; Daş et al., 2010, 2011, 2012; Schwarz et al., 2011a), and are associated with increased mortality (Hinrichsen et al., 2016). The adverse effects of ascarid infections on host performance are mediated, mainly through lower nutrient absorption (Hurwitz et al., 1972; Walker and Farrell, 1976; Schwarz et al., 2011b), impaired feed conversion efficiency and lower nutrient intake (Daş et al., 2010, 2011, 2012).

Due to the strong genetic antagonism between reproduction and growth traits (Damme, 2015), modern chicken genotypes have been developed to efficiently produce either eggs or meat, but not simultaneously in the same animal. Although both one-way production modes target mainly protein deposition in the form of either eggs or meat, divergent genotypes with different life spans are used for each purpose. Genetic selection in broilers has increased the growth rate by 400% over 50 years (Zuidhof et al., 2014). There is, however, a large body of evidence that fast growing broilers have an impaired ability to cope with physiological, behavioural and immunological challenges (Koenen et al., 2002; Rauw, 2012) that are considered undesirable consequences of selection for increased production (Rauw et al., 1998). The impaired ability of broilers to cope with the fitness related challenges may be associated with allocation of finite resources in a given environment (Glazier, 2009), because selection for increased production (e.g. growth rate) will lead to a prioritized allocation of available resources toward production at the expense of other life history traits including immunity to pathogens (Rauw, 2012).

There is evidence for the existence of different types of immune programming in broiler and layer type chickens, which appears to be in line with their productive lifespan. The study of Koenen et al. (2002) showed that broilers have a lower cytokine response and are more specialized in mounting a strong short-term humoral immune response, whereas layers rely on a strong cellular response accompanied by a long-term humoral immune response. Indeed, a meta-analysis on the trade-off between growth and immune function in poultry concluded that genetic selection for rapid growth compromises immune function (van der Most et al., 2011). In line with this, Han and Smyth (1972) showed that fast growing broilers were more susceptible to Marek's disease than slow growing broilers. Furthermore, chicken genotypes that have been selected for different digestive efficiencies (Calenge et al., 2014) and performance traits (Han et al., 2016) differ in their susceptibility to bacterial infections. Compared with bacterial and viral infections, less is known about responses of chickens selected for increased growth to parasitic challenges. As in mammals, a chicken's immune system deals with intracellular (e.g. Eimeria spp.) and extracellular (e.g. nematodes) parasites through different immune responses, i.e. Th-1 and Th2 pathways, respectively (Degen et al., 2005). In a recent study Sakkas et al. (2018) showed that fast growing broilers were similar to slow growing broilers in terms of resistance and tolerance to coccidiosis when offered nutrient adequate diets (Sakkas et al., 2018). Resistance and tolerance to nematode infections have never been studied comparatively in chickens with different performance objectives that also greatly differ in growth rates. Genotype comparisons and heritability estimates indicate that there is considerable genetic variation in resistance to nematode infections in chickens (Permin and Ranvig, 2001; Gauly et al., 2002; Schou et al., 2003; Kaufmann et al., 2011b; Wongrak et al., 2015). These results are, however, from layer type chickens exposed to experimental or naturally occurring infections. There are no such detailed studies in broilers, and in general very little is known about their responses to nematode infections (Daş and Gauly, 2014; Ruhnke et al., 2017). As the allocation of metabolic resources may be traded off between performance and defence functions in the parasitized host (Coop and Kyriazakis, 1999; Colditz, 2008; van der Most et al., 2011), we hypothesized that fast growing genotypes are more vulnerable to nematode infections compared with slow growing genotypes under the same environmental conditions. Therefore, the aim of this study was to compare male birds of meat type, layer type and dual purpose chicken genotypes in terms of resistance and tolerance to mixed nematode infections. Growth performance, which is the most relevant performance parameter for rearing male birds of all chicken genotypes, served as the base for assessment of tolerance over the same sex.

2. Materials and methods

2.1. Birds, experimental design and ethics

A total of 668 male chicks of three divergent chicken genotypes, developed for either egg production (Lohmann Brown Plus, LB) or meat production (Ross-308, R), or for both purposes (Lohmann Dual, LD), were used. Ross-308 and LB birds were used as the positive and negative control genotypes for growth performance, respectively, whereas LD birds served as an additional genotype with an intermediate growth rate. The experiment was conducted twice in succession (temporal replication), and in each iteration, there were two infected and two uninfected groups of birds (spatial replication), which were kept in separate pens within the same experimental stable. In each spatial and temporal run, infected and uninfected control birds of all three genotypes were used simultaneously. An overview of the experimental design, replication blocks, animal numbers and sampling schedule over time within a run is given in Fig. 1. Birds of each genotype were either experimentally infected (n = 430) at an age of 1 week with a total of 500 infective eggs of *A. galli* and *H. gallinarum* in equal proportions to produce mixed nematode infections, or kept as uninfected controls (n = 238). From 2 weeks p.i. onwards, randomly selected infected and control birds from each genotype were necropsied at weekly intervals up to 9 weeks p.i. to quantify infection intensity with either nematode. Uninfected control birds were examined for accidental infections to exclude any potential confounding effects on performance and immune-related parameters. By 3 weeks p.i., individual faecal samples were collected from the birds 1 day prior to necropsy during captivity in individual cages to quantify nematode egg excretion at weekly intervals. All birds were individually wing tagged on the day of infection to ensure the collection of individual data over time. Individual body weight and pen-based feed consumption (n = 4 per genotype and infection status) were determined at weekly intervals. Blood samples were collected weekly during necropsy starting at 2 weeks p.i.

The experiment was conducted in accordance with animal welfare rules (animal care and handling, stunning, necropsies) and was approved by the State ethics committee for animal experimentation (Mecklenburg-Western Pomerania State Office for Agriculture, Food Safety and Fishery, Germany; permission no: AZ: 7221.3-1-066/15). The experimental infection procedures were in line with the relevant guidelines of the World Association for the Advancement of Veterinary Parasitology for poultry (Yazwinski et al., 2003).

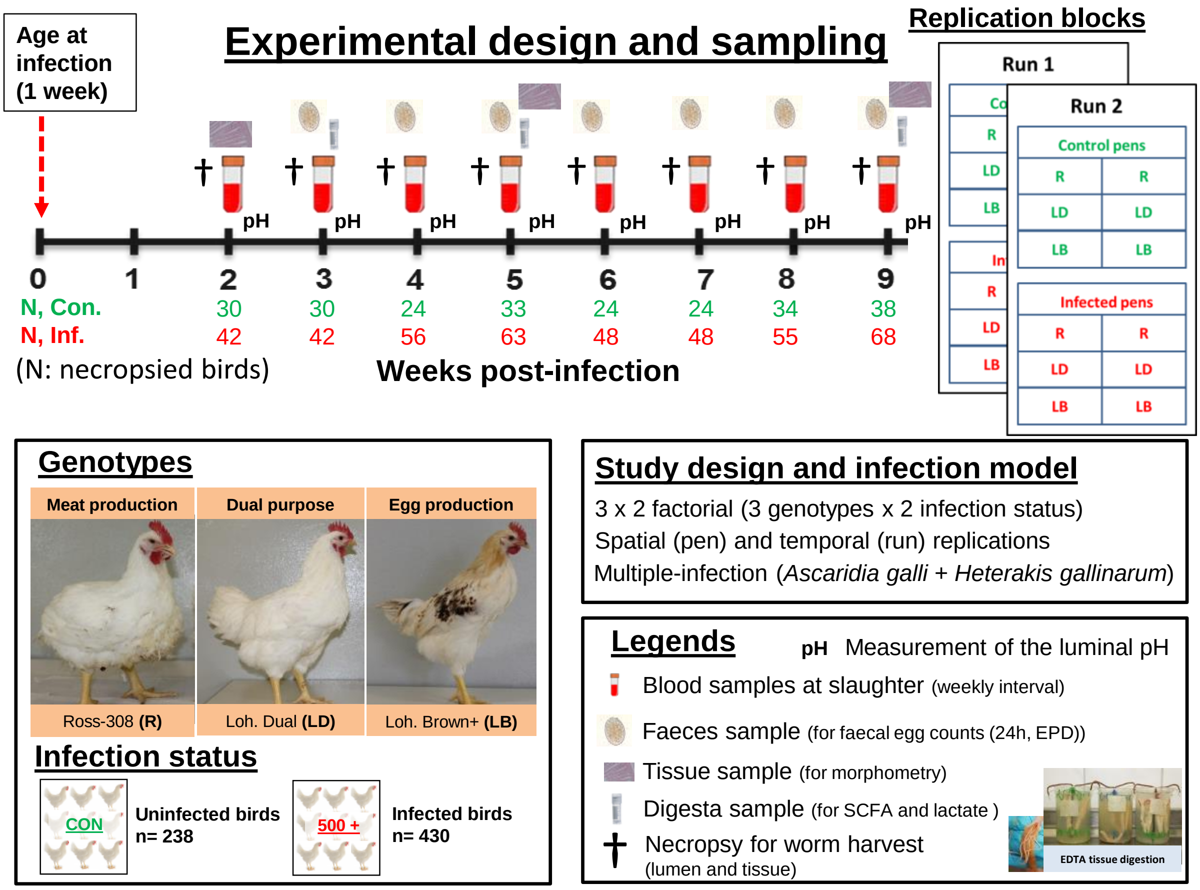

Fig. 1. Summary of the experimental design and sampling schedules. Con, control; Inf, infected; LB, Lohmann Brown Plus; LD, Lohmann Dual; R, Ross-308; SCFA, short chain fatty acid; ny, number of necropsied birds.

2.2. Housing and management

The pens containing infected and uninfected animals were in two separate rooms of an experimental research facility for poultry to avoid cross-contamination between the infected and uninfected groups of birds (Fig. 1). The birds were reared in a floor husbandry system on wood shavings as litter material. Litter was not removed during the infection period, to allow re-infections to occur. Additional litter, corrected for total body weight per m2, was added to all pens at the same time to ensure similar litter conditions for all genotypes in different pens. The climatic conditions were fully controlled by an automatic system to ensure uniform temperature, light and aeration conditions across the pens within and between rooms. The temperature was gradually decreased from 34 °C on the first day to close to 20 °C in the 10th week of life. Feed and water were given ad libitum. All genotypes were fed on the same starter (day 1–14; 12.6 MJ of metabolizable energy (ME) and 219 g of crude protein (CP) per kg of feed), grower (day 14–53; 13.0 MJ of ME and 204 g of CP per kg of feed) and finisher diets (day 53–70; 13.4 MJ of ME and 185 g of CP per kg of feed), with a transition phase of approximately 3 days between the different diets. The diets provided or exceeded age-specific nutrient requirements of the broiler birds (Aviagen, 2014; http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross308BroilerNutritionSpecs2014-EN.pdf). The detailed composition and nutrient contents of the diets are given elsewhere (Urban et al., 2018). Animal density for each genotype did not exceed 25 kg/m^2 at any time point, ensuring that standards for an ecological production system regarding the space requirements of broilers were met. The birds received no vaccinations or medical treatment, including anthelmintics, before or after infection. Mortality was recorded throughout the experimental period.

2.3. Experimental infection

The infection material was derived from the intestines of naturally infected chickens (i.e., free-range chickens) that were collected from several slaughterhouses and farms across northern Germany. The preparation techniques and incubation conditions for the infection material have previously been described by Stehr et al. (2018). On the day of infection (7 days of age), the incubation media of both *A. galli* and *H. gallinarum* were separately filtered through a sieve (36 µm mesh size), which was followed by rinsing to collect the washed eggs in saline solution (NaCl, 0.9%). Based on morphological classification (Rahimian et al., 2016), only fully embryonated eggs, which are considered infectious, were counted to determine the percentage of embryonated eggs per ml of suspension. The single infection dose for each worm species was adjusted to 250 embryonated eggs ($SD_{A.\ galli}$ = 23.7; $SD_{H.\ gallinarum}$ = 12.4) per 0.1 ml of NaCl (0.9%), which was administered to each bird in a final inoculum of 0.2 ml of NaCl, containing a total of 500 eggs with equal proportions of the two worm species. The infection dose was administered orally using a 5 cm oesophageal cannula as described previously (Daş et al., 2010), whereas uninfected control birds received a sham oral treatment with the same amount (0.2 ml) of NaCl solution.

2.4. Worm harvest

Quantification of the worm burden was performed weekly from 2 to 9 weeks p.i. Before necropsy, the birds were fasted for 3 h to allow standardized partial emptying of the intestine. Immediately post mortem, the gastrointestinal tract was removed, and the caecum and small intestine (SI) were separated. The SI was divided into the jejunum and ileum at the Meckel´s diverticulum (Svihus, 2014). The duodenum was excluded from quantification, as macroscopic examinations indicated that this intestinal section is not the normal habitat for *A.*

galli. The jejunum and ileum were opened longitudinally, and the intestinal contents, separated by section, were washed separately through sieves with mesh sizes of 36 µm and 100 µm at 2–5 weeks p.i. and 6–9 weeks p.i., respectively. The quantification of tissue-associated *A. galli* larvae was restricted to the jejunal section, which is the main preferred site of larval stages (Ferdushy et al., 2013). The procedure for larval recovery by using the EDTA-incubation method was recently described in Stehr et al. (2018).

Heterakis gallinarum worms were harvested from the lumen contents by rinsing the opened caeca in sieves (mesh sizes 20–36 mm). Both *A. galli* and *H. gallinarum* worms collected from individual birds were then separately transferred to Petri dishes for counting, sex differentiation and length measurements. Uninfected birds were also examined for the presence of worms in the SI (tissue and lumen) and caecum to exclude accidental infections with either nematode.

2.5. Worm population structure

The initial classification of the harvested worms into larvae, females and males was based on morphological characteristics. For both worm species, differentiation between male and female worms can be easily performed according to the presence of spicules. While there is no difficulty in distinguishing mature and immature females of *H. gallinarum* based on the presence of eggs in the uteri, the presence/absence of eggs in the uterus of intact *A. galli* females cannot be definitively confirmed. Therefore, we used a length cut-off (immature 43.5 mm > mature) that allowed precise separation between female worms (Stehr et al., 2018). The classified worms (larvae, immature females, mature females and males) were then individually measured to determine the worm length for each worm stage and sex. For this purpose, only intact worms (maximum of 10 per bird) from each developmental stage and sex were randomly selected and measured to determine lengths on a ruler under a stereo-microscope. The length dataset was used to calculate a new parameter, overall average worm length (OWL), combining worm length across different worm stages from each bird. Therefore, the average length (y) of each specific worm stage was multiplied by the corresponding number of worms in each developmental stage (n) to adjust their respective proportions in the total worm burden (N) as described below.

$\text{OWL} = [(\text{larvae}, y_x n) + (\text{immature}, y_x n) + (\text{mature}, y_x n) + (\text{males}, y_x n)/N]$

All *Heterakis* worms up to 4 weeks p.i. were considered to originate from the experimental infection and were therefore defined as first generation worms. By 5 weeks p.i., the number of first generation worms was calculated as the total worm burden minus the number of larvae and immature worms, as these juvenile worms must have originated from reinfection. Non-larval worms were defined as the total worm burden minus larvae and included mature and immature worms that were sexually differentiable by morphological characteristics.

2.6. Faecal egg counts

To quantify the nematode egg concentration in faeces (eggs per gram faeces, EPG), the daily total faeces were thoroughly homogenized. A random sub-sample (4 g) of homogenized faeces was then analysed with a modified version of the McMaster egg counting technique (MAFF, 1986). A saturated NaCl solution (density ≥ 1.2 g/ml) was used as the flotation liquid. The minimum detection level of the egg counting technique was 50 nematode eggs/g of faeces. By multiplying the amount of daily excreted faeces with EPG, the number of eggs excreted within 24 h (eggs per day, EPD) was then estimated (n = 320 samples). Eggs of *A. galli* and *H. gallinarum* were counted together because they could not be reliably differentiated (Kaufmann, 1996).

2.7. ELISA for ascarid-specific antibodies

An ELISA (Daş et al., 2017) was used to quantify anti-ascarid-specific IgY levels in EDTA-plasma samples collected during weekly necropsies (2–9 weeks p.i.; n = 646). Plasma was obtained from the blood samples by centrifugation at 2500g for 20 min and 4 °C, and then stored at 20 °C. The laboratory-specific intra-assay coefficient of variability (CV) and inter-assay CV for this analysis were 5.0% and 8.4%, respectively.

2.8. ELISA for immunoglobulins (Ig) IgY, IgM and IgA

Commercial ELISA Kits (IgY: Kit No. E30-104; IgM: Kit No. E30-103; IgA: Kit No. E30-102; Bethyl Laboratories, Inc, Montgomery, TX, USA) were used to analyse immunoglobulin concentrations (IgY, IgM, IgA) in EDTA-plasma samples at 2, 5 and 9 weeks p.i. (n = 273). The sample collection and preparation methods were identical to those described for the ascarid-specific antibodies. ELISA was performed according to the manufacturer’s instructions. A pooled plasma sample served as a control among all plates. The laboratory-specific intra-assay CV and inter-assay CV for the analysis ranged between 5.0–7.6% and 7.7–10.4%, respectively.

2.9. Luminal pH, short-chain fatty acids (SCFAs) and lactate (LA) in the intestines

The luminal pH of the SI and caecum was determined individually at weekly intervals (2–9 weeks p.i.) in both infected and noninfected birds at necropsy. Within 10 min post mortem, the luminal pH was measured in duplicate using a glass pH electrode directly inserted into the digesta (Spear tip, VWR GmbH, Darmstadt, Germany). For the SI, the pH was determined on both the proximal (jejunum) and distal (ileum) sites of Meckel´s diverticulum. The caecal pH was measured twice at the blind end of the left caecum. The duplicate pH measurements correlated well ($r = 0.94$, $P < 0.001$) for both the SI and caecum, and an individual locationspecific mean pH value was used for statistical analyses. SCFA and LA concentrations in both the ileal and caecal digesta were determined at the Department of Veterinary Medicine, Freie Universität Berlin, Germany. As the whole intestinal contents were used for exact quantification of the worm burden, digesta samples for SCFA and LA analyses were obtained from counterpart birds from the same study group. All the birds used in these study analyses were identically treated/infected, housed and necropsied on the same day. Digesta samples were collected from 18 fed birds (i.e., three birds per genotype and infection status) at the necropsy time-points of 3, 5 and 9 weeks p.i. ($n = 108$). Immediately post mortem, samples from the ileum and left caecum were transferred to cryo-tubes, snap frozen in liquid nitrogen and stored at 20 °C for subsequent analysis. SCFA and LA concentrations in the ileal and caecal digesta were determined via gas chromatography and HPLC as described previously (Goodarzi Boroojeni et al., 2014).

2.10. Intestinal size and histomorphometry

The length and weight of the SI and caeca were measured at 0 and 9 weeks p.i. (i.e., at an age of 7 and 63 days, respectively). For light microscopy and morphometry, the intestinal tissue (1 cm2) from the jejunum was fixed in a 4% neutral formaldehyde solution. After rinsing in water, the tissue samples ($n = 54$, collected at 2, 5 and 9 weeks p.i.) were dehydrated in a graded series of ethanol (30%, 50%, 70%, 90% and absolute ethanol), cleared in benzene and then saturated with, and embedded in, paraffin. Sections of 5 mm thickness (10 slices of each sample) were stained with H&E and observed under a light microscope as described previously (Zitnan et al., 2008). The heights and widths of 30 villi and the depths of 30 crypts were determined with the computeroperated Image C image analysis system (Imtronic GmbH, Berlin, Germany) and the IMES (interactive measurement) analysis programme, using a colour video camera (SONY 3 CCD, Sony Electronics Ltd., Tokyo, Japan) and a light microscope (Axiolab, Carl Zeiss AG, Jena, Germany).

2.11. Statistical analyses

Nematode-free control birds were excluded from the analyses of the worm burden and faecal egg count (FEC) data. Worm burden, FEC and antibody data were analysed following log transformation [Ln (y + 1)] to correct for heterogeneity of variance and produce approximately normally distributed data. All variables were then subjected to ANOVA by using the MIXED procedure in the SAS/STAT (Version 9.4) software of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA). The statistical model for all wormrelated parameters (worm burden, FEC, worm length) included the fixed effects of genotype, weeks p.i. and their interaction, plus pen and run effects. As individual performance (body weight, average daily gain) as well as antibody data were available for the uninfected control birds, the model included the effects of infections, genotype, week p.i. and their interactions, plus run and pen effects.The effect of repeated sampling of the birds (subject) over weeks was considered with the REPEATED statement of the MIXED procedure, and the structure of the block diagonal residual covariance matrix was set to AR(1), as this setting provided the best fit of the parameters (e.g., smallest Akaike Information Criterion) for the fitted models. For pen-based data (average daily feed intake, feed conversion ratio), the model was the same as for the individual performance data, but the pen effect was omitted because it was considered as the repeatedly measured experimental unit. To account for the decreasing number of birds in each pen over time (due to necropsies), the model for pen-based data included the WEIGHT statement. The covariance matrix for this model was also AR (1).

Least-squares means (LSM) and their standard errors (SE) were computed for each fixed effect in the model, and all pairwise differences in these LSMs were tested with the Tukey–Kramer test, a procedure for pairwise multiple comparisons. In addition, the SLICE statement of the MIXED procedure was used for performing partitioned analyses of the LSMs for the two- or three-way interactions (e.g., test of infections within the levels of week p.i. in each genotype). Effects and differences were considered significant at $P < 0.05$. Overall cumulative mortality was analysed with the Chisquare test by performing multiple comparisons between infected and uninfected birds as well as between genotypes.

3. Results

3.1. Host performance

In comparison with R birds (2.8%), overall mortality during the infection period was lower ($P = 0.012$) in LD (0.3%) and tended ($P = 0.09$) to be lower in LB birds (0.9%). Infections did not influence ($P = 0.763$) mortality in any genotype (i.e., 1.2% and 1.5% for control and infected birds, respectively). Body weight (BW) increased steadily over the weeks of the experiment ($P < 0.001$), and differed significantly among the genotypes in the order of R > LD > LB ($P < 0.001$; Table 1). A significant interaction among infection, genotype and time effects ($P < 0.001$; Fig. 2A) indicated a lower BW in infected R birds compared with uninfected R controls by 3 weeks p.i., which was not observed for LD or LB birds ($P > 0.05$). Similar to BW, the overall average daily gain (ADG) was significantly different among the three genotypes, in the order of R > LD > LB ($P < 0.001$). The ADG was temporarily affected in the infected R birds ($P < 0.05$) at the time points of 3, 4, 5 and 8 weeks p.i. (Fig. 2B). No significant effect of infections on the ADG was found in LB or LD ($P > 0.05$).

Table 1. Growth performance, feed intake and feed conversion efficiency in divergent chicken genotypes exposed to mixed nematode infections.

	Main effects of genotype					**Infection effect**				**Interaction, P-value, ≤**	
Item	R	LD	LB	SE	P,≤	Con.	Inf.	SE	P,≤	Inf.*wpi	Inf.*Gen*wpi
Body weight (g)	2876^a	940^b	667^c	17.12	0.001	1533	1456	16.33	0.001	0.001	0.001
Daily weight gain (g)	85.2^a	29.1^b	20.7^c	0.62	0.001	46.2	43.8	0.56	0.001	0.002	0.001
Feed intake (g/d)	178.1^a	79.8^b	57.6^c	2.13	0.001	107.9	102.5	1.90	0.040	0.231	0.905
FCR (g/g)	1.92^a	2.42^b	2.43^b	0.05	0.001	2.25	2.27	0.04	0.767	0.661	0.773

^{a,b,c}Genotypes shown with different letters differ significantly (Tukey, $P < 0.05$).

Analyses for body weight (sample size n = 3496 observations) and average daily weight gain (sample size n = 3493) are based on repeated individual bird data. Feed intake and FCR are based on repeated pen data. The number of pens used per week was four per genotype and infection group (sample size n = 216). FCR, feed conversion ratio (feed intake/weight gain); R, Ross-308; LD, Lohmann Dual; LB, Lohmann Brown Plus; Con, uninfected controls; Inf., infected; Gen, genotype; wpi, weeks p.i.; g/d, gram per day.

The overall average daily feed intake (ADFI) was different among the genotypes in the order of R > LD > LB ($P < 0.001$). The difference in the feed intake of the genotypes was fairly constant over the weeks of the experiment ($P = 0.231$; Table 1). Infected birds exhibited a significantly lower ADFI ($P = 0.040$) than the controls, without an interaction with genotype or time ($P > 0.05$). The overall feed conversion ratio (FCR) was not different between LD and LB ($P = 0.996$), but the FCR of these two genotypes was significantly higher than that of R ($P < 0.001$). Infections did not affect the overall FCR ($P = 0.767$; Table 1).

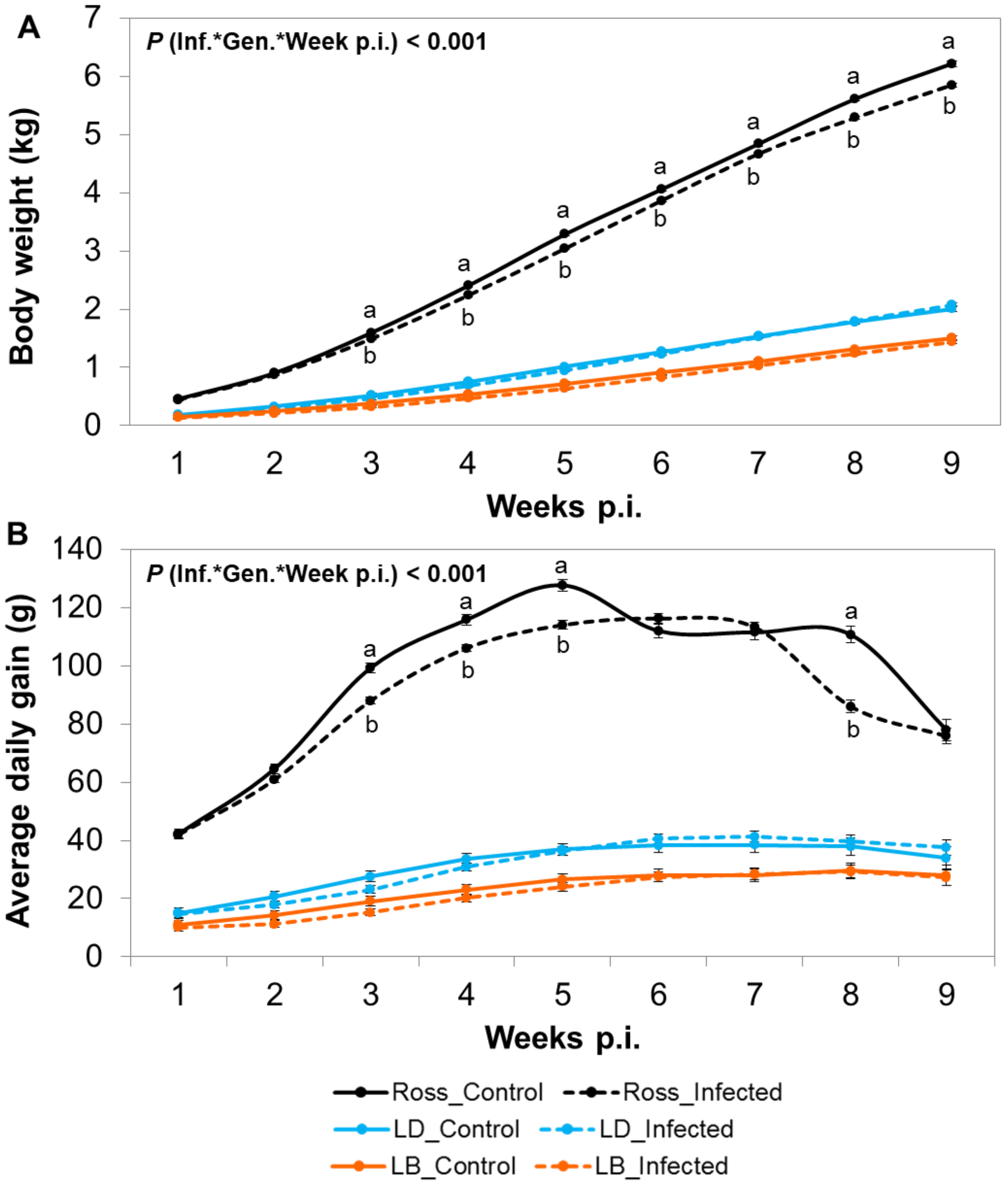

Fig. 2. Time-dependent effects of mixed nematode infections on body weight (**A**) and average daily weight gain (**B**) in divergent chicken genotypes. a,b indicate significant ($P < 0.05$) differences between infected and un-infected control birds of the same genotype at the same time point. The values are LSMEANS with SE on the error bars. (Number of birds = 668; sample size n = 3496). LB, Lohmann Brown Plus; LD, Lohmann Dual; Ross, Ross-308.

3.2. FEC

The faecal samples of all genotypes were egg-negative at 3 weeks p.i. The first egg-positive samples were encountered at 4 weeks p.i. in LD and LB birds, and at 6 weeks p.i. in R birds. The three genotypes had similar EPG and EPD levels ($P > 0.05$; Table 2). Although the FECs increased over the experimental weeks ($P = 0.005$), there was no significant difference among the genotypes at any time point ($P > 0.05$).

3.3. Worm burden

The overall average *A. galli* counts (total burden) were significantly higher in R compared with LB ($P = 0.049$), while the average *A. galli* burden of LD was not different from those of the other two genotypes ($P > 0.05$; Table 2). The genotypes did not differ in terms of *A. galli* larval counts ($P = 0.137$), whereas adult worm counts differed among the genotypes in the order of R > LD > LB ($P < 0.001$; Table 2). The overall average burden across all genotypes decreased ($P < 0.001$) over time from 95 ± 4.0 (LSM ± SE) worms/bird at 2 weeks p.i. to 3 ± 3.2 worms/bird at 9 weeks p.i. Similarly, larval counts decreased continuously over the weeks of the experiment ($P < 0.001$), and no re-infection occurred. Non-larval worm counts were higher in R than in LD and LB at the early phase of infection (3–5 weeks p.i.; data not shown), while the differences became much smaller in the subsequent weeks. The total number of tissue larvae in the jejunum did not differ among the genotypes ($P = 0.331$). Although genotype effects did not interact with time ($P = 0.101$), a three-fold higher average number of larvae was recovered from the tissue of R birds at 2 weeks p.i. than from the other two genotypes (data not shown).

The percentage of tissue larvae within the total burden was significantly higher ($P < 0.001$) in R than in LD and LB (Table 2) over the whole experimental period ($P = 0.555$). Similar to the lumen larvae, there was a significant decrease in the number of tissue larvae over time ($P < 0.001$; Fig. 3A). A significantly greater number of larvae was detected in the lumen than in the jejunum tissue ($P = 0.001$). There was a genotype-dependent worm distribution between the jejunum and ileum. As shown in Fig. 3B, the percentage of lumen larvae was similar ($P > 0.05$) between the two locations in R, whereas a higher percentage of larvae was recovered from the jejunum than the ileum in both LD and LB ($P < 0.05$). Similar to larval stages, a higher percentage of the total worm burden was found in the jejunum than in the ileum ($P = 0.001$). Although the jejunal worm burden was not significantly different among the genotypes ($P > 0.05$), a higher percentage of worms was recovered from the ileum of R birds than from those of LD and LB birds ($P < 0.05$; Fig. 3B).

Table 2. Overall effects of host genotype on faecal egg counts (FEC), worm burdens and worm length in divergent chicken genotypes exposed to mixed-nematode infections.

	Host genotype				**P ≤**		
Item	R	LD	LB	SE	Gen.	wpi	Gen.*wpi
FEC							
EPG (n/bird)	19	20	15	5.8	0.884	0.005	0.856
EPD (n/bird)	2172	1288	556	529.7	0.953	0.005	0.820
Ascaridia galli							
Total burden (n/bird)	26.9[a]	25.2[ab]	24.3[b]	2.35	0.049	0.001	0.123
Total larva (n/bird)	21.1	22.1	23.1	2.29	0.137	0.001	0.514
Non-larva worms[e] (n/bird)	5.6[a]	3.0[b]	1.3[c]	0.07	0.001	0.001	0.001
Lumen larva (n/bird)	17.7[d]	20.0	21.1[d]	2.04	0.075	0.001	0.568
Tissue larva (n/bird)	3.4	2.1	2.1	0.49	0.331	0.001	0.101
Tissue larva (%)	42.5[a]	22.4[b]	27.5[b]	3.57	0.001	0.001	0.555
OWL (mm)	16.3[a]	15.0[a]	11.3[b]	0.922	0.001	0.001	0.172
Heterakis gallinarum							
Total burden (n/bird)	8.0[b]	9.1[ab]	11.7[a]	1.17	0.031	0.001	0.201
First generation (n/bird)	7.9	8.0	9.4	1.10	0.449	0.001	0.662
Total larva (n/bird)	1.5[b]	2.8[a]	4.4[a]	0.66	0.001	0.001	0.001
Larva 1st gen. (n/bird)	3.5	4.6	5.7	2.73	0.887	0.001	0.631
Larva 2nd gen. (n/bird)	0.2[c]	1.8[b]	3.6[a]	0.39	0.001	0.001	0.001
OWL (mm)	6.49	6.43	6.19	0.213	0.531	0.001	0.002

[a,b,c] Genotypes shown with different letters differ significantly (Tukey, P < 0.05).

[d] Genotypes tend to differ (Tukey, P < 0.10).

Faecal samples were collected from 3 to 9 weeks p.i. (sample size n = 321). Sample size for worm burden data, n = 422. Analysis of overall worm length (OWL) included 304 and 378 average length measurements weighed for developmental stages of *Ascaridia galli* and *Heterakis gallinarum*, respectively.

[e] Non-larval worms, defined as the worm burden minus larvae and includes mature and immature worms that are sexually differentiable by morphological characteristics.

EPG, number of eggs per gram faeces; EPD, number of eggs excreted within 24 h; R, Ross-308; LD, Lohmann Dual; LB, Lohmann Brown Plus; Con, uninfected controls; Inf., infected; Gen, genotype; wpi, weeks p.i. Larva 1st gen./2nd gen., larval stages of the first and generation worms descending from experimental and naturally occurring reinfections, respectively.

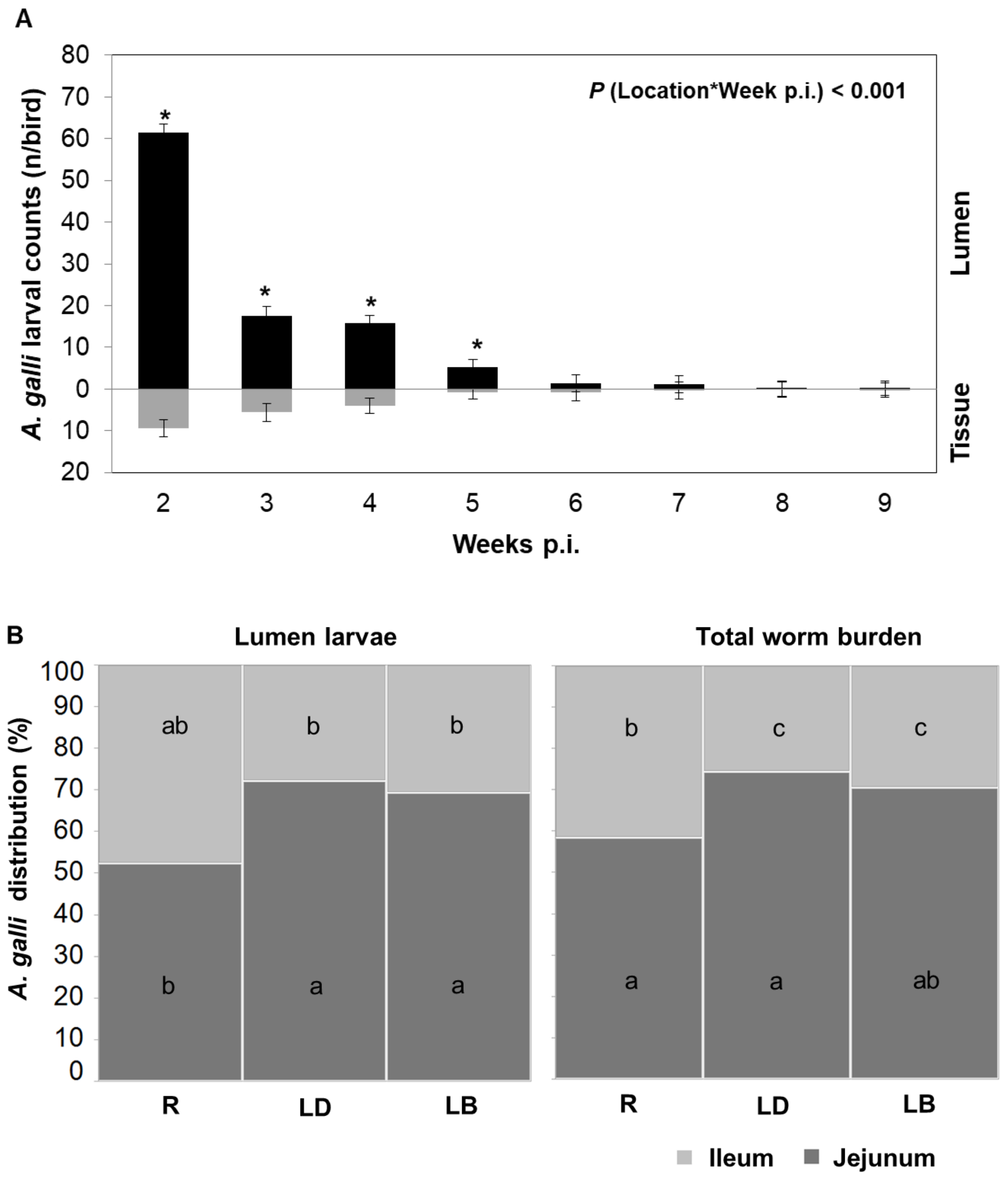

Fig. 3. Distribution of *Ascaridia galli* larvae between tissue wall and lumen in jejunum over time (**A**), and the localisation of worms between jejunum and ileum in different chicken host genotypes (**B**). (**A**) Asterisk (*) indicates a significant (Tukey, $P < 0.05$) difference between the number of larvae in the lumen and the tissue wall (sample size n = 842). (**B**) Values shown with different letters (a,b,c) differ significantly (Tukey, $P < 0.05$) (sample size n = 843). LB, Lohmann Brown Plus; LD, Lohmann Dual; R, Ross-308.

The overall average total *H. gallinarum* burden was different among the three genotypes ($P = 0.031$; Table 2). The LB had a higher number of *H. gallinarum* than R ($P = 0.034$), while the burden of LD was not different from those of LB or R ($P > 0.05$; Table 2). The numbers of first generation *H. gallinarum* worms, originating from the experimental infection, did not differ among the genotypes (P = 0.449; Table 2). Similarly, the numbers of first generation larvae did not differ (P = 0.887), whereas the numbers of larvae resulting from re-infection significantly differed among the genotypes in the order of LB > LD > R (P < 0.001) (Table 2). The overall average *H. gallinarum* burden across the three genotypes decreased from 2 to 7 weeks p.i. and increased thereafter. Almost no re-infection occurred in R birds, whereas LD and, particularly, LB birds harboured increasing numbers of larvae starting at 7 weeks p.i. (Fig. 4). At 9 weeks p.i., the highest number of *H. gallinarum* larvae was found in LB, followed by LD and R, with significant (P < 0.05) differences among all three genotypes.

The OWL of *A. galli* was significantly (P < 0.001; Table 2) higher in R and LD than in LB, with no interaction over time (P = 0.172). In contrast, the OWL of *H. gallinarum* was significantly higher (P < 0.05) in LD than in LB only at 8 weeks p.i. (Supplementary Fig. S1). At 9 weeks p.i., the OWL in both R and LD was higher compared with that of LB (P < 0.05), and the OWL of R tended to be higher than that of LD (P = 0.076).

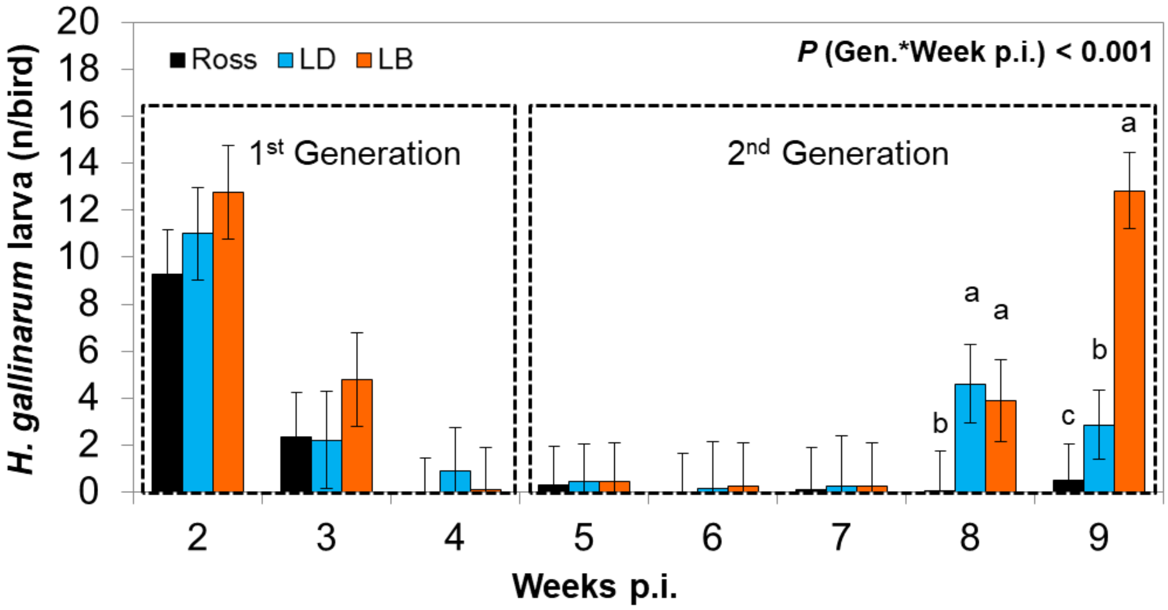

Fig. 4. *Heterakis gallinarum* larvae in divergent chicken host genotypes after experimentally-induced and naturally occurring (re)-infections. Host genotypes sharing no common letters (a–c) at a given time point differ significantly (Tukey, P < 0.05). LSMEANS and SE represent untransformed data; P-values are based on the transformed data (sample size n = 422; total number of birds necropsied at each week p.i. ranged from 42 to 68). LB, Lohmann Brown Plus; LD, Lohmann Dual; Ross, Ross-308.

3.4. Ascarid-specific IgY antibodies

Overall, average plasma ascarid-specific IgY levels tended to be higher in LB than in R ($P = 0.062$; Table 3). Independent of host genotype, infections significantly increased IgY levels ($P < 0.001$). With the exception of the time point 3 weeks p.i., infected birds exhibited significantly higher ascarid-specific IgY levels than their uninfected counterparts during the study period (i.e., from 2 to 9 weeks p.i.; Fig. 5).

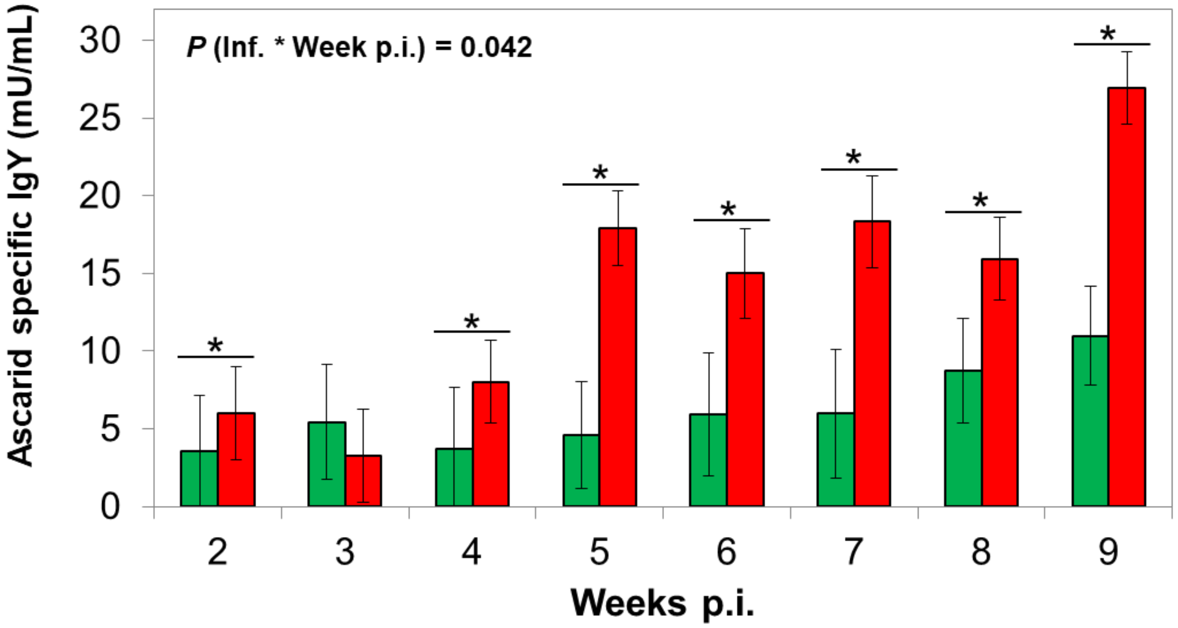

Fig. 5. Course of ascarid-specific IgY development in infected (🔴) and uninfected (🟢) control chickens. An asterisk (*) indicates a significant (Tukey, $P < 0.05$) difference between infected and uninfected birds at the given time points. LSMEANS and SE represent untransformed data; P values are based on the transformed data (sample size $n = 646$ plasma samples ($n = 72–96$ birds/week p.i.)).

3.5. Immunoglobulins

The overall IgY concentration in plasma was higher in LD and LB than in R ($P = 0.002$; Table 3). However this effect was dependent on time after infection ($P = 0.003$), with LD and LB exhibiting higher concentrations than R at 2 weeks p.i. (Supplementary Fig. S2A). At 5 weeks p.i., the IgY concentration was higher in LD than in R ($P < 0.05$), whereas LB did not differ from the two other genotypes ($P > 0.05$). No differences were found among the genotypes at 9 week p.i. ($P > 0.05$). Although infections increased the overall IgY concentration ($P = 0.002$; Table 3), this effect was only present in R and LD birds ($P = 0.048$, Supplementary Fig. S2B). Overall IgM concentrations were higher in R than in LD and LB ($P = 0.001$; Table 3), particularly at 9 weeks p.i. (Supplementary Fig. S2C).

Independent of host genotype ($P = 0.546$), infected birds had higher ($P < 0.001$) IgM concentrations than their uninfected counterparts (Table 3), due to a temporary increase at 2 weeks p. i. ($P < 0.001$; Supplementary Fig. S3D). IgA concentrations did not differ among the genotypes ($P = 0.227$; Table 3), whilst infections increased IgA concentrations in all genotypes at 2 weeks p.i. ($P = 0.002$, Supplementary Fig. S2E).

Table 3. Infection-specific and unspecific humoral immune responses in relation to host genotype and mixed nematode infections in divergent chicken genotypes.

	Main effects of genotypes					**Infection effect**				**P, ≤**	**Interaction, P-value, ≤**			
Item	R	LD	LB	SE	P,≤	Con.	Inf.	SE	P,≤	wpi	Inf.*Gen.	Inf.*wpi	Gen.*wpi	Inf.*Gen.*wpi
Asc-IgY^{d}	8.68^{c}	10.02	11.35^{c}	1.45	0.062	6.11	13.92	1.30	0.001	0.001	0.894	0.001	0.079	0.061
IgY^{e}	3.10^{b}	3.87^{a}	3.43^{a}	0.24	0.002	2.82	4.12	0.22	0.001	0.001	0.048	0.062	0.003	0.275
IgM^{e}	0.30^{a}	0.18^{b}	0.21^{b}	0.02	0.001	0.21	0.26	0.01	0.001	0.001	0.546	0.001	0.001	0.661
IgA^{e}	0.25	0.25	0.24	0.02	0.277	0.23	0.26	0.02	0.995	0.250	0.526	0.002	0.670	0.478

[a,b] Genotypes with different letters differ significantly (Tukey, $P < 0.05$). [c] Genotypes tend to differ (Tukey, $P < 0.10$).

Measurement units: [d] mU/ml, [e] mg/ml. Plasma samples for ascarid-specific IgY were taken weekly from 2 to 9 weeks p.i. (wpi) (n = 646). The samples for IgY, IgM, IgA were taken at 2, 5 and 9 wpi (n = 271).

Asc. IgY, ascarid-specific IgY; Ig, Immunoglobulin; R, Ross-308; LD, Lohmann Dual; LB, Lohmann Brown Plus; Con, uninfected controls; Inf., infected; Gen, genotype.

3.6. Intestinal size and macroscopic alterations in the caecum

At 1 week of age (0 weeks p.i., infection day), the lengths and weights of the SI were significantly higher in R than in the other genotypes ($P < 0.001$), with LD and LB showing similar weights ($P > 0.05$; Table 4). The caeca were longer in R than in LB ($P < 0.05$) and tended to be longer than in LD ($P = 0.069$), whereas caecal length was not different between LD and LB ($P > 0.05$). The total full weight of the caeca differed among the

Table 4. Size of small intestine and caeca in relation to host genotype and mixed-nematode infections in the beginning (0 weeks p.i. (wpi)) and end of the experiment (9 wpi).

		Main effects of genotypes					Infection effect				Interaction, ≤
	Item	R	LD	LB	SE	P,≤	Con.	Inf.	SE	P,≤	Inf.*Gen.
Wpi 0 **(Age: 1 week)**	**Small intestine**						-	-	-	-	-
	Length (cm)	81.8^{a}	26.8^{b}	27.4^{b}	4.73	0.001	-	-	-	-	-
	Weight (g)	18.5^{a}	8.7^{b}	6.5^{b}	1.21	0.001	-	-	-	-	-
	Caecum						-	-	-	-	-
	Length (cm)	8.2^{ad}	6.9^{abd}	5.9^{b}	0.36	0.002	-	-	-	-	-
	Weight (g)	2.8^{a}	1.9^{b}	1.1^{c}	0.19	0.001	-	-	-	-	-
Wpi 9 **(Age: 10 weeks)**	**Small intestine**										
	Length (cm)	132.9^{a}	92.1^{b}	85.7^{b}	2.81	0.001	101.8	105.3	2.53	0.243	0.587
	Weight (g)	68.8^{a}	31.0^{b}	22.4^{c}	2.41	0.001	40.5	41.0	2.18	0.851	0.157
	Caecum										
	Length (cm)	20.1^{a}	14.9^{b}	13.6^{b}	0.57	0.001	17.7	14.8	0.51	0.001	0.113
	Weight (g)	19.5^{a}	12.2^{b}	9.7^{c}	0.80	0.001	14.4	13.2	0.72	0.160	0.216

[a,b,c] Genotypes shown with different letters differ significantly (Tukey, $P < 0.05$). [d] Genotypes tend to differ (Tukey, $P < 0.10$).

Analysis for 0 wpi included 18 observations ($n = 6$ per genotype) only from the second run. Analyses for 9 wpi are based on data across spatial and temporal replications (sample size $n = 87$).

R, Ross-308; LD, Lohmann Dual; LB, Lohmann Brown Plus; Con, uninfected controls; Inf., infected; Gen, genotype.

genotypes in the order of R > LD > LB (P < 0.001; Table 4). Similar to 0 weeks p.i., at 10 weeks of age (i.e. 9 weeks p.i.), the SI was larger in R than in LD and LB (P < 0.001), with no difference between the latter two genotypes (P > 0.05). The weight of the SI differed in the order of R > LD > LB (P = 0.001; Table 4). SI length was not affected by infection at 9 weeks p.i. (P = 0.851). Caecum length was similar between LD and LB (P > 0.10), whereas the caeca of R birds were larger (P < 0.001). Full caecum weight was in the order of R > LD > LB (P < 0.001). Infected birds exhibited shorter caeca than did uninfected controls at 9 weeks p.i. (P < 0.001), although caecal weight was not influenced by the infection (P = 0.160). The worm burden of both species did not correlate with the length or weight of their predilection sites (P > 0.05). Macroscopic alterations (e.g., mucosal bleeding, thickened caecal wall and fibrinous content) in the caeca were observed in 13.1%, 18.9% and 19.2% of the infected birds in the R, LD and LB genotypes, respectively. The percentage of macroscopically affected caeca decreased across the weeks of infection in all three genotypes (from 45.2 % at 2 weeks p.i. to 4.4% at 9 weeks p.i.).

3.7. Intestinal morphometry

Villus height (VH; P < 0.001, Table 5) and the ratio of villus height to crypt depth (P = 0.005) were significantly different among the genotypes, with R exhibiting higher (P < 0.05) values than LD and LB. Villus width was greater for R and LD than for LB (P = 0.001). Crypt depth was not different among the genotypes (P = 0.653). An interaction between genotype and week p.i. (P = 0.002) indicated that the differences in VH among the genotypes were time-dependent. At 2 weeks p.i., VH followed the order of R > LD > LB, whereas at 5 weeks p.i., the VH of R was significantly higher (P < 0.05) than those of LD and LB. At 9 weeks p.i., the genotypes did not differ for VH (P > 0.05). Villus width differed among the genotypes only at 9 weeks p.i., with LB exhibiting a narrower villus width than R and LD (P < 0.05). Villus width and crypt depth were not influenced by infection (P > 0.05; Table 5). A significant interaction among the effects of infection, genotype and week p.i. (P = 0.013), however, indicated a tendency (P < 0.10; Supplementary Fig. S3) towards a shorter VH in the infected LD birds at 2 and 9 weeks p.i. compared with the corresponding uninfected controls. The VH of R and LB was not affected by infection (P > 0.05). Additionally, the VH:crypt depth ratio was significantly lower in infected birds (P = 0.048).

Table 5. Morphometric measurements of the small intestine in relation to host genotype and mixed nematode infections.

	Main effects of genotypes					**Infection effect**				**P, ≤**	**Interaction, P-value, ≤**			
Item	R	LD	LB	SE	P,≤	Con.	Inf.	SE	P,≤	wpi	Inf.*Gen.	Inf.*wpi	Gen.*wpi	Inf.*Gen.*wpi
Villus height (µm)	1311^{a}	1173^{b}	1128^{b}	22.0	0.001	1226	1182	18.0	0.098	0.001	0.048	0.786	0.002	0.013
Villus width (µm)	146.5^{a}	144.2^{a}	123.3^{b}	4.10	0.001	134.7	141.3	3.34	0.172	0.001	0.440	0.341	0.041	0.335
Crypt depth (µm)	116.3	119.6	116.0	2.99	0.653	114.4	120.2	2.44	0.099	0.125	0.832	0.516	0.357	0.223
Villus:crypt ratio	11.40^{a}	9.93^{b}	9.82^{b}	0.36	0.005	10.80	9.96	0.29	0.048	0.009	0.211	0.663	0.080	0.087

^{ab}Genotypes shown with different letters differ significantly (Tukey, P < 0.05).

Tissue samples from jejunum were collected from six birds (three infected, three uninfected controls) of each genotype at each sampling point (week p.i.). Sample size n = 54 (six birds *x* three genotypes *x* three time points).

Villus:crypt ratio, villus height to crypt depth ratio; R, Ross-308; LD, Lohmann Dual; LB, Lohmann Brown Plus; Con, uninfected controls; Inf., infected; Gen, Genotype; wpi, weeks p.i.

3.8. Luminal pH in the small intestine (SI) and caecum

Luminal pH in SI was higher in R than in LB at 7 and 8 weeks p.i., whereas LD did not differ from the other two genotypes (P = 0.007; Fig. 6A). Infection lowered the luminal pH in the SI (P < 0.001; Table 6) independent of any interactions with host genotype (P = 0.627) and week p.i. (P = 0.234). The intra-caecal pH was different among the genotypes in the order of R > LB > LD (P = 0.001; Table 6). An interaction between genotype and time (P < 0.001) indicated significant alterations in caecal pH among the genotypes by time (Fig. 6B). Infection elevated the intra-caecal pH in LD and LB but not in R birds (P = 0.015). Independent of host genotype, infection increased intra-caecal pH from 2 to 5 weeks p.i. (Fig. 6C). Moderate negative correlations between the *A. galli* larval burden and the pH of the SI were detected at 2 weeks p.i. (r = 0.46; P = 0.002) and 7 weeks p.i. (r = 0.43; P = 0.003), whereas no significant correlation was determined at other time points (P > 0.05). The intra-caecal pH was positively correlated with the *H. gallinarum* larval burden from 2 to 4 weeks p.i. (r = 0.45 to r = 0.29; P < 0.05); however, at 6 and 8 weeks p.i. moderate-negative correlations (r = 0.38 and r = 0.39, respectively, P < 0.05) were detected.

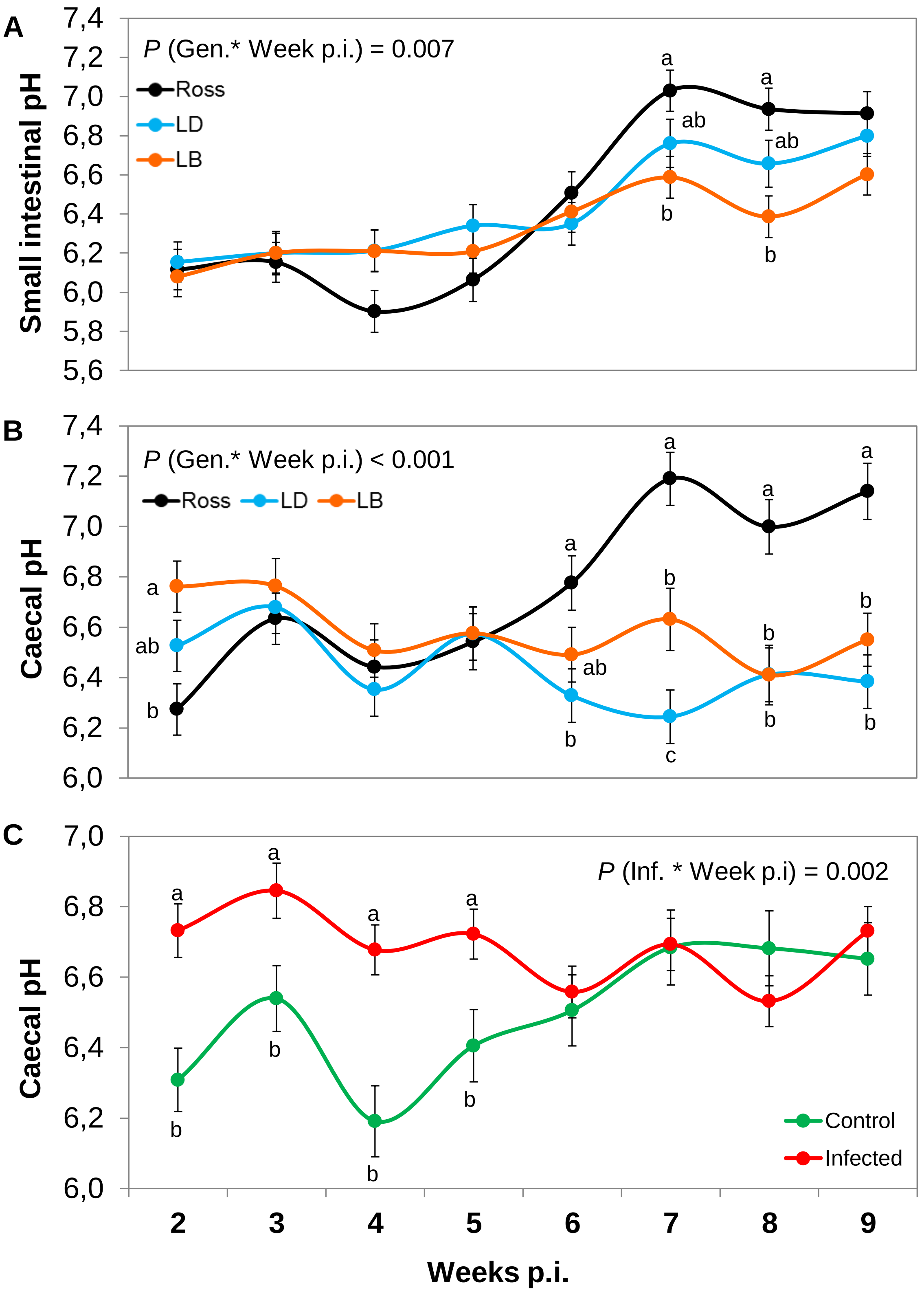

Fig. 6. Time-dependent alterations in the luminal pH of the small intestine (**A**) and caecum (**B**) in relation to chicken genotype or infection (**C**) effects over time. Values with different letters at the same time point indicate significant (Tukey, P < 0.05) differences. Sample size for each intestinal segment n = 559. LB, Lohmann Brown Plus; LD, Lohmann Dual; Ross, Ross-308.

Table 6. Biochemical parameters describing intestinal environment in ileum and caeca in divergent chicken genotypes exposed to mixed nematode infections.

	Main effects of genotypes					**Infection effect**				**P-values for further effects**				
Item	R	LD	LB	SE	P,≤	Con.	Inf.	SE	P,≤	wpi	Inf.*Gen.	Inf.*wpi	Gen.*wpi	Inf.*Gen.*wpi
Ileum														
SCFA^{e}	2.59	2.28	2.53	0.20	0.504	2.45	2.49	0.16	0.878	0.029	0.143	0.628	0.092	0.807
Lactate^{e}	40.92^{a}	23.62^{b}	18.36^{b}	4.21	0.001	25.48	29.80	3.42	0.371	0.007	0.827	0.470	0.395	0.243
pH	6.45^{ad}	6.43^{a}	6.34^{ad}	0.04	0.064	6.53	6.28	0.04	0.001	0.001	0.627	0.234	0.007	0.368
Caecum														
SCFA^{e}	57.04	50.40	50.09	3.79	0.306	51.49	53.53	2.72	0.622	0.415	0.051	0.037	0.056	0.393
Lactate^{e}	9.27	9.70	7.86	1.00	0.339	8.74	9.14	0.78	0.679	0.008	0.757	0.115	0.337	0.197
pH	6.75^{a}	6.44^{c}	6.59^{b}	0.04	0.001	6.50	6.69	0.04	0.001	0.021	0.015	0.002	0.001	0.275

^{abc}Genotypes shown with different letters differ significantly (Tukey, $P < 0.05$). ^{d}Genotypes tend to differ (Tukey, $P < 0.10$).

^{e}Measurement unit: mmol/g digesta.

Digesta samples for short chain fatty acid concentration (SCFA) and lactate were taken from three birds per genotype and infection status at the necropsy time-points of 3, 5 and 9 weeks p.i. (wpi) (n = 108). Analyses on the intestinal pH are based on 559 observations.

SI: small intestine; R, Ross-308; LD, Lohmann Dual; LB+, Lohmann Brown; Con, uninfected controls; Inf., infection; Gen, genotype.

3.9. SCFAs and LA

The SCFA concentration in the ileum did not differ among the genotypes ($P < 0.05$), whereas LA concentration was significantly higher in R than in LD and LB birds ($P < 0.05$; Table 6). Infection had no significant effect on the concentrations of SCFA ($P = 0.878$) and LA ($P = 0.371$) in the ileum. The concentrations of SCFA ($P = 0.306$) and LA ($P = 0.339$) in the caecum were not different among the genotypes. Lower SFCA concentrations were quantified in the infected birds than in the controls at 9 week p.i. (data not shown). Detailed results for individual SCFA and LA levels are summarized in Supplementary Table S1.

4. Discussion

To test the hypothesis that chicken genotypes selected for increased growth rate are more vulnerable to the most common nematode infections, we compared male birds of a fast growing genotype (i.e., R) with those of an extremely slow growing genotype (i.e., LB), representing layer type chickens. In addition, male birds of a dual purpose genotype (LD) with an intermediate growth potential were included in the study design. Due to sex dependency in egg production, male birds of layer type chickens are not under production pressure for what their genotype has been selected for. This applies partly to the LD birds, too. Thus, the comparison of three genotypes with extremely different growth rates provided a unique experimental model for assessing the consequences of selection for increased growth rate on response to nematode infections in chickens. The contrasted genotypes were reared in an environment designed fully for broilers (e.g. diets and husbandry conditions). Following experimental infection, measurements on host responses related to resistance, tolerance and susceptibility to mixed nematode infections were then carried out. To imitate naturally occurring field infections we used an experimental multiple species infection model with *A. galli* and *H. gallinarum*. *Heterakis gallinarum* is the main vector for transmission of *Histomonas meleagridis* (McDougald, 2005) which induces caecal lesions of similar severity in broiler and layer type chickens (Lotfi et al., 2014). We observed macroscopic caecal tissue alterations in some birds (overall <20%) that are characteristic for the protozoan parasite (McDougald, 2005; Hess et al., 2006), confirming the involvement of *H. meleagridis* in the mixed infection as it occurs under natural conditions following *H. gallinarum* infection (Grafl et al., 2011). The duration of the infection period (9 weeks) allowed one of the nematode species (*H. gallinarum*) to induce reinfection, additionally enabling an evaluation of host susceptibility to naturally occurring secondary infection.

Our results showed that tolerance to mixed nematode infections, which is the ability of the host animal to perform well despite an infection (Best et al., 2008; Råberg et al., 2009; Doeschl-Wilson and Kyriazakis, 2012), was higher in LB and LD than in R as the latter responded to infection with an impaired growth performance. This suggests that selection for increased growth rate may be associated with an impaired ability to tolerate nematode infections. Impaired tolerance in R birds was however associated with a relative nutrient scarcity due to the infection-induced lower feed intake. Resistance, the ability of the host animal to reduce the pathogen load, e.g., worm burden, FEC (Best et al., 2008; Råberg et al., 2009; Doeschl-Wilson and Kyriazakis, 2012) was dependent on both host genotype and worm species. The R was less resistant to *A. galli* compared with LB, whereas LD was in an intermediate position. These differences suggest that resistance to *A. galli* may be negatively associated with selection for increased growth rate. Resistance to *H. gallinarum* depended not only on host genotype, but also on the type of infection (i.e., primary versus secondary). *Heterakis gallinarum* burden with the first generation worms resulting from the experimental infection was similar in three genotypes. Susceptibility to naturally occurring re-infection with *H. gallinarum* was, however, higher in LB and LD than in R. In the following sections, we will address potential factors influencing tolerance, resistance and susceptibility to re-infection in different genotypes with respect to selection objectives and partitioning of resources in a given environment. Infections reduced feed intake in all three genotypes. Mechanisms for the helminth-induced depression in feed intake are not yet conclusively elucidated. Defence strategies, actively managed by the host itself, to cope with parasites (Exton, 1997; Kyriazakis et al., 1998), changes in the hormonal regulation of feed intake (Yang et al., 1990; Zaralis et al, 2008) as well as anorexic effects stimulated by the immune system during parasite challenge (Colditz, 2008) play important roles for the impairment in the voluntary feed intake. The activation of the adaptive immune system (Colditz, 2008) and the increase in abundance of specific cytokines (e.g., IL 1, IL 6, IL 8, TNFα, IFNα) during the acute phase response are well known to induce anorexic effects (McCarthy, 2000; Plata-Salamán, 2001; Rauw, 2012). Such cytokines were also partly found to be upregulated during an *A. galli* infection in chickens (Dalgaard et al., 2015). In our study, we found the first noticeable drop in both feed intake and weight gain between 2 and 3 weeks p.i., the time period that corresponds well to the activation of the adaptive arm of the avian immune system following nematode infections (Schwarz et al., 2011a,b; Stehr et al., 2018).

Although all three genotypes responded to the infections with a reduced feed intake, only growth performance of infected R birds was impaired. In contrast to R, infected LB and LD birds were able to maintain their growth performance compared with their uninfected counterparts. The infection-induced impairment in growth performance of the fast but not that of the slower growing genotypes is in agreement with previous data from a murine-nematode system. Coltherd et al. (2009, 2011) experimentally infected mouse lines divergently selected for high or low body weight with the intestinal nematode *Heligmosomoides bakeri*. The nematode infection reduced body weight gain in the high body weight line only. Furthermore, infected mice of the high body weight line were able to maintain their growth performance when fed high but not low protein diets (Coltherd et al., 2009, 2011).

Taken together, these results not only support the hypothesis that selection for high growth performance impairs host tolerance to gastrointestinal nematodes, but also provide evidence that there is a nutritional basis of tolerance to nematode infections which depends on host genotype. The finding that the infection-induced impairment in feed intake was associated with lower growth performance in the fast growing genotype but not in slower growing genotypes might indicate such an interaction. Nutrient and energy requirements of layer and broiler genotypes in the growing phase differ, with the latter requiring more nutrient-dense diets (Jeroch et al., 2013). The birds of all three genotypes were fed the same age-specific diets, which were designed in accordance with the nutrient requirements of commercial broiler chickens. This implies that excessive amounts of surplus nutrients and energy were provided to slower growing genotypes compared with the fast growing genotype. As a consequence, the nutrient and energy dense diets might have compensated the adverse effect of lower feed intake on performance of the slower growing genotypes. Surplus nutrient supply, particularly protein, indeed has great potential to compensate adverse effects of parasite infection on growth and improve resistance in different host-parasite systems (Coop and Kyriazakis, 2001; Liu et al., 2005; Coltherd et al., 2009, 2011). In line with this view, Daş et al. (2010) reported that a lysine-enriched diet reduced the impact of an *A. galli* infection on growth performance in chickens. These results collectively suggest that nutrient concentrations in excess of the physiological requirements can reduce the impact of an infection on performance traits, thereby enhancing host tolerance to parasite infection. Feed conversion efficiency was not affected by the infections in any of the genotypes, indicating that overall nutrient utilisation was not impaired by the infection. The size of the SI, the primary absorption site of nutrients (Denbow, 2015) which is also the predilection site of *A. galli*, was not influenced by the infection. Nevertheless, structural alterations of the intestinal wall (e.g., shorter villus, mucosal thickness, mucosal lesions) during *A. galli* infection are well known (Dänicke et al., 2009; Marcos-Atxutegi et al., 2009; Luna-Olivares et al, 2015) and are likely caused by the larval stages penetrating the tissue wall (Luna-Olivares et al., 2012). In our study, infected birds tended to have a shorter villus and greater crypt depth in the jejunal section which resulted in a smaller villus:crypt ratio, but alterations were rather insubstantial as the FCR remained un-affected in all three genotypes. The higher mortality and the impaired tolerance to mixed nematode infections in R birds confirm that the fast-growing genotypes have lower fitness than slower growing genotypes (Rauw et al., 1998; Julian, 2005; Bessei, 2006; Olkowski, 2007; Raynal-Ljutovac et al., 2007; Oltenacu and Broom, 2010). This observation is in line with the theory of resource partitioning in genotypes intensively selected for a particular performance trait, i.e. increased growth rate in chickens (Rauw, 2012).

The *A. galli* burden was higher in R than in LB. Similarly, a higher number of *A. galli* survived beyond larval stages in R than in LB. The overall *A. galli* length was also higher in R than in LB birds. When compared with R and LB, LD was in an intermediate position in terms of both survival and growth of *A. galli*. These results collectively suggest that resistance to *A. galli* may be negatively associated with selection for increased growth rate in chickens. In contrast, first generation burden with *H. gallinarum*, originating from the experimental infection, was similar in the three genotypes. The larger *A. galli* burden in the fast growing R birds than in the slow growing LB birds might at least partly be due to differences in immune functions. The level of ascarid-specific IgY is not strongly associated with a direct protection in chickens (Daş et al., 2018), but is indicative of the activation of the adaptive cell-mediated immune responses (Harris and Gause, 2011; Stehr et al., 2018). Cell-mediated immune responses differ between layers and broilers following challenge with lipopolysaccharide, trinitrophenyl-conjugated keyhole limpet hemocyanin or human serum albumin (Leshchinsky and Klasing, 2001; Koenen et al., 2002; Parmentier et al., 2010). Although we did not measure cell-mediated immune responses, differences in both infection-specific and -unspecific humoral immune responses between R and LB birds are in line with the lower resistance of R birds to *A. galli*. The LB birds tended to have higher ascarid-specific IgY levels than R birds. Similarly, the concentration of worm-unspecific IgY was higher in LB than in R, which might be associated with the lower *A. galli* burden in the slow growing genotype. The larger *A. galli* burden in R than in LB might furthermore be related to the differences in the intestinal environment of fast and slow growing genotypes. *Ascaridia galli* embeds itself in intestinal tissue (Luna-Olivares et al., 2012), likely to escape the expulsion reaction. The tissue-associated phase of *A. galli* was almost completed at approximately 5 weeks p.i. in all three genotypes. This finding is in line with previous data describing the duration of the histotrophic phase for this nematode (Herd and McNaught, 1975). A higher percentage of larvae was isolated from the tissue wall of R birds than from those of LD and LB birds. Non-larval *A. galli* counts were also higher in R than in LD and LB, likely indicating a more favourable intestinal environment for worm survival in R birds. The larger intestines as well as higher nutrient intake in R birds might have resulted in less competition among worms for space and nutrients. This hypothesis is further supported by the higher OWL of *A. galli* in R than in LB. Furthermore, the greater quantity of lactic acid in the small intestines of R birds might have been associated with the higher *A. galli* worm burdens in this genotype. The higher lactic acid concentration suggests a higher abundance of lactic acid-producing bacteria (e.g., Lactobacillus spp.) in the digesta, which might have promoted worm development as known for a helminth-microbiota system in mice (Reynolds et al., 2014).

Host genotype-dependent differences in resistance to *A. galli* did not apply to *H. gallinarum*, as the burden with first generation worms was similar in the three genotypes. The intra-caecal environment is known to affect larval establishment, growth and fecundity of *H. gallinarum* (Springer et al., 1970; Daş et al., 2011). Chicken genotypes differ in their intestinal environments, particularly with progressing age (Zhao et al., 2013; Schokker et al., 2015; Walugembe et al., 2015). Differences in the intra-caecal pH of the three genotypes confirmed the age-dependent differences by 6 weeks p.i. However, in the early infection period (2 weeks p.i.), there was only a transient pH difference between LB and R. The similar metabolite profiles (i.e., SCFA and LA) in the caecal digesta of different genotypes suggest negligible differences in the predilection site of *H. gallinarum*. Thus, the absence of differences in the caecal environment of the host genotypes may be associated with similar first generation burdens with *H. gallinarum* in the three genotypes.

Although the three genotypes did not differ in the number of *H. gallinarum* larvae originating from experimental infection, there were considerable differences in terms of secondary *H. gallinarum* infection (i.e. re-infection). The finding that re-infection occurred only for *H. gallinarum* but not for *A. galli* can be ascribed to the longer prepatent period of *A. galli* than that of *H. gallinarum* (Ramadan and Abou Znada, 1991; Daş et al., 2014; Daş and Gauly, 2014). The worm burden of the genotypes with first and second generation *H. gallinarum* worms provided crucial information about susceptibility to experimentally induced and naturally occurring (re)infection. When a fully controlled experimental infection was induced, the number of worms did not differ among the genotypes, whereas susceptibility to naturally occurring infection was highest in LB and lowest in R. Although secondary infections are expected to be influenced by the immune response acquired during the primary infection (Anthony et al., 2007), outcomes of naturally occurring re-infection may not be solely immune-function dependent. Unlike experimentally induced infections, the occurrence and magnitude of re-infections depend on the ingestion of embryonated eggs from the environment (i.e., from pen litter) by the host animal. The similar EPD levels in the three genotypes indicated comparable pen contamination levels with nematode eggs. This implied a similar exposure risk for the occurrence of re-infection in the three genotypes. The extremely low re-infection observed in R might have been at least partly related to the number of ingested eggs actively picked up from the pen environment, likely due to behavioural differences. In comparison to layer type chickens, broilers spend less time eating (Masic et al.,

1974) and are less active (Lindqvist et al., 2006; Tickle et al., 2018). Furthermore, broilers show less ground foraging and contrafreeloading (e.g. selectively pick up from the ground) behaviours than layers (Lindqvist et al., 2006). Such behavioural differences might have influenced the number of infectious eggs taken up from the pen environment by the three genotypes, and thus may explain different levels of secondary infection with *H. gallinarum*. The finding that host responses to experimental and naturally occurring infections differed in association with host genotype is an important outcome with practical implications. Furthermore, the divergent responses of the genotypes to experimentally induced or naturally occurring infection indicate that experimentally induced infection may not necessarily be representative of naturally occurring infection. This is particularly important when host genotypes with different body sizes and behavioural patterns are compared. Thus, we propose that the assessment of host genotypes in terms of parasite resistance should not be limited to experimental infections only, but also include naturally occurring reinfection. Other factors related to experimental designs when comparing genotypes of different body size are the environmental conditions and the infection dose. We compared fast and slower growing genotypes in an environment (i.e., diet, climatic conditions, space requirements etc.) fully designed for the fast growing genotype. Despite the large differences in body size, the three genotypes were also given the same infection dose. Therefore, we appreciate that the outcomes of the experiment may differ when genotypes are constrained in genotype-specific environments (e.g. fed on genotype-specific diets) or given an infection dose adjusted to body size (e.g. see Coltherd et al., 2009, 2011). As the growth performance was only impaired in the large size broiler birds, an adjusted infection dose would even imply a higher infection pressure on R birds. However, whether growth performance of the slower growing genotypes would also be impaired by the infections, if less a nutrient- and energy-dense diet was offered, needs to be clarified in further studies.

Our data collectively suggest that resistance and tolerance to mixed nematode infections are sensitive to growth rate in chickens. These differences amongst genotypes may be partly associated with a mismatch between the actual nutrient supply and genotype-specific nutrient requirements.

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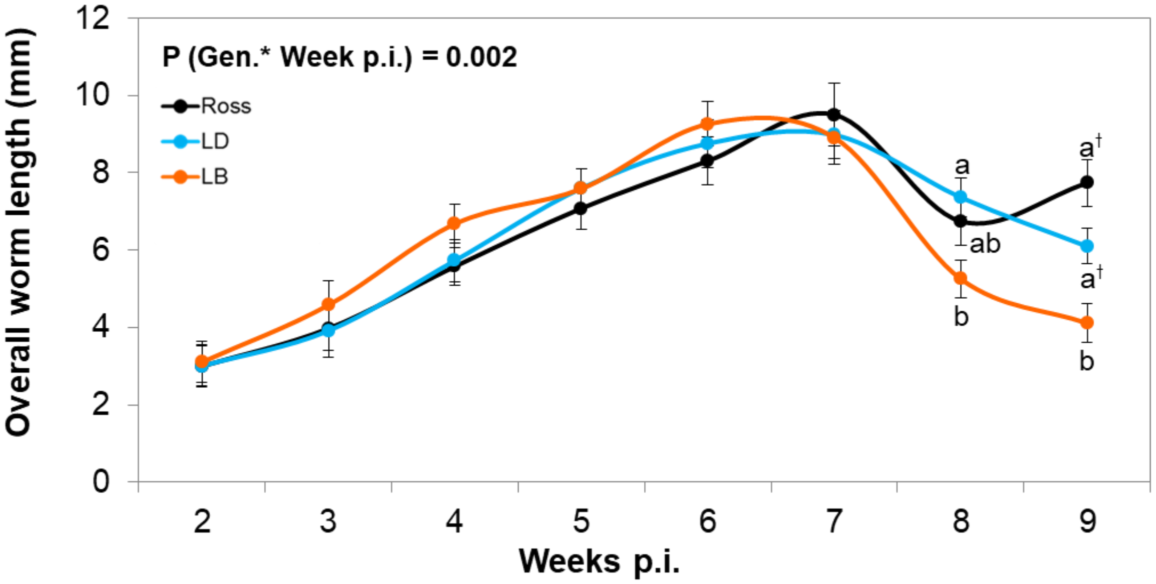
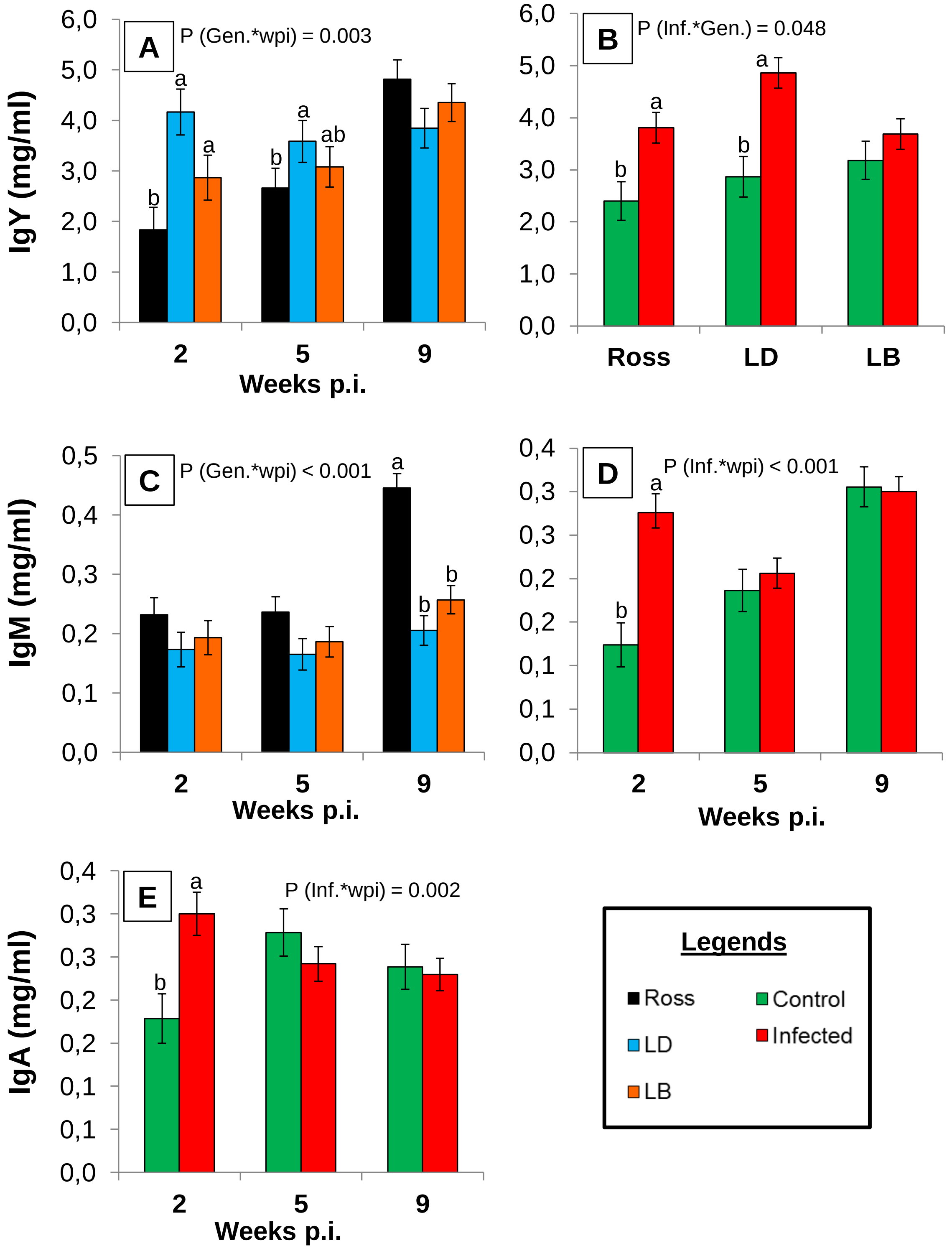
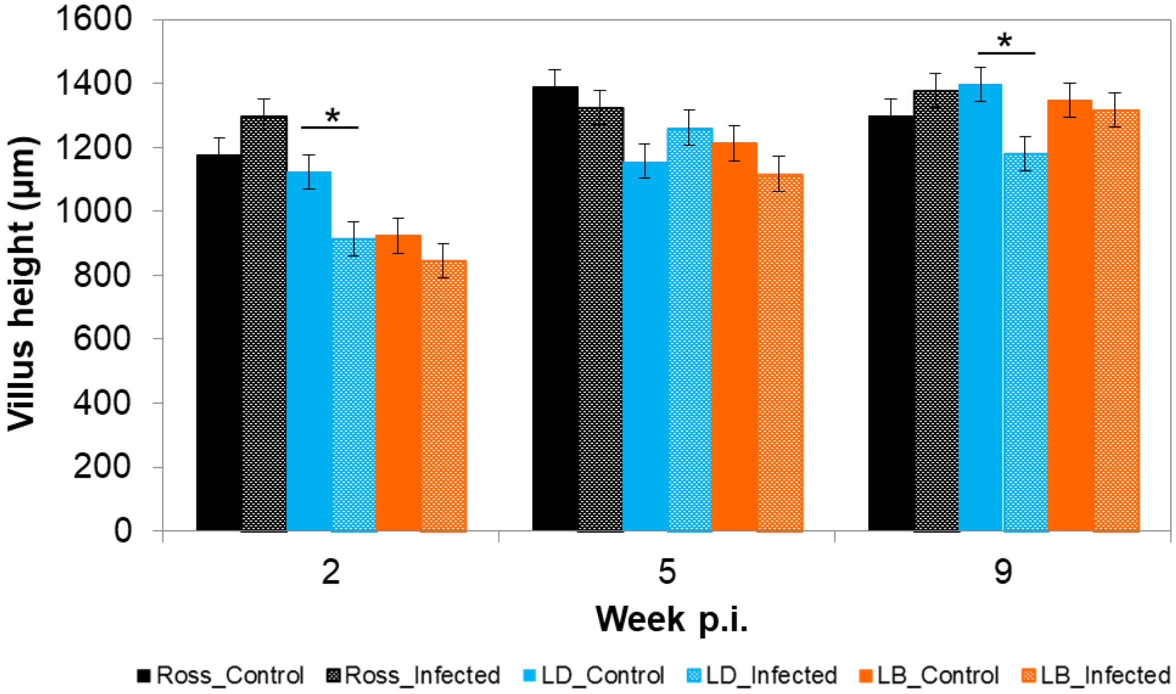
Supplementary Data



Supplementary Fig. S1. Changes in the overall average length of *Heterakis gallinarum* worms in relation to host genotype and time. Values with different letters for the same time point indicate significant (Tukey, P<0.05 following a significant interaction) differences. Values indicated with † show a tendency (P>0.10) between the two genotypes. Analyses are based on pooled data across spatial and temporal replications (sample size, N=304).



Supplementary Fig. S2. Interactions of plasma immunoglobulin concentrations (IgG, IgM, IgA) in relation to host genotype, mixed nematode infection and time. Values with different letters at the same time point show significant (Tukey, P<0.05 following a significant interaction) differences. Sample size, N=271 blood samples collected at 2, 5 and 9 wpi.



Supplementary Fig. S3. Changes in villus height in relation to host genotype, mixed nematode infection and time. Values indicated with * at the same time point show tendencies (Tukey, P<0.10 following a significant interaction). Tissue samples were collected from 18 birds (9 infected, 9 uninfected controls) at each sampling point (2, 5 and 9 wpi). Sample size: N=54 (18 birds x 3 time points).

Supplementary Table S1. Further biochemical parameters describing gastrointestinal environment in highly divergent chicken genotypes exposed to mixed nematode infections.

	Main effects of genotypes					**Infection effect**			
Item, µmol/g US	Ross	LD	LB	SE	P,≤	Con.	Inf.	SE	P,≤
Ileum									
Acetate	2.45	2.19	2.39	0.19	0.607	2.32	2.36	0.16	0.875
Propionate	0.12	0.07	0.12	0.02	0.168	0.10	0.11	0.02	0.677
Butyrate	0.02	0.006	0.006	0.005	0.227	0.01	0.01	0.005	0.439
Valeriate	0.01	0.02	0.02	0.002	0.226	0.02	0.01	0.002	0.035
Caecum									
Acetate	41.85	36.30	35.71	2.75	0.195	36.83	39.08	2.27	0.455
Propionate	5.35	2.97	3.58	0.84	0.093	3.50	4.44	0.69	0.306
Butyrate	8.31	9.95	9.77	1.02	0.422	9.88	8.80	0.84	0.336
Valeriate	1.53	1.18	1.02	0.18	0.120	1.28	1.20	0.15	0.673

Abbreviations: R: Ross-308; LD: Lohmann Dual; LB+: Lohmann Brown; Con; Uninfected controls; Inf.: Infection; Gen: Genotype; P,≤: P-value

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CHAPTER FOUR

Resistance and tolerance to mixed nematode infections in relation to performance level in laying hens

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Abstract

Modern layer type chickens have been genetically developed to perform high under optimal environmental conditions. We hypothesized that high-performance is associated with a higher sensitivity to environmental challenges in laying hens. By using the most common nematode infections as an environmental stressor, we assessed performance-level associated host responses in a high (i.e. Lohmann Brown Plus, LB) and in a lower performing, a so-called dual-purpose chicken genotype (i.e. Lohmann Dual, LD). The hens were infected with 1,000 eggs of *Ascaridia galli* and *Heterakis gallinarum* at 24 weeks of age and monitored for 18 post infection weeks (wpi). While infections had no significant effect on feed intake (P=0.130) and body weight in both genotypes (P=0.392), feed conversion efficiency was negatively affected by infections (P=0.017). Infections reduced both laying rate and egg weight and thereby per capita egg mass in both genotypes (P<0.05). While infected LB hens decreased significantly (P<0.05) laying rate in the early infection period (i.e. by 3 wpi), the decrease in LD hens appeared much later (i.e. by 14 wpi). Resistance to the experimental infection was not different between the genotypes for both worm species (P>0.05), whereas LB hens were more susceptible (P<0.05) to re-infections than LD hens. Changes in humoral immune responses (i.e. of ascarid-specific IgY antibodies in plasma and egg yolks) of the two genotypes over time reflected closely the corresponding changes in larval counts of the hens, descending from both experimental and naturally occurring infections in both genotypes. Infections caused a shift in egg size classes, leading to smaller frequency of larger eggs in both genotypes. Infections reduced egg weight (P=0.018) and led to a reduced fat content in the egg yolks (P=0.045). The proportion of polyunsaturated fatty acids (PUFA), especially n-6-PUFA, was also lower in the egg yolks of the infected hens (P=0.032). Our data collectively support the hypothesis that tolerance to nematode infections in laying hens is dependent on host-performance level. The impairment in host tolerance was both genotype and time dependent, likely due to differences in genetic programming for production peak and persistency of the two genotypes. Resistance to infections was dependent on the type of infection (i.e., experimentally vs. naturally re-infection). The two genotypes exhibited similar levels of resistance after a fully controlled experimental infection, but the high performing hens were more susceptible to naturally occurring re-infections. Infections negatively affected economically important egg-quality traits, including egg weight, fat content and fatty acid profiles in egg yolks.

Keywords: Egg production, Multi-species infection, Resistance, Susceptibility, Tolerance, Egg quality

1. Introduction

Farm animals have continuously been selected for increased production efficiency (Rauw et al., 1998). Comparing modern broiler genotypes with those used in 1957 their growth has been increased by about 400% accompanied by an improvement (50%) in feed conversion efficiency (Zuidhof et al., 2014). In the same time period, modern laying hens increased their productivity (i.e., laying rate) by about 230% (LTZ, 2015). About 85-90% of the improvement, in broilers for instance, is attributed to changes in genetics through genetic selection (Zuidhof et al., 2014). As a consequence of selection for increased production efficiency, high performance in chickens is yet already often associated with metabolic disorders (Rauw et al., 1998; Julian, 2005; Leeson, 2007), causing health and welfare problems in both broilers and laying hens. In layers disorders mainly include the skeletal system (i.e., osteoporosis; Rodenburg et al., 2008; Habig and Distl, 2013), reproductive system (i.e., inflammation of the fallopian tube; Grafl et al., 2017) as well as liver health (i.e., fatty liver syndrome; Shini et al., 2019). Since an increased persistency in lay performance by extending productive life of the hens is still a current breeding goal (Bain et al., 2016), even a higher physiological load, i.e., performance associated physiological pressure, may be expected for future hens. Animals being continuously under high physiological pressure often fail to mount effective immune responses against pathogenic stressors, likely due to a disturbed resource allocation (Rauw, 2012). Thus long-term selection for increased performance may have made laying hens more vulnerable to pathogenic and/or environmental challenges.

Modern layer type chicken genotypes have been selected for the performance of female birds only, whereas males are usually killed as day-old-birds for economic reasons as they exhibit a poor growth performance and feed utilization efficiency (Damme and Ristic, 2003; Kaufmann and Andersson, 2013). Only in Germany, approximately 46 million day-old male birds are killed annually (Destatis, 2017). Globally, there are 7.5 billion laying hens (FAO, 2016), implying the maximum number of male birds that might potentially be killed after hatch. The growing societal debate regarding ethical issues emerging from this practice demands suitable alternatives to culling (Leenstra et al., 2011; Bruijnis et al., 2015). Using chicken genotypes with commercially viable both egg and meat production potential, i.e. dual-purpose, through the use of both sexes may be such an alternative. Such an approach has indeed received growing attention mainly as an alternative to culling of male birds

(Krautwald-Junghanns et al., 2018). The use of dual-purpose genotypes may also mitigate the high-performance associated health and welfare problems in both broilers and laying hens. Although performance of both male and female birds of dual-purpose genotypes is lower than that of classical broilers and laying hens, respectively, the potential of health and welfare associated improvement (Giersberg et al., 2017; Harash et al., 2019) and a smaller dependency on the extremely high nutrient-dense diets (Urban et al., 2018a, b) may enable dual purpose genotypes to be adopted by the poultry industry.

Nematode infections of laying hens are highly prevalent in organic and free-range systems (Kaufmann et al., 2011a; Wongrak et al., 2014; Thapa et al., 2015; Wuthijaree et al., 2017). These infections have drastically re-emerged since the EU-wide ban of conventional cage systems. The infections are associated with mortality and welfare problems and likely contribute to economic losses in laying hens (Hinrichsen et al., 2016). The infections can directly impair host performance by affecting feed intake, feed conversion efficiency and growth (Daş et al., 2010, 2011, 2012), whereas effects of the infections on laying performance and egg quality traits have only rarely been examined. Several studies have demonstrated genotype-dependent differences in host resistance (ability to reduce pathogen burden) against nematode infections in laying hens (Permin and Ranvig, 2001; Kaufmann et al., 2011b; Wongrak et al., 2015). Host tolerance against nematodes, i.e. the ability to perform well despite infections, has to our knowledge not been studied comparatively in layer chickens, as it additionally requires performance measurements of the infected host. By using male birds of divergent chicken genotypes, we recently demonstrated that high-performing broilers are less tolerant to nematode infections than lower performing dual-purpose or layer-type genotypes (Stehr et al., 2019). Whether performance level is associated with an impaired tolerance in laying hens has yet to be clarified.

The impact of infections on host animal may be performance-level associated as high-performing genotypes have a higher production-pressure that may interfere or be traded-off with defense functions. Because the dual purpose genotypes are expected to perform lower than high-performing genotypes (Mueller et al., 2018; Siekmann et al., 2018), high-performance associated health and welfare problems maybe overcome through the use of dual-purpose genotypes. Thus we hypothesized that high-performance is associated with a higher sensitivity to environmental challenges in laying hens. We used nematode infections as an environmental stressor to compare a high performing genotype with the lower performing dual-purpose genotype so that performance-level associated host-responses in terms of tolerance and resistance to infections could be quantified. We also quantified alterations in basic external and internal quality traits of eggs in order to investigate whether basic egg quality parameters are affected by the infections in high and lower performing genotypes.

2. Materials and methods

2.1. Hens, experimental design and ethics

Hens (N=181) of a high performing layer genotype (Lohmann Brown Plus, LB; n=110) and of a dual-purpose genotype (Lohmann Dual, LD; n=71) were used in this study. The hens were obtained as 17 weeks-old pullets from a research farm (Farm for Education and Research in Ruthe, University of Veterinary Medicine Hannover). Following the arrival, hens of each genotype were randomly allocated to six pens. The hens received wing-tags to ensure hen-individual repeated-measurements over time. After the entry to laying period (i.e. laying rate >50%), hens in three pens per genotype were experimentally infected at an age of 24 weeks, while the remaining hens in other three pens were kept as uninfected controls. Starting from 2 weeks post infection (wpi) infected and uninfected hens of both genotypes were randomly collected from each pen and necropsied at timed intervals (i.e. 2, 4, 6, 10, 14 and 18 wpi) to quantify infection intensity with either nematode. Total number of hens necropsied at each wpi ranged from 29 to 34. The experimental design of the study was a 3-factorial arrangement of treatments (infection x genotype x wpi).

Ethical approval of the experiment was obtained from the relevant state ethics committee for animal experimentations (Mecklenburg-Western Pomerania State Office for Agriculture, Food Safety, and Fisheries, Germany; permission no.: AZ.: 7221.3-1-080/16). The experiment was conducted in accordance with animal welfare rules (animal care and handling, stunning, necropsies) and all sampling procedures were performed by trained/authorized staff. Experimental infection procedures were also in line with the relevant guidelines of the World Association for the Advancement of Veterinary Parasitology for Poultry (Yazwinsky et al., 2003).

2.2. Housing and management

The hens were kept in pens on wood shavings as litter material. The pens of infected and uninfected hens were in two separated rooms in a poultry research facility to avoid cross-contamination. On the day of infection (i.e. at 24 weeks of age) the litter was renewed, and thereafter it was not removed during the 18 week infection period to allow naturally occurring reinfections. Additional litter was added to all pens proportionally to ensure similar conditions for all genotypes and infection status in different pens. The climatic conditions in rooms were fully-controlled through an automatic system ensuring the same temperature, light and aeration conditions across the pens within and between rooms. At the beginning of the experiment stock density in all the pens was max. 6 hens per m^2. As the timed necropsies were performed, stock density decreased proportionally in all the pens throughout the post infection weeks. The beaks of the hens were kept intact, and the hens were provided with pecking stones placed in each pen. All hens were fed a commercial laying-hen diet that contained 11.2 MJ metabolizable energy, 170 g crude protein and 3.6 g Calcium per kg feed (i.e. as-fed basis). Feed and water were offered for ad libitum intake. Lighting (light: 14 h; light intensity: 10-15 lux) and temperature (18-20 °C) regimes were as suggested by the breeding company (LTZ, 2018). During the growing period (17 weeks), the hens had been subjected to a vaccination program that included immunization against major bacterial and viral diseases (e.g., Salmonella, ND, IB, etc.) as well as coccidiosis (Paracox 8) at recommended ages. During the experimental period hens received no further vaccinations or medical treatments, including anthelminthics. Eggs were collected daily from the nests in the mornings. The eggs were then weighed individually and average egg weight in each pen was determined. Furthermore, frequency of eggs falling into different egg-size classes (i.e., S < 53 g; M ≥ 53 and < 63 g; L ≥ 63 and < 73 g; XL > 73 g) according to the EU weight standards for "Class A" eggs (Anonymous, 2008) were determined. Individual body weight of hens and pen-based feed intake were measured at weekly intervals.

2.3. Experimental infection

The infection material was collected from worms that were isolated from intestines of naturally infected chickens (i.e. free range chickens). Preparation techniques, incubation conditions and the preparation of the final inoculum have been described in detail by Stehr et al. (2018). On the day of infection, the separately incubated eggs of *A. galli* and *H. gallinarum* were adjusted to a final dosage of 0.4 ml/hen containing 1,000 embryonated eggs in equal proportions. Hens to be infected were given the infection dose orally by using a 5-cm esophageal cannula, whereas uninfected control hens received a sham-oral treatment with the same amount (0.4 ml) of NaCl (0.9%) as a placebo.

2.4. Worm harvest and larval recovery

By starting 2 wpi, randomly selected hens (n= ca. 30 per wpi) were necropsied at wpi 2, 4, 6, 10, 14 and 18 to quantify worm burdens with both species. To exclude any potential confounding effects of accidental infections on performance and immune-related parameters, intestines of uninfected control hens were also subjected to parasitological examinations. All hens to be necropsied were fasted for 3 hours for a standardized emptying of gastrointestinal tract. Immediately post mortem, the gastrointestinal tract was removed and the small intestine and caeca were separated. The intestine was opened longitudinally and the intestinal content was washed through sieves (mesh size: 36 µm and 100 µm at 2-6 wpi and 10-18 wpi, respectively). Tissue-associated *A. galli* larvae were recovered by using a slightly modified EDTA-incubation method (Kringel et al., 2002; Katakam et al., 2010; Ferdushy et al., 2012). The procedures of the EDTA-incubation have been described by Stehr et al. (2018). Briefly, after removing the luminal contents, the intestinal tissue was squeezed through a pair of pencil-pincer under running lukewarm tap water to remove accidentally attached luminal worms. Immediately following this step, the washed tissue was hung into a preheated 400 ml EDTA-solution (10 mM EDTA, 0.9% NaCl) for an overnight incubation (> 22 hours at 40°C). Thereafter the EDTA-solution was passed through a 20 µm sieve to collect the tissue larvae.

H. gallinarum were harvested in the lumen contents only as described for *A. galli*, but the worms were collected on smaller mesh sized sieves (20-36 µm). Worms of both species collected from each host were then placed in petri dishes for counting, sex differentiation and length measurements using a stereo microscope. Uninfected control hens were also examined for the presence of worms in small intestines (tissue and lumen) and caeca to check for accidental infections with either nematode.

2.5. Worm population structure

The worms were classified into larvae, females and males based on morphology. While differentiation of female *H. gallinarum* for sexual maturity is easily performed under a stereo-microscope, the larger and thicker body of *A. galli* makes it difficult to differentiate eggs and egg-like structures in the uterus. Therefore a length cut-off (43.5 mm) was used to determine mature *A. galli* females as described (Stehr et al., 2018). All *Heterakis* worms until wpi 4 were considered to descend from the experimental infection, thus defined as first-generation worms. By wpi 5, first generation worms were calculated as the total worm burden minus immature worms as the latter must have descended from re-infections.

2.6. Faecal egg counts (FEC)

As no nematode egg excretion was expected at wpi 2, FECs were determined from wpi 4 to 18. Faecal samples were collected one day prior to necropsies. Uninfected control and infected hens were placed in individual cages to collect faecal samples from the hens (N=151). The daily total faeces was thoroughly mixed and a random sub-sample (2 g) was analyzed with the Mini-FLOTAC egg counting technique (Maurelli et al., 2014) using a saturated sodium chloride solution as the flotation liquid (density ≥ 1.2 g/ml). The minimum detection level of the Mini-FLOTAC technique was 10 eggs/g faeces. After quantification of nematode egg concentration in faeces (eggs per gram faeces, EPG), total number of eggs excreted within 24 h (eggs per day, EPD) from each host was then estimated by multiplying the amount of total daily faeces with the EPG. Eggs of *A. galli* and *H. gallinarum* were not differentiated, and counted together since a reliable differentiation cannot be made (Kaufmann, 1996).

2.7. ELISA for quantification of ascarid-specific antibodies in plasma and egg yolks

To quantify the development of ascarid-specific IgY antibodies in plasma, blood samples were repeatedly taken from individual hens at weekly intervals from wpi 0 to 18. For this purpose, 10 infected and 10 uninfected hens per genotype (i.e., N = 40 hens in total) were randomly selected at the beginning of the experiment (wpi 0). Blood (N=677) was collected from the wing vein (Vena cutanea ulnaris) into vials containing potassium-EDTA (Kabe Labortechnik GmbH, Nümbrecht-Elsenroth, Germany). For quantification of ascarid specific IgY in egg yolks (N=667), 10 eggs were randomly collected from the pens of infected and uninfected hens of each genotype at weekly intervals. On the sampling day, the eggs were opened and egg yolks were collected. A sub-sample of the egg yolks (250 µL) was diluted with 1.5 ml of purified water (pH = 2.5) and homogenised by using a vortexer. Plasma samples were centrifuged at 2,500 g for 20 min, and the supernatant was stored at -20°C for later analysis. Egg yolk samples were centrifuged at 12,000 g for 15 min. Ascarid-specific IgY levels in plasma and egg yolk samples were then determined with an ELISA as described (Daş et al., 2017).

2.8. External and internal egg quality

External (i.e., colour, thickness and weight of the egg shell; percentage weight of the egg shell; breaking strength; elasticity) and internal (i.e., yolk colour, weight and percentage of the egg yolk; height of the egg white; Haugh unit) egg quality traits were investigated on a total of 503 eggs collected at the time points 2, 4, 6, 10 and 14 wpi. The analyses were performed at the University of Hohenheim as described by Simons (2017), Grashorn et al. (2016) and Grashorn (2018). After weighing of the eggs, shell colour was measured according to CIE-

L*a*b* using the Chroma meter Minolta CR-300. Shell stability was determined in a compression test using an Instron model 5565 controlled by the software Bluehill series 3. Elasticity of the shell was determined under a load of 9.8 Newton (N) and maximum force to break the shell was recorded, thereafter. Head speed was 5 mm/min. Then, eggs were broken on a glass plate. Albumen was removed from the shells, whereas, membranes were left. Shells were dried for 24 h at 60 C° in a drying cabinet. Albumen height (mm) was determined 1 cm aside the yolk by using an Ames gauge. Yolk colour was measured both with the Minolta CR-300 (CIE-L*a*b*) and the the DSM Yolk Colour Fan (DSM-YCF, 2005; 15 colour blades) for an overall colour assessment. Yolks were grabbed from the plate by hand, albumen and chalazae were removed manually. Dried shells and yolks were weighed. Proportion of shells and yolks was determined by dividing weights through egg weights. Albumen proportion was calculated by subtraction method. Finally, Haugh units were calculated by the formula: HU = 100*log*(albumen height – 1.7*egg weight**0.37 + 7.6).

2.9. Fatty acid profiles in egg yolks

To characterize infection-induced alterations in fatty acid profiles of the egg yolks in each genotype, individual eggs were collected during captivity in the cages shortly before necropsy at wpi 2, 4, 6, 10, 14 and 18. The eggs (N=163) were opened and the egg yolk was separated from the albumen. In a further step, the egg yolk was carefully rolled on a filter paper to remove any albumen residues, ensuring a precise separation between the both egg components. The egg yolks were then stored at -20 °C until analyses.

After homogenization of frozen egg samples and the addition of C19:0 as an internal standard, total egg lipids were extracted in duplicate using chloroform/methanol (2:1, v/v) and the Ultra Turrax T25 (IKA, Staufen, Germany) at 3 x 15 sec, 15,777 g and room temperature. The detailed sample preparation procedure has been recently described by Kalbe et al (2019). Briefly, the final extraction mixtures were stored at 5°C for 18 h in the dark and subsequently washed with 0.02% CaCl2 solution. After centrifugation (2,500 rpm, 5 min), the organic phase was dried with Na2SO4 and K2CO3 (10:1, wt/wt), and the solvent was subsequently removed under gentle nitrogen stream at room temperature. The lipid extracts were dissolved in 150 µL of toluene for methyl ester preparation. Next, 1 ml of 0.5 M sodium methoxide in methanol was added to the samples, which were shaken in a 60ºC water bath for 10 minutes. Subsequently, 0.5 ml of 14% boron trifluoride (BF3) in methanol was added to the mixture, which was then shaken for an additional 10 minutes at 60ºC. The fatty acid methyl esters

(FAMEs) were extracted three times in 2 ml of n-hexane. The FAMEs were re-suspended in 100 µl of n-hexane and stored at -18 ºC until used for gas chromatography (GC) analysis.

The fatty acid analysis of egg lipids was performed using capillary GC with a CP-Sil 88 CB column (100 m x 0.25 mm, Agilent, Santa Clara, CA, United States) that was installed in a PerkinElmer gas chromatograph CLARUS 680 with a flame ionisation detector and split injection (PerkinElmer Instruments, Shelton, United States). The detailed GC conditions were described by Herdmann et al. 2010. Briefly, the initial oven temperature was 150°C, which was held for 5 min; subsequently, the temperature was increased to 175 °C and then to 200 °C at a rate of 2 °C min-1 and held for 10 min. Finally, the temperature was increased to 225 °C at a rate of 1.5°C min-1 and held for 25 minutes. Hydrogen was used as the carrier gas at a flow rate of 1 mL min-1. The split ratio was 1:20, and the injector and detector were set at 260°C and 280°C, respectively. The quantification of fatty acids was done by the use of C19:0 as internal standard. For the calibration procedure the reference standard mixture 'Sigma FAME' (Sigma-Aldrich, Deisenhofen, Germany), the methyl ester of C18:1cis-11, C22:5n-3 and C18:2cis-9,trans-11 (Matreya, PA, USA), C22:4n-6 (Sigma-Aldrich, Deisenhofen, Germany) and C18:4n-3 (Larodan, Limhamn, Sweden) were used. The five-point calibration of single fatty acids ranged between 16 and 415 mg/mL and was checked after GC analysis of five samples. Groups of saturated-, monounsaturated- and polyunsaturated fatty acids were then summarized. Sum of saturated fatty acids (SFA) included the sum of C10:0, C11:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C23:0. Sum MUFA (monounsaturated fatty acids) were calculated as the sum of C14:1cis-9, C16:1cis-9, C17:1cis-9, C18:1cis-9, C18:1cis-11, Sum C18:1trans, C20:1cis-11, and sum PUFA (polyunsaturated fatty acids) included the sum of C18:2n-6, C18:2cis-9,trans-11, C18:3n-3, C18:3n-6, C18:4n-3, C20:2n-6, C20:3n-6, C20:4n-6, C22:5n-6, C22:4n-6, C22:5n-3, C22:6n-3.

2.10. Statistical analyses

Data were modelled differently by considering experimental units (e.g. a hen or pen), measurement intervals (daily or weekly) and repetition over the same experimental unit and time (single or repeated measurement) for each variable. For all individually measured parameters (e.g. worm burden, body weight) the experimental unit was a hen, whilst it was a pen for the variables measured at pen level (e.g. feed intake, laying rate of all animals in a pen). Pens were considered as blocks or replicate for the statistical analysis of individual (e.g. worm burden) or pen-based (e.g. feed intake) variables, respectively. Nematode-free control hens were excluded from the analyses of the worm burden and FEC data. Worm burden, FEC and ascarid-specific antibody data were analysed following a log transformation [Ln (y +1)] to correct for heterogeneity of variance and produce approximately normally distributed data. For the analysis of fatty acid profiles of the egg yolks, percentage of individual or groups of fatty acids in the total fat in the egg yolk was used as the raw data.

All data were then subjected to analysis of variance by using the MIXED procedure in the SAS/STAT (Version 9.4) software of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA). The statistical model for worm burden and FEC included fixed effects of host genotype, wpi and their interaction, plus pen effects. As individual body weight, egg quality data (external and internal egg quality, egg yolk fatty acids) and antibody data were also available for the uninfected control hens, the statistical model for such variables included the effects of infection, genotype, wpi and their interactions, plus pen effect. The model for daily measured pen data (laying performance, egg weight and per capita egg mass production) were analyzed with repeated measures ANOVA including the effects of genotype, infection, wpi, all possible interactions among these three factors and the effect of day within a week. Body weight and plasma ascarid-antibody data, which were measured repeatedly from the same individual hen over the experimental weeks, were also analysed using repeated measures ANOVA including the effects of genotype, infection, wpi, all possible interactions among these three factors and plus pen effects. Egg yolk ascarid-antibody levels, which were measured on randomly selected eggs, were analyzed using a statistical model including the effects of infection, genotype, wpi, their interactions and pen effect.

The effects of repeatedly sampled hen or pen (subject) over time were accounted for in the relevant models with the inclusion of REPEATED statement in the MIXED procedure. The structure of the block diagonal residual covariance matrix was set to AR(1) as this setting provided the best fit of the parameters (e.g., smallest AIC) for the fitted models. In addition, to account for the decreasing number of hens in each pen over time (because of necropsies), the models for the pen-based data included the WEIGHT statement. Least-squares means (LSM) and their standard errors (SE) were computed for each fixed effect in the model, and all pairwise differences in these LSMs were tested with the Tukey-Kramer test, a procedure for pairwise multiple comparisons. In addition, the SLICE statement of the MIXED procedure was used for performing partitioned analyses of the LSMs for the two- or three-way interactions (e.g., test of infection within the levels of wpi in each genotype). Effects and differences were considered significant at $p<0.05$.

3. Results

3.1. Host performance

Out of 181 hens, only one infected LD hen died throughout the experimental period. The overall laying rate of LD hens (78.6%) was significantly lower ($P<0.001$; Table 1) compared with that of LB hens (93.4%). On average, infections impaired laying rate in both genotypes by 6.8% ($P<0.001$). Infection-induced effects on laying rate of the two genotypes were however time dependent. As indicated by a significant interaction between infection, genotype and wpi ($P=0.010$; Fig. 1A), LB hens responded with an impaired laying rate already in the early phase of infection (3-5 wpi; $P<0.05$), whereas infected LD hens laid less number of eggs than their uninfected control counterparts in the advanced infection period (i.e. by 14 wpi; Fig. 1). Infected hens had also a lower egg weight than did the uninfected controls in both genotypes ($P=0.018$; Table 1). The overall average egg weight was lower ($P<0.001$) in LD than in LB hens during the first 15 wpi, but thereafter no significant difference was quantified (Fig. 2). LB hens had a greater daily egg mass production (DEM) than LD hens, ($P<0.001$; Table 1), and infections reduced ($P<0.001$) egg mass production in both genotypes by about 9%, though in a time-dependent manner ($P=0.008$; Fig. 1B). In line with laying rate, infections in LB hens reduced DEM significantly from 3 to 5 wpi. In the following DEM increased again, but was still lower than that of controls, even if statistically not significant ($P>0.05$). In LD hens DEM was not different between controls and infected hens until wpi 14, whereas it decreased thereafter significantly in the infected hens.

The infection-induced decrease in the egg weight of both genotypes resulted in an apparent shift in the frequency of eggs in different egg-size classes (Supplementary Figure S1A). On average, infected hens laid less frequently XL (0.9 vs. 3.5%) but more S-size eggs (13.3 vs. 10.7%). The shift in the frequency of eggs from larger to smaller egg-size classes was apparent in both genotypes with their own distribution patterns over the experimental weeks (Supplementary Figure S1B). With progressing time (i.e. wpi), infected LB hens laid higher number of M-size eggs at the expense of XL eggs. Similarly, infected LD hens laid S-size eggs more frequently while decreasing frequency of L-size eggs (Supplementary Figure S1B).

Table 1. Effects of host-genotype and mixed-nematode infections on main performance parameters in laying hens.

	Genotype				**Infection**				**Interactions, P-Value, ≤**				
Item	**LD**	**LB**	SE	P,≤	**Con.**	**Inf.**	SE	P,≤	wpi	Inf*G.	Inf*wpi	G*wpi	Inf*G*wpi
Laying rate, %	78.6	93.4	0.645	0.001	89.4	82.6	0.637	0.001	0.001	0.720	0.161	0.001	0.010
Egg weight, g	58.3	63.2	0.175	0.001	61.1	60.4	0.171	0.018	0.001	0.732	0.901	0.001	0.800
Daily egg mass, g/hen	45.5	59.0	0.398	0.001	54.7	49.8	0.390	0.001	0.001	0.990	0.016	0.001	0.008
Feed intake, g/day	93.2	122.4	1.671	0.001	109.6	105.9	1.625	0.130	0.001	0.626	0.168	0.428	0.505
FCR, g/g	2.06	2.09	0.023	0.394	2.03	2.12	0.022	0.017	0.002	0.231	0.147	0.004	0.545
Body weight, g/hen	1,738	1,891	28.55	0.001	1,830	1,799	28.32	0.392	0.001	0.251	0.001	0.198	0.258

Laying performance (N=1512) and egg weight (N=1490) and per capita egg mass production (N= 1511) were determined at daily intervals on pen level. Feed intake (N=206) and feed conversion ratio (i.e. FCR, N=196) were determined at weekly intervals at pen level. Body weight was measured individually at weekly intervals (N=1831).

Abbreviations: LD: Lohmann Dual; LB: Lohmann Brown Plus; Con.: Control birds; Inf.: Infected birds; wpi: Week post infection; I.: Infection group; G: Genotype; FCR: feed conversion ratio (g feed intake/ g egg mass)

LB hens consumed a significantly higher amount of feed than LD hens (P<0.001; Table 1). Average daily feed intake of infected hens was about 3.4% less than that of un-infected hens, although this difference was not significant (P=0.130; Table 1). Overall feed conversion ratio (FCR, i.e., feed / egg mass) was similar between the two genotypes (P=0.394), although time dependent genotype differences were also observed (P=0.004). The FCR was lower in LD than in LB hens from wpi 1 to 6 (P<0.01), whereas it was lower in LB hens from wpi 14 to 16 than in LD hens (P<0.05; data not shown). Infections impaired the feed conversion efficiency (P=0.017) in both genotypes across the whole experimental period.

LB hens had higher body weights than LD hens (P<0.001; Table 1). Infections had no effect on body weight (P=0.564). Although body weights of both genotypes changed over time (P<0.001), there was no significant interaction between the effects of genotype and infection (P=0.251). A triple interaction indicating time dependent infection effects on body weights in any genotype was also absent (P=0.258).

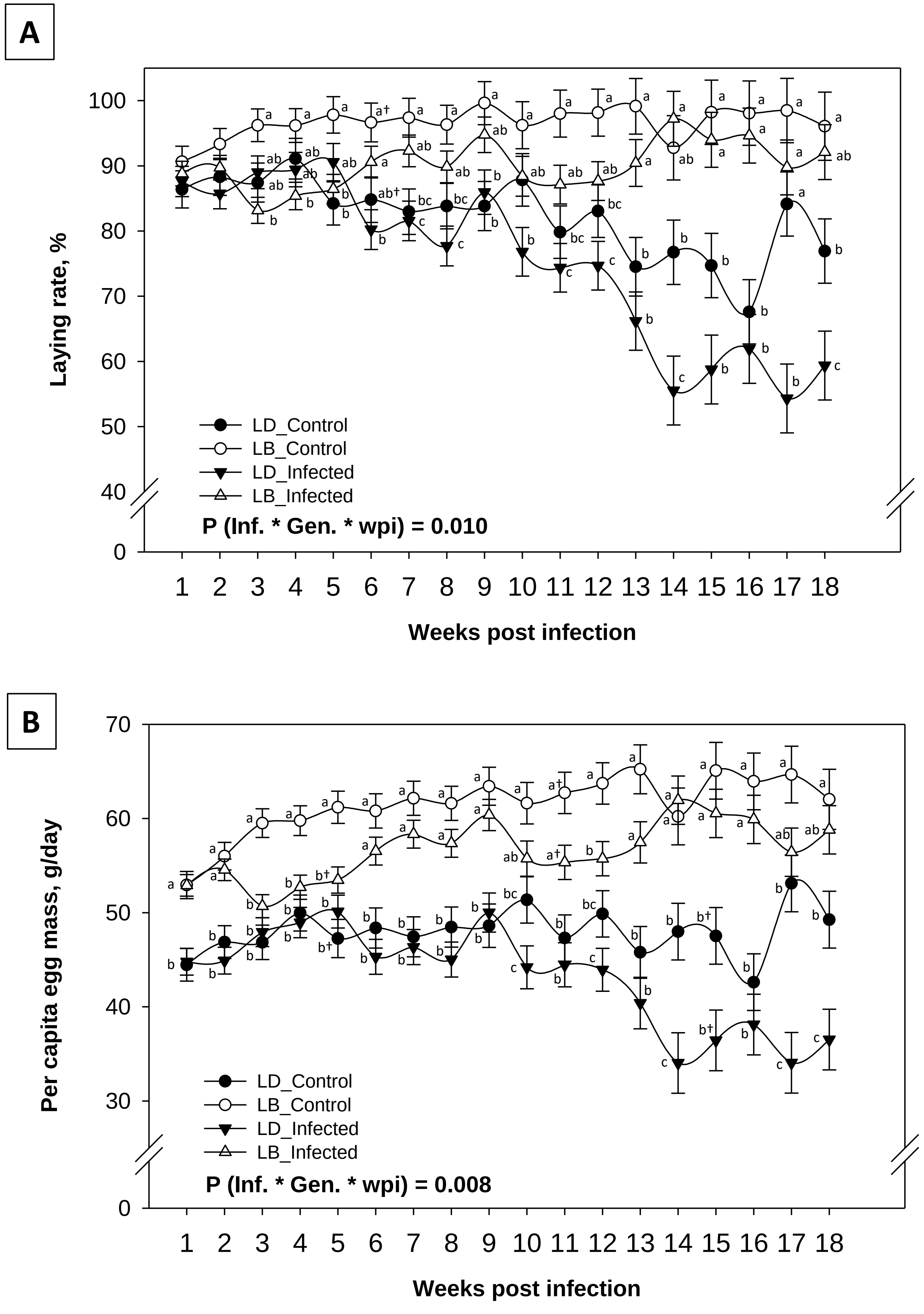
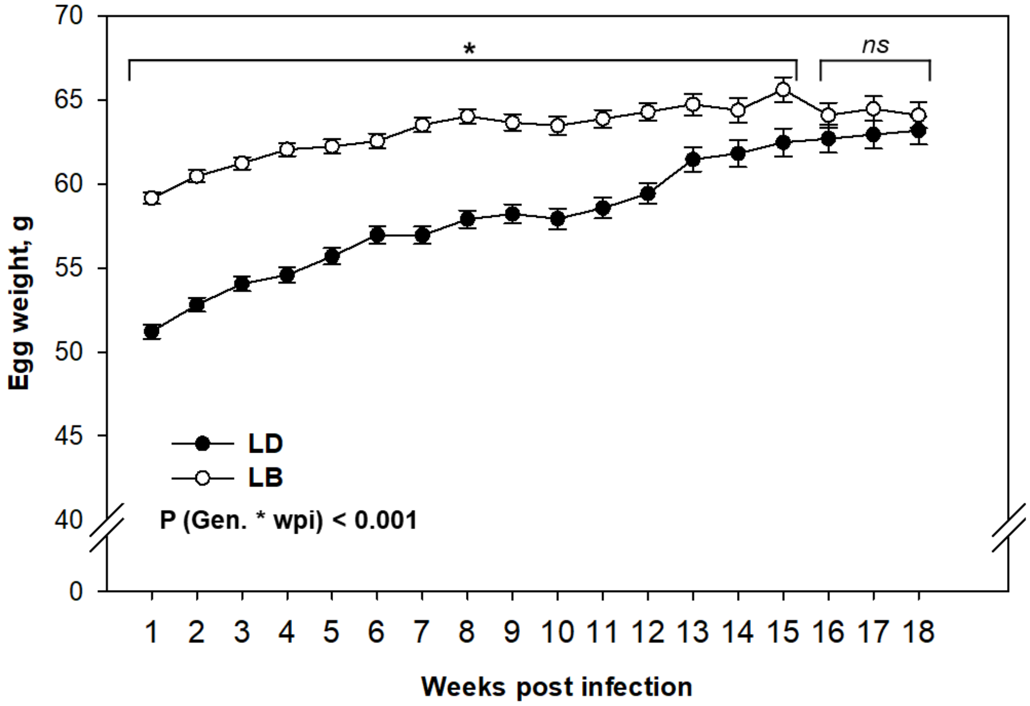


Fig. 1. Laying rate (**A**) and per capita egg mass production (**B**) in high (LB) or lower (LD) performing laying hens exposed to mixed nematode infections.



*: Indicates significant differences (Tukey, p<0.05) between two genotypes until wpi 15
ns: no significant difference (Tukey, p>0.05) between two genotypes by wpi 16.

Fig. 2 Time-dependent alterations in egg weight in a high (LB) or lower (LD) performing host genotype.

3.2. Faecal egg counts (FEC)

All faecal samples from control hens were negative for the presence of nematode eggs. For infected hens, the first positive faecal samples were found at wpi 4. The two genotypes tended to differ in EPG (P=0.058; Table 2), with LD hens having a numerically higher EPG than did LB at wpi 4 only (P=0.066; data not shown). Similar to EPG, the overall EPD did not differ between the genotypes (P=0.165), but a significantly higher (P=0.026) number of nematode eggs was excreted through the total faeces of LD hens than those of LB hens within a day (i.e. EPD) at wpi 4 only.

3.3. Worm burdens

The overall average *A. galli* counts were not significantly different between the two genotypes (P=0.112; Table 2). Contrarily, both lumen and tissue larva counts as well as total *A. galli* larval counts were higher in LB than in LD hens (P≤0.002). Non-larval worm counts were not different between the genotypes (P=0.529). The percentage of tissue larvae in the small intestine was not different between the two genotypes (P=0.967). The overall average *A. galli* burden across the two genotypes decreased (P<0.001) over time from 31±4.2 (MEANS ± SE) worms/hen at 2 wpi to 9±1.5 worms/hen at 18 wpi (P=0.001). Similarly, *A. galli* larval counts continuously decreased over the experimental weeks (P<0.001). Apparent re-infections with this worm species were low at the end of the 18-week study period (0.6 and 2.2 larvae/hen in LD and LB, respectively).

Table 2. Overall average faecal egg counts and worm burdens in two laying hen genotypes exposed to mixed-nematode infections.

	Host genotype			**P-Values, ≤**		
Item	LD	LB	SE	Gen.	wpi	Gen.*wpi
FEC						
EPG, n/bird	576	545	103.8	0.058	0.001	0.066
EPD, n/bird	46,388	90,669	15,185	0.165	0.001	0.026
A. galli						
Total burden, n/bird	11.3	15.0	1.76	0.110	0.001	0.267
Total larva, n/bird	3.1	8.3	1.25	0.001	0.001	0.106
Non-larva worms*, n/bird	6.8	5.9	1.30	0.529	0.001	0.110
Lumen larva, n/bird	2.5	6.2	0.96	0.001	0.001	0.110
Tissue larva, n/bird	0.6	2.1	0.45	0.002	0.001	0.123
Tissue larva, %	15.7	15.1	14.84	0.967	0.758	0.334
H. gallinarum						
Total burden, n/bird	131.7	141.0	16.15	0.453	0.305	0.293
First generation, n/bird	131.6	137.6	16.11	0.563	0.236	0.400
Non-larva worms*, n/bird	100.9	104.4	12.31	0.415	0.001	0.346
Total larva, n/bird	30.6	36.2	10.26	0.001	0.001	0.001
Larva 1st Gen.*, n/bird	93.7	100.4	31.88	0.534	0.001	0.816
Larva 2nd Gen.*, n/bird	0.1	4.2	0.93	0.001	0.001	0.001

Values for all count variables are least-squares means and their standard errors (SE). P-values are based on analysis of log-transformed data.

Worm burdens were determined at wpi 2, 4, 6, 10, 14 and 18. Sample size for worm burden data was N = 108. Faeces samples were collected by starting wpi 4 one day prior to necropsies. Sample size for FEC data was N=91 (excludes samples from uninfected controls).

* Non-larva worms: defined as worm burden minus larvae, and included mature and immature worms that are sexually differentiable by morphological characteristics.

* Larva 1st Gen.: defined as larval burden until 4 wpi.

* Larva 2nd Gen.: defined as larval burden by 5 wpi.

Abbreviations: FEC: faecal egg counts; EPG: number of eggs per gram faeces; EPD: number of eggs excreted within 24h; LD: Lohmann Dual; LB: Lohmann Brown Plus; wpi: Weeks post infection; Gen.: Genotype

The overall average total *H. gallinarum* burden was not different between the LD and LB hens (P=0.410; Table 2). Despite a numerical decrease of approximately 50% over time (i.e. 187±36 worms / hen at wpi 2 to 96±14 worms / hen at wpi 10), *H. gallinarum* burden did not change significantly over the experimental weeks (P=0.262). While the number of first-generation *H. gallinarum* larvae did not differ between the two genotypes (P=0.654), there was a significant difference in the number of larvae originating from re-infections (P<0.001). The LB hens had increasingly higher number of second-generation larva than LD hens by wpi 14 (Fig. 3). Non-larva worm counts were not significantly different between the genotypes (P=0.413).

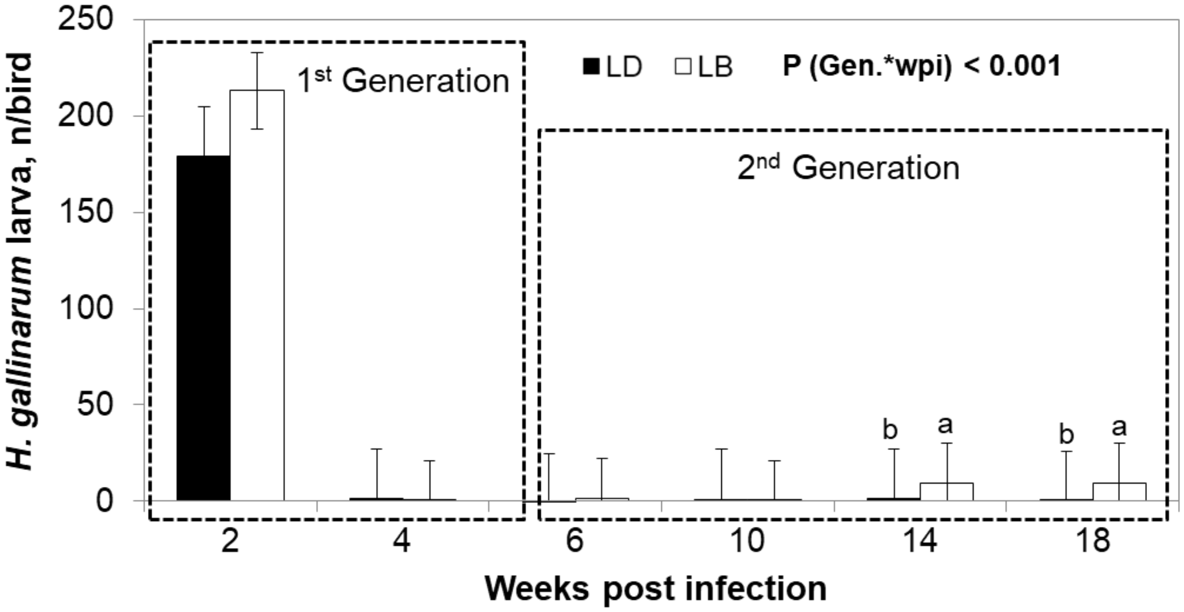


Fig. 3. *Heterakis gallinarum* larvae in high or lower performing host-genotypes after experimentally induced and naturally-occurring (re)-infections.

3.4. Ascarid-specific IgY antibodies in plasma

Overall average plasma ascarid-specific IgY levels were significantly (P<0.001) higher in LB than in LD hens (73.1 vs. 26.9 mU/mL ± 17.17). Infections increased (P<0.001) the IgY levels in both genotypes (22.0 vs. 78.0 mU/mL for controls and infected hens, respectively). The infection-induced increase in the ascarid-specific IgY levels were however both time- and genotype-dependent (P=0.003; Fig. 4A). As response to experimental infections, both LB and LD hens showed very similar patterns of ascarids-specific antibody development at the beginning of the experiment, though at different levels. Infected LB hens showed a second and stronger increase also in the end of the experiment, which was delayed and less pronounced in the infected LD hens. As shown in Fig. 4A, infected LB hens had higher IgY than the uninfected controls for the first time by wpi 2 (P<0.05) with a peak occurring at wpi 3 (P<0.001). Thereafter, IgY levels of infected LB hens decreased (P<0.05) until wpi 6 and remained fairly constant at a low level until wpi 10. During this period of time (i.e. wpi 6-10) IgY levels were not different between infected and control hens (P>0.05). By wpi 11 the IgY levels of infected LB hens increased more notably, with a plateau phase from wpi 14 to 15 followed by a decrease. During this second phase (wpi 11 to 18), IgY levels were continuously higher in infected than in control LB hens (P<0.001). In contrast to LB hens, infections increased IgY levels in LD only at wpi 2 and 3 (P<0.001). From wpi 4 to 16 IgY levels of infected LD hens remained at a low level, which was not significantly different from that of controls (P>0.05). By wpi 17 IgY levels increased again slowly in the infected LD hens (P<0.05). Infected hens of LB tended to have higher plasma IgY levels than LD hens by wpi 3 (P=0.054), whereas in the following time period until wpi 10 no differences were found (P>0.05). By 11 wpi IgY levels increased again in infected LB hens (P<0.001), being continuously higher than in the infected LD hens (P<0.001).

3.5. Ascarid-specific IgY antibodies in egg yolks

Development of ascarid-specific IgY antibody levels in egg yolks was highly similar to the overall pattern observed for the same antibodies in the plasma (Fig. 4A vs Fig.4B). Overall average ascarid-specific IgY levels in egg yolks were significantly (P<0.001) higher in the LB than in LD hens (40.0 vs. 11.9 mU/mL). Infections increased ascarid specific IgY in egg yolks (7.1 vs. 44.8 mU/mL for controls and infected hens, respectively) (P<0.001), although an interaction between infection, genotype and time effect (P<0.001) implied significant changes over the weeks. Infections increased IgY in egg yolks firstly by wpi 3 (P<0.001) in both genotypes (Fig. 4B). Thereafter IgY levels of infected hens behaved differently depending on host genotype. Although egg yolk IgY levels of infected LB hens decreased significantly from wpi 3 to 10 (P<0.001), IgY levels were continuously higher in infected than control LB hens (P<0.05) during this time period, with the exception of wpi 10 (P=0.058; not indicated in Fig. 4B). By wpi 11 IgY levels of the egg yolk in the infected LB hens increased strongly until wpi 15 (P<0.001) and thereafter decreased in the following weeks, which was still significantly higher than that of uninfected controls of the same genotype. Contrarily, IgY in egg yolks of LD hens were only higher in infected hens than in controls from wpi 3 to 5 (P<0.001). Infected LD hens showed slightly increasing levels of IgY by wpi 16, but the increase was not significant when compared with that of their uninfected control counterparts. Egg yolk IgY of infected hens were not different between the two genotypes until wpi 10 (P<0.05), although infected LB hens had temporarily higher IgY in egg yolks than LD at wpi 9 (P>0.05). By wpi 11 IgY levels of infected LB hens increased again, while IgY in egg yolks of infected LD hens remained at to the level of controls (P>0.05).

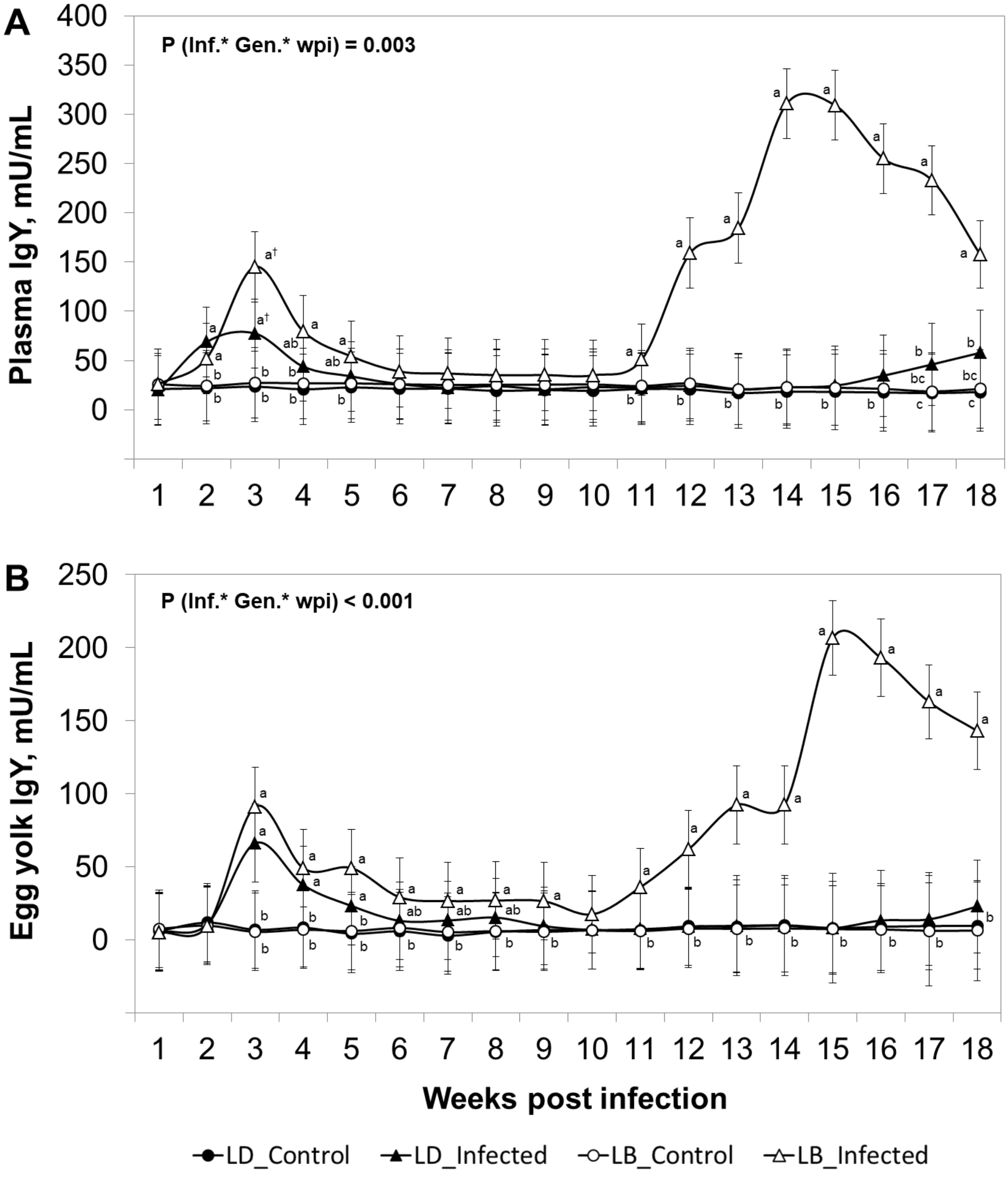


Fig. 4. Development of ascarid-specific IgY in plasma (**A**) and egg yolks (**B**) as response to mixed *Ascaridia galli* and *Heterakis gallinarum* infections in two chicken genotypes with different performance levels.

3.6. External and internal egg quality

With the exceptions of the elasticity and the percentage weight of egg shell ($P>0.05$), hen genotype significantly affected all external and internal egg quality traits ($P<0.001$; Table 3). LB hens had heavier egg yolks than LD hens ($P<0.001$), but the proportion of yolk to egg weight was higher ($P<0.001$) in LD than in LB hens. Egg shells of LB hens were thicker ($P<0.001$) and more resistant ($P<0.001$) to breaking than those of LD hens. Additionally, egg shells of LD hens were significantly lighter and had lower redness (a*) and yellowness values (b*) than that of LB hens. Albumen height and Haugh units were higher in LD than in LB eggs. Yolk color, measured with the DSM Yolk Colour Fan (DSM-YCF), was slightly darker in LB than LD eggs, being in line with a higher redness value for egg yolks of LB. Infections had no effect on most egg quality traits ($P>0.05$). A significant interaction between infection and genotype ($P=0.024$) for the DSM-YCF-scale indicated lighter egg yolk colors in infected LB hens than in their uninfected counterparts, whereas no effect was observed in LD hens. The redness values (a*) were significantly lower in infected than in controls of LB ($P<0.001$), but vice versa for LD hens ($P<0.05$). A significant interaction between infection, genotype and time ($P=0.044$) indicated lower b* values (yellowness) for yolk color in the infected LD hens than in controls only at wpi 6 ($P<0.01$), whereas b* values were not affected by infections in LB hens ($P>0.05$).

3.7. Egg yolk fatty acid profiles

The two genotypes differed greatly in egg yolk fatty acid (FA) profiles. The percentage of saturated FA (SFA) was significantly higher in the egg yolks of the LD than that of LB hens ($P<0.001$; Table 4) at wpi 10, 14, 18 ($P<0.01$; data not shown). Although LD hens tended to have a higher percentage of SFA in egg yolks at wpi 6 ($P=0.054$), no significant differences were observed between LD and LB in the preceding weeks (i.e. wpi 2 and 4). The LB hens had a higher ($P<0.001$) percentage of monounsaturated FA (MUFA) than LD hens in the egg yolks, whereas percentage of polyunsaturated FA (PUFA) was higher in LD than in LB hens ($P<0.001$). The differences in percentage of PUFA were mainly due to the lower percentage of n-6 PUFA ($P<0.001$) in the egg yolks of LB hens. The n-3 PUFA partly contributed to the genotype differences in total PUFA as indicated by a significant interaction between genotype and time effects ($P<0.001$). The n-3 PUFA was significantly higher in LD than in LB at wpi 2 and 6 ($P<0.05$), whereas egg yolks of LB hens had a higher proportion of n-3 PUFA at wpi 18. ($P<0.001$; data not shown). The fat content of the egg yolks was not different between the two genotypes ($P=0.230$), whereas infections reduced the fat content significantly (5.4 vs.

5.7g; P=0.045), mainly due to the impact induced at 2 wpi (P<0.001; data not shown). The proportion of MUFA tended to be higher in infected hens (P=0.067). The infections had no effect on the proportion of SFA (P=0.646), but reduced the proportion of PUFA in the egg yolks (P=0.032), accompanied by a lower proportion of n-6 PUFA (P=0.032) but not n-3 PUFA (P=0.525; Table 4). Infection- and genotype-dependent changes in individual FA of the egg yolks are summarized in Supplementary Table S1.

Table 3. External and internal egg quality traits in relation to host genotype and infection effects.

	Main genotype effect				**Infection effect**				**Interactions, P-Value, ≤**				
	LD	**LB**	SE	P,≤	**Con.**	**Inf.**	SE	P,≤	wpi	I.*Gen	I.*wpi	Gen*wpi	I.*Gen*wpi
Albumen height, mm	6.31	5.81	0.09	0.001	6.14	5.99	0.08	0.185	0.002	0.499	0.966	0.961	0.817
Yolk weight, g	14.6	15.4	0.14	0.001	15.1	14.8	0.13	0.095	0.001	0.319	0.320	0.799	0.694
Yolk proportion, %	25.9	24.5	0.20	0.001	25.3	25.0	0.19	0.158	0.001	0.491	0.379	0.989	0.366
Egg shell weight, g	5.82	6.45	0.05	0.001	6.12	6.15	0.05	0.579	0.001	0.627	0.936	0.147	0.560
Egg shell thickness, µm	408.0	423.1	2.37	0.001	414.6	416.5	2.21	0.513	0.121	0.556	0.566	0.170	0.635
Egg shell proportion, %	10.3	10.4	0.07	0.752	10.3	10.4	0.06	0.075	0.057	0.959	0.468	0.133	0.127
Breaking strength, N	42.5	47.1	0.86	0.001	44.7	44.9	0.80	0.791	0.010	0.292	0.166	0.248	0.472
Elasticity, mm	0.05	0.04	0.01	0.377	0.04	0.05	0.01	0.868	0.001	0.736	0.845	0.454	0.442
Haugh Unit	79.8	73.7	0.65	0.001	77.0	76.5	0.61	0.459	0.001	0.368	0.951	0.935	0.857
Shell color													
L*	65.5	55.1	0.29	0.001	60.4	62.2	0.27	0.582	0.001	0.896	0.432	0.561	0.135
a*	16.3	22.6	0.20	0.001	19.4	19.5	0.19	0.535	0.006	0.925	0.237	0.623	0.083
b*	27.2	29.3	0.29	0.001	28.3	28.2	0.27	0.849	0.779	0.659	0.059	0.181	0.115
Yolk color													
DSM-YCF[1]	13.2	13.6	0.05	0.001	13.5	13.4	0.04	0.060	0.001	0.024	0.441	0.334	0.742
L*	55.3	56.3	0.20	0.001	55.8	55.7	0.18	0.745	0.045	0.452	0.423	0.601	0.979
a*	7.92	8.90	0.11	0.001	8.45	8.37	0.10	0.500	0.001	0.001	0.400	0.288	0.496
b*	36.6	36.8	0.29	0.669	37.0	36.3	0.27	0.050	0.080	0.924	0.230	0.147	0.044

Eggs were collected at the wpi 2, 4, 6, 10 and 14 (N=503).

[1] Measured with the DSM Yolk Colour Fan (DSM-YCF) scale.

Abbreviations: LD: Lohmann Dual; LB: Lohmann Brown Plus; Con.: Control birds; Inf.: Infected birds; wpi: Week p.i.; I: Infection group; Gen.: Genotype; L*: Lightness; a*: redness; b*: yellowness.

Table 4. Effects of host genotype and mixed-nematode infections on fatty acid profiles and fat contents of egg yolks.

	Host genotype				**Infection**				**Interactions, P-Value, ≤**				
Item	**LD**	**LB**	SE	P,≤	**Con.**	**Inf.**	SE	P,≤	wpi	I.*Gen	I.*wpi	Gen*wpi	I.*Gen*wpi
SFA, %	34.38	33.28	0.131	0.001	33.79	33.87	0.126	0.646	0.001	0.288	0.608	0.006	0.985
MUFA, %	42.27	45.36	0.256	0.001	43.52	44.11	0.247	0.067	0.001	0.507	0.754	0.868	0.942
PUFA, %	23.35	21.36	0.258	0.001	22.69	22.02	0.249	0.040	0.001	0.231	0.654	0.115	0.916
n-3 PUFA, %	1.76	1.71	0.026	0.103	1.74	1.72	0.025	0.525	0.001	0.836	0.076	0.001	0.148
n-6 PUFA, %	21.52	19.59	0.239	0.001	20.88	20.23	0.231	0.032	0.002	0.203	0.567	0.169	0.958
Fat content, g	5.62	5.46	0.105	0.230	5.67	5.41	0.102	0.045	0.006	0.989	0.011	0.343	0.617

Egg yolk samples were collected at the wpi 2, 4, 6, 10, 14 and 18 (N=163).

Abbreviations: SFA: saturated fatty acids; MUFA: mono-unsaturated fatty acids; PUFA: poly-unsaturated fatty acids; n-3 PUFA: omega-3-PUFA; n-6 PUFA: omega-6 PUFA; LD: Lohmann Dual; LB: Lohmann Brown Plus; Con.: Control birds; I.: Infected birds; wpi: Week post infection; IG: Infection group; Gen.: Genotype

SFA: sum of C10:0, C11:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C23:0.

MUFA: sum of C14:1cis-9, C16:1cis-9, C17:1cis-9, C18:1cis-9, C18:1cis-11, Sum C18:1trans, C20:1cis-11.

PUFA: sum of C18:2n-6, C18:2cis-9,trans-11, C18:3n-3, C18:3n-6, C18:4n-3, C20:2n-6, C20:3n-6, C20:4n-6, C22:5n-6, C22:4n-6, C22:5n-3, C22:6n-3.

4. Discussion

High performance is associated with several welfare and health problems in different farm animal species, including chickens (Rauw et al., 1998; Julian, 2005). Because genetic selection is performed under optimal environmental conditions to exploit the greatest genetic potential, high performing animals are likely more sensitive to environmental challenges. The aim of this study was to test the hypothesis that high performing laying hens are more sensitive to the gastrointestinal nematode infections, which are highly common in the field (e.g. Kaufmann et al., 2011a; Thapa et al., 2015). Using a chicken-nematode host-parasite system we compared hens of a high- (i.e., LB) and a lower-performing (i.e., LD) chicken genotype to assess impact of common nematode infections on host tolerance. Moreover, genotype-specific host responses to infections were quantified for a period of 18 weeks, in which both resistance to experimental infections and susceptibility to naturally occurring infections were assessed using a wide range of parasitological and immunological parameters. In order to quantify the impact of nematode infections on host performance, the study was performed during the first third of laying period. During this period, laying performance is at its maximum, thus the production-associated physiological pressure was also expected to be highest in both genotypes. The results clearly demonstrated that the performance-dependent physiological pressure affected the hosts' ability to tolerate the nematode infections. Both genotypes responded to the infections with a reduction in laying performance (i.e., laying rate and egg mass production) and a lower feed conversion efficiency, while feed intake was not significantly affected. The two genotypes did not differ in terms of resistance to experimental infections, but the high performing LB hens were more susceptible to naturally occurring reinfections. In the following sections we discuss the most important results of the present study with regard to host performance level as well as dynamics of infections.

4.1 Host performance

Laying performance, measured as both laying rate and egg mass production, was reduced in infected hens of both genotypes. Such adverse effects of parasitic challenges on laying performance are already known from other chicken parasites, e.g. coccidiosis (McDougald et al., 1990; Lensing et al., 2012), histomonosis (Liebhart et al., 2013) and ectoparasites (Mullens et al., 2009), whereas most nematode-infection studies reported only negative effects on nutritional parameters (i.e., feed intake and feed conversion efficiency; Daş et al., 2010, 2011, 2012) but not on the laying performance (Gauly et al., 2002; Dahl et al., 2002; Sharma et al., 2018a). Studies describing a drop in laying rate during nematode infections are rare (Permin et al., 1998). However, studies investigating infection effects on laying performance mostly deal with mono-species infections even though multi-species infections occur naturally in the field (Wongrak et al., 2014; Thapa et al., 2015). The drop in laying rate for both genotypes in this study may therefore be a consequence of the multi-species infection model, which in addition is clearly more representative for the conditions in the field. The infection with two worm species may therefore have developed a more severe physiological pressure on the hens than only a single worm species does. The higher pressure potentially occurs from the double immunological burden, as both worm species separately induce local immune responses in their respective predilection sites (Stehr et al., 2018).

The reduction in laying rate in this study was most probably associated with the immunological status of the birds. As shown with the LB hens, the first drop in laying performance (i.e., 2 to 3 wpi) corresponds well to the onset of the adaptive immune response (measured as ascarid-IgY level), already known from other studies (Daş et al., 2018; Stehr et al., 2018). Similarly, meat-type chickens (high-performing in growth) responded with a reduced growth rate at the same time point as described recently (Stehr et al., 2019). However, in contrast to Stehr et al. (2019), where growth rate and feed intake were reduced in equal shares, feed intake was not affected by infections in both genotypes. Thus, the drop in laying rate may be associated with the infection induced immune responses that are metabolically/nutritionally costly (Colditz, 2008). Available nutrients may have been allocated away from host performance traits (i.e. laying rate) towards mounting immunity against the nematode species (e.g. worm expulsion and tissue repair). The worse feed conversion efficiency of infected hens compared to controls supports this assumption. In previous mono-species-infection studies with *A. galli* hens increased their feed intake (Gauly, 2007), likely to compensate for an imbalanced nutrient in a specific diet (Daş et al., 2010).

Laying performance in both genotypes was reduced at different time points. The high-performing LB hens firstly responded with a drop in laying rate in the early phase of infection (i.e., by wpi 3), whereas infected LD hens laid less numbers of eggs only in the advanced infection period. The fact that LB hens dropped laying rate much earlier than LD did was likely associated with different levels of production pressure in the two genotypes. LB hens already encountered a high physiological pressure in the beginning of the laying period as total egg mass production was already on a high level for this genotype. This was obviously not the case with LD hens as total egg mass production was much lower in the early laying period. However, when egg weight and thus the total egg mass production of LD hens increased with progressing age, likely due to a genetic programming, infected LD hens were not able to maintain their performance level at that of non-infected LD hens. Collectively, these results are collectively indicative for a negative association between a performance related physiological pressure and host tolerance.

4.2 Host resistance and susceptibility

In both host genotypes the numbers of first-generation worms of both species, originating from the experimental infection, decreased over the study period. The results are fully in line with our recent data collected from male birds of the same genotypes (Stehr et al., 2018, 2019), and therefore confirm that worm expulsion, the first effective mechanism of the host animal to control worm burdens (Lawrence, 2003; Stehr et al., 2018), takes place independent of worm species, host genotype as well as host sex.

Burdens of the first generation worms with both *A. galli* and *H. gallinarum* were not different between the two host genotypes, whereas number of larvae resulting from the naturally occurring re-infections was higher in LB than in LD hens. These results indicate that the genotypes did not differ in terms of resistance to fully controlled experimentally performed infections, whereas LB hens are more susceptible to naturally occurring re-infections. Considering that most ascarids descending from a single infection are expelled in a few weeks (Stehr et al., 2018), final worm burdens of hens under field conditions are mainly determined by the continuously occurring re-infections (Daş et al., 2018). Thus, the higher re-infection in LB birds may indicate higher final worm burdens for LB than LD hens over time. Similar observations were made with the hens' male counterparts in a previous study (Stehr et al., 2019). Whether behavioural differences between the two genotypes, suggested by Giersberg et al. (2017) and Malchow et al. (2018), contributed to a different intake of nematode eggs from the pen environment needs to be elucidated in further studies. The fact that re-infections with *H. gallinarum* were clearly higher than with *A. galli* was probably based on to the shorter prepatent period of *H. gallinarum* than that of *A. galli* (Ramadan and Abou Znada, 1992; Daş et al., 2014). Although the infection period should have given sufficient time to induce significant re-infections, the re-infection level was generally low with both species. Well controlled aeration conditions and the addition of extra litter material may have reduced the overall humidity and composting activity in the pen litter, which can consequently affect the embryonation and infection ability of the nematode eggs (Katakam et al., 2014; Tarbiat et al., 2015; Thapa et al. 2017).

4.3 External and internal egg quality traits

The infections significantly affected a number of external and internal egg quality traits, probably leading to disadvantages for farmer, consumer and the chicken's offspring. In contrast to several mono-species-infection studies (Gauly et al., 2002; Sharma et al., 2018a), we demonstrated that egg weight was significantly reduced by nematode infections in chickens. To our knowledge comparable observations have been described only with protozoan parasites (i.e., Eimeria maxima coccidian; Lensing et al., 2011), ectoparasites (i.e., northern fowl mite; Vezzoli et al., 2016) and viral infections (e.g., Newcastle disease virus; Quinn et al., 1956) in chickens before. However, the decrease in egg weight resulted in a lower egg mass per hen (i.e., combination of laying rate and egg weight) and additionally caused a considerable shift in egg size classes towards smaller sized eggs. Infection also reduced yolk color, indicating a lower pigmentation of egg yolks, in most cases associated with changes in the yolk carotenoid concentrations (Laudadio et al., 2014; Grashorn, 2016). Lighter egg yolks were also found during northern red mite challenges in chickens (Vezzoli et al. 2016). Carotenoids are likely associated with immune functions of the host functions of the host, but results are ambiguous (Sepp et al., 2011; Butler and McGraw, 2013). Deposition of carotenoids into egg yolks may thus be disturbed under metabolically costly immune challenges. The lighter yolks and carotenoid concentrations in yolks are further associated with the immunological status, i.e. the healthy status of the birds (Sepp et al., 2011; Lucas et al., 2014), potentially indicating an impaired chick health, if their mothers were infected with nematodes. However, this needs to be elucidated in future nematode-infection studies. Nevertheless, while egg mass per hen as well as egg size are of economic importance for farmers (Golden et al., 2012), both egg size and egg yolk color are important quality criteria in consumer's perception (Grashorn, 2016), thus being of crucial importance for marketing of the eggs. Thus, strategies to reduce worm burdens benefit not only the animals itself, by increasing animal health and welfare, but also ensure the profitability of a laying flock.

The fatty acid profile of egg yolks can be affected by several factors such as age (Lesic et al., 2017), lipid source in diet (Oliveira et al., 2010) and housing system (Anderson, 2011). Whether nematode infections in chickens have an impact on the fatty acid profile, however, has so far never been studied. Analysis of the fatty acid profile in egg yolks revealed significant alterations in the total fat content and proportion of PUFA due to the infections. Both fat content and proportion of PUFA, especially n-6 PUFA, were significantly reduced in egg yolks, whereas the proportion of MUFA tended to be higher in infected hens, which is likely a consequence of a lower proportion of PUFA. Because feed intake was not significantly reduced by infections, the alterations were not directly based on a lower consumption of fatty acids via feed. However, the infection-induced worsened feed conversion efficiency may indicate a diverted distribution of fat components away from yolk deposition towards metabolic processes. Fatty acids, such as n-6 PUFA, may thus have been involved in tissue repair and regeneration processes (Silva et al., 2018), because tissue dwelling *A. galli* larvae damage the intestines (Marcos-Atxutegi et al., 2009; Dänicke et al., 2009; Schwarz et al., 2019; Luna-Olivares et al, 2015). As fatty acids are transferred from the mothers to offspring via deposition in yolks, the fatty acids are considered as functional yolk nutrients (Cherian, 2015). Thus, a lowered concentration of such nutrients or a disbalance of fatty acids composition might be indicative of an impaired reproductive fitness, as they serve as a source of energy or are essential nutrients for development of the chick embryo (Speake et al., 1998).

4.4 Ascarid specific IgY

The level of ascarid-specific IgY antibodies in plasma of the hens fully conform to the course of infections. As known from other recent studies (Daş et al., 2018; Stehr et al., 2018), plasma IgY increased first by 2 wpi and peaked at wpi 3, demonstrating the first humoral immune response to the experimentally induced infection. When *A. galli* larvae obviously have migrated back from tissue to lumen, IgY concentrations decreased again (by 4 wpi). During the primary response, LB hens exhibited higher IgY levels than LD did, even if not significant. However, different IgY levels fully conform to the higher number of larvae for LB than LD in both tissue and lumen. IgY levels were not different between infected and controls from wpi 6 to 10 in both genotypes, indicating that an antigenic stimulus was absent during this time period even though adult worms of both species were present. This is in line with the overall assumption that ascarid-specific antibodies are not induced by adult worms but rather by larval stages (Marcos-Atxutegi et al., 2009; Daş et al., 2018), which live in close contact to the intestinal tissue, known for both worm species (Vatne and Hansen, 1965; Stehr et al., 2018). This is also reflected by the increase in IgY at wpi 11 in LB hens undoubtedly induced by naturally occurring re-infections with *H. gallinarum*. The IgY level of infected LD hens increased only slightly by wpi 17, but was on a very low level if compared with those of LB hens, being in line with the lower re-infection level.

The pattern of IgY antibodies in egg-yolks was comparable to that in plasma. Both primary (i.e. experimental) and secondary infections (i.e. re-infections) were observed in egg yolks, too, although the overall IgY level in egg-yolks was lower compared to that in plasma.

In contrast to plasma, the IgY levels in egg-yolks of infected LB hens were significantly different to that of controls during the whole study period (except wpi 10), indicating that IgY antibodies are more stable in eggs yolks than in plasma. Because more than one follicle is formed on the ovary at the same time, it is possible that egg-yolk IgY do not fully represent the current immune status of the birds, as there exists a temporal offset between IgY transfer from blood into egg yolks and egg deposition. However, the analysis of egg-yolks instead of blood samples is more host-friendly (Daş et al., 2017; Sharma et al., 2018b) and is still a valuable tool to determine qualitatively the infection status of the hens. The dynamics of ascarid-specific antibodies in plasma and egg yolks deserve further studies, where relationships with worm burdens should insensitively be investigated.

5. Conclusions

Our data collectively support the hypothesis that tolerance to nematode infections in laying hens is dependent on host-performance level. The impairment in host tolerance was both genotype and time dependent, likely due to differences in genetic programming for production peak and persistency of the two genotypes. Resistance to infections was dependent on the type of infection (i.e., experimentally vs. naturally re-infection). The two genotypes exhibited similar levels of resistance after a fully controlled experimental infection, but the high performing hens were more susceptible to naturally occurring re-infections. Infections negatively affected economically important egg-quality traits, including egg weight, fat content and fatty acid profiles in egg yolks.

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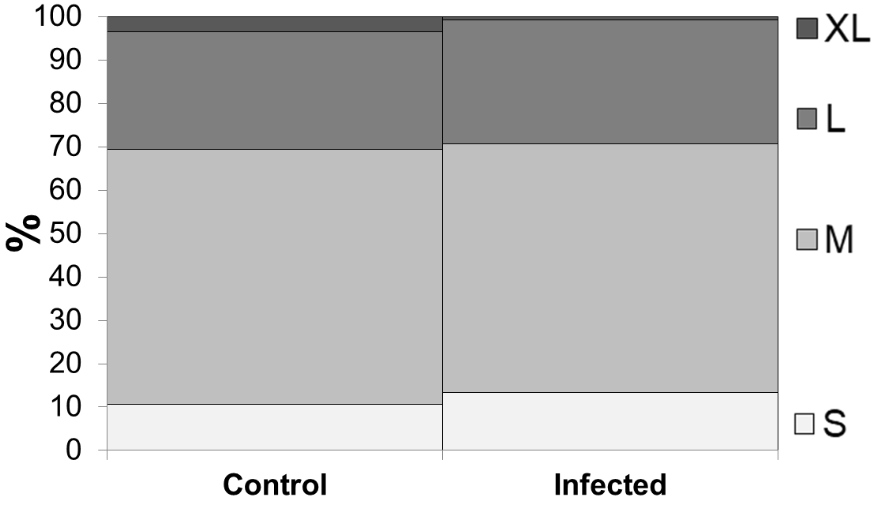
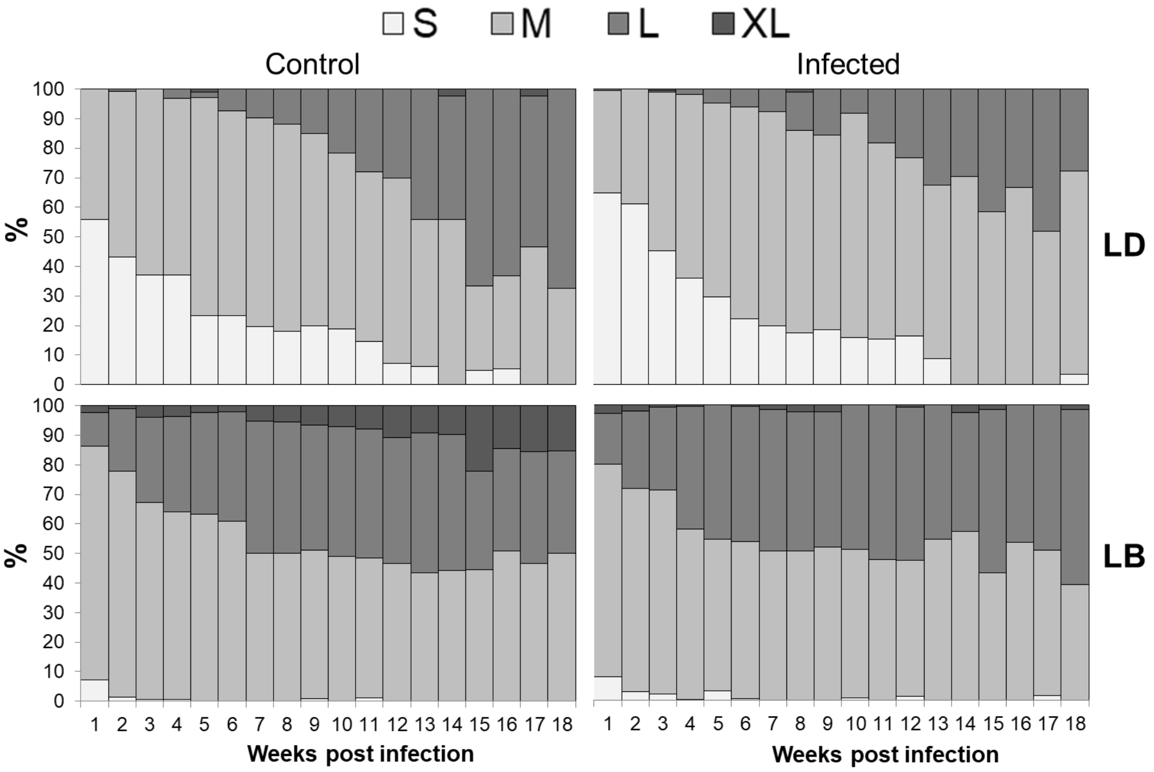
Supplementary Data



Supplementary Figure S1A



Supplementary Figure S1B

Supplementary Fig. S1. Distribution of eggs falling into size classes in uninfected control and nematode-infected chickens across both genotypes (**A**) and in each genotype over time (**B**).

Supplementary Table 1. Effects of host genotype and a mixed nematode infection on individual- and groups of fatty acids (%) in egg yolks of laying hens.

	Main effect of genotype				**Infection effect**				**Interactions, P-Value, ≤**				
Item	**LD**	**LB**	SE	P,≤	**Con.**	**Inf.**	SE	P,≤	wpi	IG*Gen	IG*wpi	Gen*wpi	IG*Gen*wpi
C10:0	0.013	0.015	0.0007	0.019	0.013	0.014	0.0007	0.078	0.001	0.211	0.401	0.048	0.429
C11:0	0.018	0.020	0.0007	0.040	0.017	0.020	0.0007	0.017	0.038	0.090	0.001	0.680	0.599
C12:0	0.006	0.002	0.0006	0.001	0.004	0.003	0.0006	0.774	0.001	0.796	0.490	0.050	0.548
C14:0	0.343	0.305	0.0047	0.001	0.325	0.323	0.0046	0.833	0.001	0.225	0.511	0.188	0.399
C15:0	0.085	0.066	0.0019	0.001	0.079	0.073	0.0019	0.058	0.429	0.030	0.583	0.319	0.732
C16:0	25.40	24.45	0.107	0.001	24.94	24.91	0.103	0.789	0.001	0.058	0.910	0.024	0.991
C17:0	0.324	0.264	0.0061	0.001	0.302	0.286	0.0059	0.034	0.001	0.046	0.563	0.143	0.790
C18:0	8.10	8.06	0.086	0.679	8.01	8.14	0.083	0.243	0.021	0.537	0.622	0.434	0.772
C20:0	0.021	0.021	0.0008	0.821	0.022	0.021	0.0008	0.347	0.011	0.166	0.289	0.396	0.055
C21:0	0.027	0.026	0.0008	0.819	0.028	0.027	0.0009	0.530	0.001	0.430	0.158	0.987	0.276
C22:0	0.034	0.035	0.002	0.805	0.032	0.036	0.0024	0.265	0.001	0.814	0.009	0.053	0.744
C23:0	0.011	0.016	0.0006	0.001	0.012	0.014	0.0006	0.006	0.002	0.310	0.950	0.116	0.550
Sum SFA	34.38	33.28	0.131	0.001	33.79	33.87	0.126	0.646	0.001	0.288	0.608	0.006	0.985
C14:1*cis*-9	0.057	0.057	0.0019	0.932	0.057	0.057	0.0019	0.677	0.001	0.033	0.423	0.172	0.820
C16:1*cis*-9	2.38	2.62	0.056	0.001	2.47	2.53	0.054	0.364	0.002	0.060	0.547	0.018	0.911
C17:1*cis*-9	0.131	0.125	0.0025	0.052	0.130	0.126	0.0025	0.245	0.077	0.082	0.796	0.688	0.840
C18:1*cis*-9	37.35	39.86	0.244	0.001	38.31	38.90	0.236	0.056	0.001	0.906	0.749	0.881	0.954
C18:1*cis*-11	1.95	2.28	0.026	0.001	2.14	2.08	0.025	0.094	0.001	0.182	0.041	0.118	0.430
Sum C18:1*trans*	0.219	0.213	0.0037	0.198	0.215	0.216	0.0037	0.869	0.001	0.905	0.772	0.444	0.552
C20:1*cis*-11	0.194	0.210	0.0028	0.001	0.203	0.201	0.0027	0.502	0.002	0.198	0.559	0.010	0.335
Sum MUFA	42.27	45.36	0.256	0.001	43.52	44.11	0.247	0.067	0.001	0.507	0.754	0.868	0.942

C18:2*n*-6	17.50	15.78	0.224	0.001	16.89	16.39	0.217	0.081	0.001	0.208	0.724	0.099	0.871
18:2*cis*-9,*trans*-11	0.064	0.064	0.0009	0.571	0.064	0.064	0.0008	0.774	0.001	0.595	0.289	0.518	0.234
C18:3*n*-3	0.561	0.499	0.0116	0.001	0.530	0.530	0.0112	0.970	0.001	0.714	0.751	0.083	0.036
C18:3*n*-6	0.125	0.097	0.0029	0.001	0.112	0.110	0.0028	0.556	0.582	0.217	0.096	0.019	0.664
C18:4*n*-3	0.007	0.008	0.0008	0.161	0.008	0.008	0.0008	0.987	0.001	0.312	0.385	0.553	0.395
C20:2*n*-6	0.165	0.148	0.0034	0.001	0.160	0.153	0.0033	0.108	0.056	0.880	0.874	0.725	0.553
C20:3*n*-6	0.156	0.138	0.0028	0.001	0.149	0.146	0.0027	0.322	0.248	0.447	0.969	0.719	0.798
C20:4*n*-6	2.47	2.62	0.036	0.001	2.258	2.52	0.035	0.270	0.302	0.257	0.029	0.519	0.376
C22:5*n*-6	0.832	0.555	0.020	0.001	0.723	0.664	0.018	0.018	0.001	0.547	0.116	0.789	0.223
C22:4*n*-6	0.264	0.259	0.010	0.652	0.278	0.245	0.010	0.010	0.104	0.457	0.094	0.538	0.134
C22:5*n*-3	0.126	0.124	0.005	0.726	0.130	0.121	0.005	0.144	0.001	0.453	0.060	0.427	0.696
C22:6*n*-3	1.06	.107	0.019	0.665	1.07	1.06	0.019	0.594	0.001	0.528	0.122	0.001	0.301
Sum PUFA	23.28	21.30	0.258	0.001	22.63	21.95	0.249	0.040	0.001	0.230	0.651	0.115	0.916
Sum *n*-3 PUFA	1.76	1.71	0.026	0.103	1.74	1.72	0.025	0.525	0.001	0.836	0.076	0.001	0.148
Sum *n*-6 PUFA	21.52	19.59	0.239	0.001	20.88	20.23	0.231	0.032	0.002	0.203	0.567	0.169	0.958
Fat content	5.62	5.46	0.105	0.230	5.67	5.41	0.102	0.045	0.006	0.989	0.011	0.343	0.617

Egg yolk samples were collected at the weeks p.i. 2, 4, 6, 10, 14 and 18 (N=163).

Abbreviations: LD: Lohmann Dual; LB: Lohmann Brown Plus; Con.: Control birds; Inf.: Infected birds; wpi: Week p.i.; IG: Infection group; Gen.: Genotype

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CHAPTER FIVE

General Discussion

5.1 Nematode infections

Gastro-intestinal nematode infections are a concrete problem for laying hens in the field (Kaufmann et al., 2011a; Wongrak et al., 2014; Thapa et al., 2015; Wuthijaree et al., 2017) and constitute a health risk for broiler chickens kept outdoors (Abdelqader et al., 2008; Katoch et al., 2012; Ferdushy et al., 2016) and indoors (Wilson et al. 1994; Jansson et al., 2010; Yazwinski et al., 2013) as well. The nematodes *A. galli* and *H. gallinarum* are most prevalent in chickens (Kaufmann et al., 2011a). Since the increasing research for nematode infections in chickens, several studies reported a genotype difference in resistance to nematodes (Permin and Ranvig, 2001; Kaufmann et al., 2011b; Wongrak et al., 2015), that is probably based on several physiological, immunological and behavioural differences between the genotypes. Thus, the use of chickens more resistant and tolerant genotypes to nematode infections would be a sustainable way to control nematode infections.

Based on the knowledge that long-term selection for high performance in farm animals has been accompanied with several health problems (see Chapter 1.6), we tested the hypothesis that selection for increased performance in chickens impairs their ability to cope with nematode infections. Therefore we separately performed two experiments with male and female birds to study the impact of selection for high performances (i.e., growth or laying performance, respectively) on resistance and tolerance to a mixed nematode infection with *A. galli* and *H. gallinarum.* In the male study three divergent genotypes selected either for meat- or egg production or for both purposes were used, whereas in the female study only hens of a layer line and a dual-purpose line were comparatively investigated. The use of divergent chicken genotypes as well as different host sexes provided an excellent animal model to study the consequences of selection for high performances on health as the genotypes could not be more different in the traits they have been selected over a long time period. As birds of both sexes were used, this study additionally allowed us to determine whether host resistance and tolerance to nematode infections is dependent on the performance direction that the genotypes have been selected for (i.e., growth or laying performance). Our results conform to the above proposed hypothesis. While in the male study host tolerance was sensitive to growth rate as demonstrated by an impaired growth performance in the meat-type chickens only, impaired tolerance in the female birds was both genotype and time dependent, likely due to differences in genetic programming for production peak and persistency of the two genotypes. The high performing meat-type chickens also had a lower ability to resist the nematode infection at least with one worm species (i.e., *A. galli*) when compared with the slower growing layer and dual-purpose genotypes, likely due to growth dependent conditions in the intestinal environment. Female birds, however, did not differ in their resistance to nematode infections. However, in both studies natural re-infections were highest for the layer-type chickens, likely because of behavioural differences (Malchow et al., 2018) that may increase risk of reinfections to occur. These results conform to the performance dependent susceptibility to non-parasitic infections (Calenge et al., 2014; Han et al., 2016) and the several behavioural, physiological and immunological problems in the high performing meat- and layer-type chickens (for further details see chapter 1.6.1 and 1.6.2).

A further part of this thesis addresses the worm expulsion phenomenon that has been described several times in the mammalian host (Vallance et al., 1999; Dawson et al., 2005; Miquel et al., 2005; Masure et al., 2013; Kringel et al., 2015; Oeser et al., 2015; Sun et al., 2016), whereas only a few studies have so far suggested the existence of a worm expulsion in the avian host exposed to mono-infections only (Tompkins and Hudson, 1999; Permin and Ranvig, 2001; Ferdushy et al., 2013). We therefore performed a detailed study to characterize the extent and duration of worm expulsion and the accompanied immune responses following an experimental infection with the most common nematodes. Our results clearly demonstrated a strong expulsion taking place for both nematodes, where juvenile worms were specifically targeted. A few worms that were able to survive and reached maturity had the chance to induce re-infections thereby likely passing their genetic material to the next generation worm populations. This expulsion phenomenon, which explains low establishment rates following experimental infections and the inability to induce patent infections with *A. galli* in chickens (Permin et al., 1997; Daş et al., 2011; Schwarz et al., 2011a), was considered to be mediated mainly on host immune responses (Dold and Holland, 2010), although–differences among worms' ability to survive may also exist (Johnston et al., 2005; Nejsum et al., 2009).

In the following sections, we will discuss the importance of the present results in a wider spectrum and their practical significance.

5.2 Worm expulsion and potential implication for population dynamics of the worms

As confirmed with all genotypes in this study the worm expulsion is a consistently occurring defence mechanism in the chicken host, which occurs independent of the worm species, host genotype, host age as well as host sex. Worm expulsion is the first effective defence reaction of the host to cope with the worms (Dold and Holland, 2010). The rigorous reduction in worm burdens within a short time period found in this study likely minimizes the negative effects that occur from the infections found in the present as well as previous studies (Schwarz et al, 2011b; Daş et al., 2010, 2011b, 2012; Sharma et al., 2018a) and is an effective mechanism of the host animal to reduce the physiological and metabolic costs (Coop and Kyriazakis, 1999; Colditz, 2008; van der Most et al., 2011; Rauw, 2012) that inevitable emerge from parasitic challenges. Especially the elimination of juvenile worms, or more precisely larvae (hatched or un-hatched), can be considered as a highly efficient strategy of the host to minimize the negative effects as early as possible. Compared to the adult worms the larvae are most harmful for the host as they have direct contact to the intestinal wall (Marcos-Atxutegi et al., 2009; Daş et al., 2017, 2018) and induce costly immune responses (Colditz, 2008). These costs are extended through the diverted use of nutrients needed to repair the intestinal tissue injury, caused by the tissue penetrating larvae (Luna-Olivares et al., 2012). Injury of the intestinal wall may also impair the overall absorption of dietary nutrients.

As exemplary shown with results of the male study (Chapter Three), the worm expulsion pattern, however, were different between the genotypes and worm species, indicating host-genotype and worm species-specific expulsion patterns. The curves shown in Fig. 2 predicted almost similar expulsions of *A. galli* for the lower performing genotypes (LB and LD), whereas expulsion in the high performing genotype (R) differed at least in the early phase. The duration of worm expulsion in the meat-type chicken was estimated to be shorter as compared to the slower growing genotypes, already starting with a higher estimated *A. galli* burden at day one of infection. The more efficient expulsion is not surprising in this genotype as environmental conditions are expected to be worse for juvenile worms, potentially due to the faster digesta transit time in the high feed consuming meat-type chickens (Denbow, 2015). The higher proportion of tissue larvae in this high performing genotype in the early infection period confirms this assumption and may explain the larger *A. galli* burden predicted at infection day one.

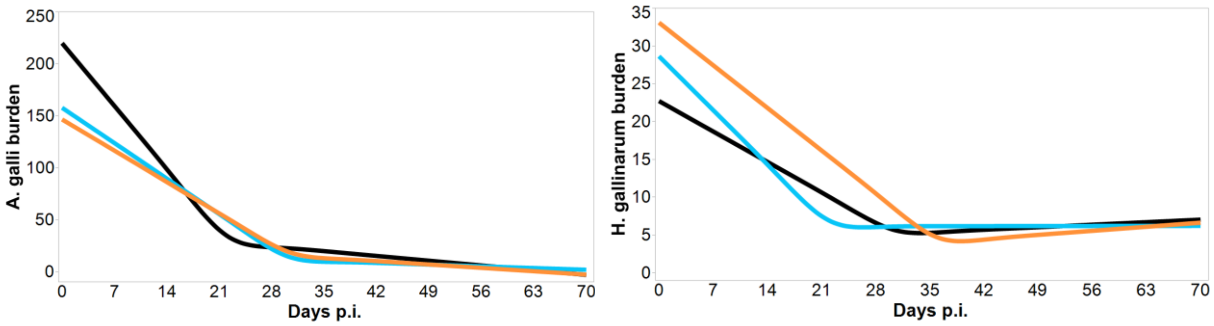

Fig. 2. The extent and duration of worm expulsion by meat-type chickens (R ■), layer-type chickens (LB 🟧) and dual-purpose chickens (LD 🟦) co-infected with *Ascaridia galli* (left) and *Heterakis gallinarum* (right). The data presented here are based on total worm burdens of the first worm generations originating from the experimental infection. The expulsion curves are based on outcomes of segmented regression as described in Chapter Two. The lines represent the predicted expulsion curves for each genotype. The worm expulsion is described before and after a worm and genotype specific breakpoint, that serves as a threshold time point that identifies different worm expulsion patterns.

Expulsion patterns of *H. gallinarum* were slightly different between the three genotypes as well (Fig. 2). However, particularly remarkable is the lower number of worms estimated for day one of infection if compared with *A. galli* burdens. The involvement of *H. meleagridis* might have accounted, at least partly, for the lower *H. gallinarum* burdens. *H. meleagridis* impairs the incidence, establishment and development of *H. gallinarum* (Lund, 1968; Schwarz et al., 2011b; Daş et al., 2011a), likely due to heavy destructions in the caeca (Hess et al., 2006) as well as local immune responses (Powell et al., 2009). The alterations in the predilection site of *H. gallinarum*, induced by *H. meleagridis*, may provide unfavourable conditions for *H. gallinarum* to survive in their habitat, finally causing lower *H. gallinarum* burdens or even their complete absence (Lund, 1968). The results of the female study support this hypothesis, too. Although expulsion of *H. gallinarum* undoubtedly took place in females as well, the higher establishment rates with *H. gallinarum* indicate that expulsion occurred in a lesser degree than found in the male birds (Fig. 3). Because a co-infection with *H. meleagridis* apparently did not occur in the female birds (i.e., based on the absence of caecal alterations typically induced by *H. meleagridis*), it is most likely that higher establishment rates with *H. gallinarum* in females than males result from the *H. meleagridis*-free infection.

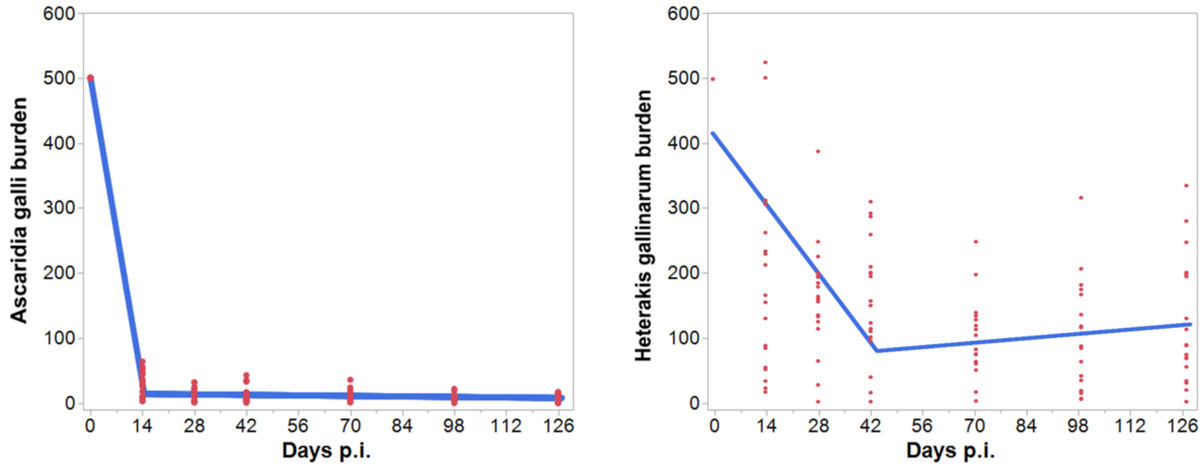

Fig. 3. The extent and duration of worm expulsion by laying hens co-infected with *Ascaridia galli* (left) and *Heterakis gallinarum* (right). The data presented here are based on total worm burdens of the first worm generations originating from the experimental infection. Data of both genotypes were combined. Each dot is an independent observation. The expulsion curves are based on outcomes of segmented regression. The line represents the predicted expulsion curves for the respective worm species. The worm expulsion is described before and after a worm and genotype specific breakpoint, that serves as a threshold time point that identifies different worm expulsion patterns.

Assuming that a co-infection with *H. meleagridis* was absent in the female birds, expulsion patterns with *H. gallinarum* may be more undisturbed in the female birds. Thus, comparing expulsions of *A. galli* and *H. gallinarum* with the data obtained in the female study, worm expulsion is much stronger with *A. galli* than *H. gallinarum*. As shown with Fig.

3 overall worm burdens of *H. gallinarum* were higher than that of *A. galli* also in the advanced infection period, although the infection dose given was similar for both worm species. The differences found are possibly related to differences in the physiology of the intestinal niche of the respective worm species (i.e., small intestine and caeca). While digesta passes the small intestine in about 1-2 hours, caecal emptying occurs less frequently (Svihus, 2014). In broiler chickens a 24-hour feed deprivation did not significantly reduce the caecal content (Hinton et al., 2000; Warriss et al., 2004), indicating a relatively long retention time in the caeca. Additionally, colonic antiperistalsis transports digesta back into the caeca (Svihus, 2014). Thus, the caeca constitutes a more untouched / closed niche, in which *H. gallinarum* is only rarely affected by intestinal peristaltic movements, whereas *A. galli* needs to escape from continuous gut peristalsis.

As co-infections with *H. meleagridis* occur naturally in the field frequently (Grafl et al., 2011) the concurrent infection with this parasite does not diminish the value of the data presented with the male study but rather represents more in detail what occurs under natural conditions. Additionally, our data point to possibility that the expulsion of worms may be associated with involvement of other pathogens, too. The very low *H. gallinarum* burdens predicted on the infection day one are likely underestimated by the segmented regression model. As the hatchability of *H. gallinarum* is not affected by *H. meleagridis* (Lund and Burtner, 1957) and the *Histomonads* are liberated through moulting of the larvae earliest by day 4 p.i. (Lund, 1968), the *H. gallinarum* burden might not have already been affected at the first day of infection. Although our modelling was based on concrete experimental data, the above discussed issue clearly demonstrates the significance of parasitological examinations to perform a reliable modelling. Parasitological examinations are thus crucial to describe complex situations how they take place under natural circumstances and therefore cannot be replaced by modelling / simulations without concrete data.

That only a low number of worms survived until the end of the study period, implies that some worms had specific characteristics that enabled themselves to escape from the host induced expulsion mechanisms (Lawrence, 2003; Patel et al., 2009; Dold and Holland, 2010). This host-parasite interaction might be related to the genetic background of the worms. In a few studies it has been demonstrated that expulsion of the worms was associated with their genetic constitution (Johnston et al., 2005; D´Elia et al., 2009). In a study by Nejsum et al. (2009) the host-parasite interaction between the roundworm *A. suum* and the pig host was studied more in detail. It has been shown that the pigs harboured a worm infra-population with a host individual genetic composition, although the infection material was composed of genetically different eggs in equal proportion for each pig (Nejsum et al., 2009). Masure et al. (2005) suggested that larvae with a high metabolism, an indicator for the motility, were able to counterbalance the expulsion only. The fact that mainly larval stages were targeted in our study may confirm this suggestion. Does it mean that the infection material was composed of worm eggs with a different background? The infection material used in this study was obtained from birds of different genotypes, ages, sex and locations. Whether this has led to a diverse genetic composition and different quality of the infection material can not clearly be answered with our data as diversity in genetic composition of the worms was not investigated. However, there is evidence that the geographical source of *A. galli* isolates influences the outcome of an infection (Abdelqader et al., 2007), implying that locally adapted worms are more likely to establish in locally adapted hosts than worms from another location. Furthermore, Lippens et al. (2017) found that the fitness prospect of the worms is dependent on the age of their parents. The worms used to produce the infection material in our study, were of different size and thus likely of a different age. Whether this is an indicator for the different quality of the eggs remains questionable. To clarify the role of worm genetics in worm expulsion by chicken genetic investigations of the worms in an infra-population needs to be included in further studies. However, the strong selection pressure induced by host animal on the worms found in this study is in line with the basics of “survival of the fittest” (Darwin, 1859), when applied to our observations: only those worms that were able to withstand the expulsion mechanisms, probably because of their genetic background, can induce a patent infection.

How does the worm expulsion influence the worm population dynamics? Due to the limited period of time in our studies, only *H. gallinarum* was given sufficient time to induce re-infections. Thus it was not possible to determine how the expulsion affects the population dynamics if (re)-infections occur continuously as it happens in the field. However, under natural conditions in the field high worm burdens with both *A. galli* and *H. gallinarum* are usually found in un-dewormed chickens (Kaufmann et al., 2011; Wongrak et al., 2014; Thapa et al., 2015; Wuthijaree et al., 2017). Interestingly there are very high worm burdens despite the existence of an efficient worm expulsion mechanism. Based on our results we assume that worm expulsion or the selection pressure on worms is a continuous process that enables only a small number of worms to establish in the host successfully. Under natural conditions in the field, where environmental contamination with nematode eggs is high, birds are continuously exposed to infections. Considering that only a small percentage of ingested eggs are able to establish successfully in the host, it is reasonable to assume that the worm burden with mature worms grows slowly step by step which implies that the naturally infected host animal harbours an infra-population that consists of different worm generations. Nevertheless, the few survivor worms that reached maturity probably produce an offspring that is better adapted to the host intestinal conditions, as they may carry superior genetic material of the successful parents, leading to a higher infection success in the long run. Several re-infections, which determine the final worm burden (Daş et al., 2018), are thus needed to induce high worm burdens and consistent patent infections.

5.3 Infection induced impairment in feed intake in relation to host tolerance

The nematode infections induced a significant reduction in feed intake only in the birds of the male study but not in those of the female study. Whether the host-sex specific performance direction (i.e., growth rate vs. laying performance) had an impact on the host responses differently found between the two studies remains unknown. However, literature review clearly demonstrates a variety of results ranging from an unaffected feed intake (Sharma et al., 2018b), a reduction in feed intake (Daş et al., 2010, 2011b, 2012) or even an increase in the voluntary feed intake of infected birds (Gauly, 2007). Nevertheless, the mixed nematode infection in the male study induced a reduction in feed intake in all three genotypes that has already been shown to occur in mono-species infections in chickens (Daş et al., 2010, 2011b, 2012). The infection induced anorexia is a common feature that occurs in several host-parasite systems (Kyriazakis et al. 1998; Coop and Kyriazakis, 1999; Kipper et al., 2011; Kyriazakis, 2014). A meta-analysis in pigs shows that the anorexia is in large part responsible for the lower growth performance in parasite-infected animals (Kipper et al., 2011) which conforms to the observations made in the male study. However, an impaired feed conversion (Daş et al., 2010, 2011b, 2012), a lower nutrient absorption (Hurwitz et al., 1972; Walker and Farell, 1976; Schwarz et al., 2011a) as well as direct effects of the worms (Kipper et al., 2011) affect the host performance as well. The reduction in feed intake is somewhat paradoxical as the nutritional demand increases through the infection (Coop and Kyriazakis, 1999; Colditz, 2008; Rauw, 2012). But why does the host still respond with anorexia to an infection? The reduction in feed intake in the male study was likely associated with the activation of the adaptive arm of the immune system. The expression of specific cytokines (McCarthy, 2000; Plata-Salamán, 2001; Rauw, 2012) that are partly upregulated during an *A. galli* infection (Dalgaard et al., 2015) may be responsible for the induction of anorexia (Rauw, 2012). The drop in weight gain of the meat-type chickens, due to the lower feed intake, occurred simultaneously with the onset of the adaptive immunity, which supports the assumption that the anorexia was immune related. Some authors suggest that the host actively reduces the feed intake, a behavioural strategy to cope with the infection (Exton, 1997; Kyriazakis et al. 1998; Kyriazakis, 2014). In this case Kyriazakis et al. (1998) proposed two hypotheses: first, the lower feed intake promotes host immune responses by reducing the intake of immunosuppressive nutrients, and second, the host is more selective in the diet to minimize the risk for ingestion of parasite eggs. Changes in feed intake regulating hormones, which were found during parasite challenges, were ascribed a lesser importance in inducing heavy direct anorexic effects (Yang et al., 1990; Roberts et al., 1999; Zaralis et al, 2008).

As shown in the male study, the slow growing genotypes (LD and LB) highly tolerated the infection despite the lower feed intake, probably due to the nutrient-dense broiler diet that supplied the slower growing genotype with a sufficient quantity of nutrients to compensate the lower feed consumption. In contrast, in the high performing genotype the anorexia induced a lower availability of nutrients for the host, which in addition to the already existing parasite infection is a huge environmental stressor (Schaible and Kaufmann, 2007). However, comparing different genotypes on the same diet is somehow problematic, as each genotype has specific nutritional needs. Comparing different genotypes on genotype-specific diets would be more significant for determining the real resistance and tolerance against nematode infections. However, as indicated with present results an adequate nutrient supply to the infected host can increase the host tolerance. Especially the deficit in protein intake that undoubtedly occurred for the meat-type chickens in the male study due to anorexia is known to affect host tolerance negatively (Clough et al., 2016). Additionally, infections with *A. galli* impair the absorption of protein in the intestine (Hurwitz et al., 1972; Walker and Farell, 1976; Schwarz et al., 2011a). In chickens an increased supply with the amino acid lysine improved the tolerance to an *A. galli* infection (Daş et al., 2010), indicating that a nutritional intervention to maintain host performance is possible. Also in other host species the level of protein supply was associated with both host tolerance and resistance to parasites (Knox and Steel, 1996; Sykes and Coop, 2001; Kyriazakis and Houdijk, 2006). When given free choice to a high and low dense protein diet sheep infected with helminths preferred the high protein diet to meet the higher protein requirements that arise from the parasite infection (Kyriazakis et al., 1994). The higher nutrient requirement in face of parasite infections mainly results from the upregulation of pathways that are related to the induction of nutritional costly immune responses (Colditz, 2008; Rauw, 2012). Regarding infections with *A. galli* and *H. gallinarum* such costs likely emerge from production of humoral (Marcos-Atxutegi et al., 2009; Schwarz et al., 2011a; Norup et al., 2013; Daş et al., 2017, 2018) and cell-mediated immune responses

(Degen et al., 2005; Schwarz et al., 2011a,b; Pleidrup et al., 2014; Dalgaard et al., 2015) and additional nutrients needed for the repair of the tissue damage (Herd and McNaught, 1975; Ferdushy et al., 2012; Luna-Olivares et al., 2012).

The activation of components to maintain resistance and tolerance against the worms consequently results in the allocation of nutritional resources away from other physiological processes which are thus no longer available for performance related traits, i.e. growth and reproduction (Rauw, 2012); a trade-off function based on the "resource allocation theory" (Beilharz et al., 1993). Especially when resources are limited, e.g. in the case of anorexia induced reduction in nutrient availability, the impairment in host performance is better recognisable as shown for the meat-type chickens in the male study. Based on a large body of evidence the long-term artificial selection for high performances in farm animals has increased the risk of several physiological and metabolic disorders (Rauw et al., 1998; van der Most et al., 2011; Hocking, 2014) being in accordance with the assumption of a disturbed resource allocation of high performing genotypes during stressors (Rauw, 2012). Rauw (2012) therefore suggested that "artificial selection may result in preferential allocation of resources to the traits selected for, leaving animals lacking in ability to respond adequately to other demands". Referring to the observations made by our own in the male study the loss in performance in the high performing meat-type chickens was mainly based on the lower feed intake, as percentage loss in both was almost the same. However, it is not excludable that a disturbed nutrient allocation contributed to the lower tolerance of this genotype in addition. In contrast to the male study, the reduction in laying performance in the female study was not due to a lower feed intake, but was most likely associated with nutritional needs for immune responses confirmed by a worse feed conversion than controls, indicating an altered nutrient utilization. The allocation of nutrients for immune responses or rather the prioritization of nutrients from performance traits towards immune functions may have induced a nutritional deficiency to maintain overall laying performance. These results also indicate that birds under high physiological challenges may need higher amounts of essential nutrients to balance the loss of nutrients for other physiological purposes already mentioned above. However, what does the genotype dependent reduction in laying performance tell us? First, tolerance to nematode infections is performance dependent, demonstrated by a high tolerance of LD hens to the infections in the first weeks of infection, when the performance level is low. Second, as LD hens time-delayed reduced the laying performance, it clearly shows us that even if the overall performance level is different between genotypes, each genotype has a specific production peak at which the birds are vulnerable to environmental stressors. As shown with our data, the hens failed to tolerate the nematode infections when they reached their individual performance peak (e.g., total egg mass production), probably based on the different genetic programming for egg production. In contrast to our results, Sakkas et al. (2018) recently found no association between host resistance and tolerance and the performance level of broiler chickens exposed to coccidiosis. The different results found between our study and Sakkas et al. (2018) may be related to the genotypes used. In our study, genotypes of totally divergent performance directions have been used (layer- vs. meat- vs. dual-purpose-type chickens), whereas Sakkas et al. (2018) used broiler chickens with slightly different growth performance only, probably with a closer genetic relationship.

5.4 Host resistance and susceptibility to nematode infections

Both resistance against the worms and the susceptibility to naturally occurring infections fundamentally determine the final worm burdens of a host (Stear and Wakelin, 1998). Stear and Wakelin (1998) defined the term “resistance” as the ability of the host to control the parasites through immunological mechanisms, known as the specific resistance or acquired immunity, whereas the term “susceptibility” was defined as the trait of the host to provide an environment that is suitable for the parasite, often called non-specific or natural resistance. However the term “susceptibility” can also refer to the ability of the host animal to prevent from infections that in our study was specifically referred as the “susceptibility to re-infections” (e.g. the risk of a host animal to ingest parasite eggs). The data of the experimental infection make it possible to discuss differences between the acquired resistance (immunity) and natural resistance, whereas burdens of the second generation worms provide an insight into mechanisms that determine the susceptibility to natural re-infections.

The data with all genotypes shows that the worm expulsion was responsible for the elimination of the majority of worms. The worm expulsion is based on the acquired immunity of the host animal that is considered to be the most effective resistance mechanism of the host to cope with the parasites (Lawrence, 2003; Patel et al., 2009; Dold and Holland, 2010). The development of Th2 immune responses are known to mediate the worm expulsion (Lawrence, 2003; Patel et al., 2009; Dold and Holland, 2010; Grencis, 2015) as also shown with one of the genotypes in this study (Chaper Two). However, regarding the male study the differences in worm burdens found between the three genotypes were likely not exclusively based on the acquired immunity of the host, even if very slight differences in the humoral immune response between the genotypes that are related to host performance (Leshchinsky and

Klasing, 2001; Koenen et al., 2002; Parmentier et al., 2010; van der Most et al., 2011; Simon et al., 2014) may indicate a contribution to the different host resistance at least in parts.

Physiological differences between genotypes other than direct immune functions demonstrate that the natural resistance of a host to nematodes plays an important role in determining the worm burdens, too. We assume that the physico-chemical properties of the intestinal environment play a key role in natural resistance to the worms. As demonstrated with birds of the male study, worm burdens differed between the fast growing meat-type chickens and the extremely slow growing layer-type chickens (LB), whereas LB and LD birds harboured similar worm burden. Similarly, LB and LD hens in the female study did not differ in their worm burdens. As intestinal conditions (intestinal size, metabolite profile) were much more comparable between LB and LD birds than with the meat-type chickens (Chapter Three) the results seem not remarkable. The intestinal environment of the meat-type chicken may have provided more suitable conditions for the worms at the early phase of infection. Such interactions between the worms and their intestinal niche have often been described previously (Hauck, 2017; Kers et al., 2018). Concentrations of *Lactobacilli* or its metabolic product lactate, that was clearly highest for the meat-type chickens compared to the other genotypes, are further known to affect intestinal parasites positively (Walk et al., 2010; Reynolds et al., 2014; Jiang et al., 2016) although Petkevicius et al. (2004) also reported a negative influence of SCFA and lactate on worm burdens in pigs. However, the higher ileal lactate concentrations in the meat-type chickens in our study may have favoured the worms, indicating a worm- or host-species dependency. In studies by Daş et al. (2011b, 2012) non-starch polysaccharides (NSP) enriched diets benefited the worms in many ways. It was shown that the NSP increased the microbial fermentation and thereby caused a higher abundance of metabolites like SCFA (Daş et al., 2011b, 2012). However, the larger intestine as well as the higher feed intake in the meat-type chicken, additionally, may have reduced the worm competition for space and nutrients. Such a density dependent establishment of worms is known from other host parasite systems (Brown, 1986; Michael and Bundy, 1989).

Both the acquired immunity and components of the natural resistance (i.e., intestinal environment) of a host have undoubtedly decisive effects on the outcome of an infection probably with a different share. Differences in worm burdens between chicken genotypes have often been reported previously (Permin and Ranvig, 2001; Gauly et al., 2002; Schou et al., 2003; Kaufmann et al., 2011b; Wongrak et al., 2015). Whether these differences were based on the acquired immunity or the natural resistance of the birds was however not clarified. Nevertheless, the heritability estimated for worm burdens in chickens point out that selection for nematode resistance in chicken is possible (Kaufmann et al., 2011b; Wongrak et al., 2014). However, does selection for increased host resistance bring the desired success in reducing worm burdens in the long run? The selection for increased host resistance, the ability of the host to limit the pathogen burden due to several mechanisms (Best et al., 2008), will reduce worm burdens for a foreseeable time period (usually limited to study period), but probably will not reduce the worm burdens to a level that is harmless for the host in the long run (several weeks or months). Some authors suggested that the prevalence of the worms will decrease in a population if frequency of resistance genes is increased (Kutzer and Armitage, 2016). But does this assumption conform to the observations made by our own? Based on Darwin’s theory of fitness and the results of the worm expulsion study, the worms that survive the resistance mechanisms of the host will pass their genetic material to the next generation that therefore may have a greater fitness to survive the resistance mechanisms. The selection for resistance may thus be simultaneously accompanied by a selection in worm burdens, leading to a higher pathogenicity of the survivor worms and more successful re-infections by time. The resistance genes will consequently lose their advantage then (Kutzer and Armitage, 2016). The selection for increased host tolerance as well may thus have a higher relevance for farm animals than the selection for increased host resistance, as selection for tolerance will increases host robustness and maintain the health and performance of the host (Kutzer and Armitage, 2016). As host tolerance is rather host- than pathogen-specific, selection for an improved tolerance may have also some beneficial effects concerning other infectious pathogens (Doeschl-Wilson and Kyriazakis, 2012).

The modulation of the worm’s habitat by feeding dietary components may constitute an alternative approach to reduce the susceptibility to the nematodes without performing genetic improvement towards parasite resistance. However, previous approaches to reduce worm burdens by using dietary components as a nutritional intervention strategy have not yielded in desired success for nematode species in chickens. The idea to feed inulin enriched diets to reduce worm burdens as effectively shown in pigs at least with specific worm species (Petkevicius et al., 1997; 2001; 2003) did not work in the chicken host infected with *A. galli* or *H. gallinarum* (Daş et al., 2011b, 2012). Allicin from garlic, an antimicrobial agent (Wallock-Richards et al., 2014), had no deworming effect (Velkers et al., 2011). Citrus peels ethanolic extracts, however, have had anthelmintic effects on *A. galli* (Abdelqader et al., 2012). Nevertheless, further studies are needed to find efficient nutritional approaches.

Next to the acquired and natural host resistance the susceptibility to re-infections influences the worm population dynamics. Although in the male study meat-type chickens were less resistant to *A. galli*, most likely due to the intestinal environment, which apparently provides a better habitat for the worms, the re-infections observed with *H. gallinarum* were close to zero for this genotype but highest for the layer-type chickens, which in reverse had the lowest *A. galli* burden. Even if a host genotype provides a more suitable environment for worms than other genotypes do, this genotype is not necessarily predisposed to re-infections and *vice versa*. The higher susceptibility to re-infections found in both male and female layer-type chickens compared to the dual-purpose and meat-type chickens was likely based on behavioural differences. There is evidence that dual-purpose chickens are less active than the layer-type chickens (Malchow et al., 2018). Comparing layer- and meat-type chickens, the latter are less active and show less ground foraging behaviour (Lindqvist et al., 2006; Tickle et al., 2018). Such a relationship between host behaviour and parasite load is well known (Ezenwa et al., 2016), although the host behaviour in reverse can also function as an active defence strategy of the host (Hart, 2011). As re-infections determine final worm burdens (Daş et al., 2018) the behaviour of the host may play a key role in determining the worm burden and the growth of the worm population. However, studies comparing genotypes with such disparate behaviours have apparently not been performed yet. Nevertheless, the susceptibility to re-infections may also be of importance considering the costs of an infection. As it is already known mainly the larvae induce pronounced humoral host immune responses (Marcos-Atxutegi et al., 2009; Daş et al., 2017, 2018). Birds that harbour larger number of larvae, which descend from continuous re-infections, may therefore have higher immunological costs (Colditz, 2008). Next to the acquired immunity, the components of the natural resistance and the susceptibility to naturally occurring re-infections are sustainable approaches to affect host resistance efficiently as well. The two latter are possible strategies to improve host resistance in a more natural way without selecting directly for host immunity, considering that selection for increasing host immunity can negatively affect host performance (van der Most et al., 2011).

5.5 Implications for the use of a dual-purpose genotype

The results of the present thesis clearly show that both male and female chickens of a dual-purpose genotype have benefits in their ability to cope with nematode infections as compared to conventionally used performance-specified genotypes. The male birds of the dual-purpose genotype had a higher tolerance to the infections than the high performing meat-type chickens, demonstrated by an impaired growth rate in the meat-type chickens only. The higher tolerance of the male dual-purpose chickens than the classical meat-type chickens is of advantage if the increasing attention for animal welfare concerns (Boogaard et al., 2006; Tonsor et al., 2009; Van Loo et al., 2014) should lead to a prolonged fattening time of meat-type chickens in the future that increases the risk for nematode infections. The female dual-purpose hens, however, tolerated the infection only in the first weeks of laying period, whereas performance was only impaired in the advanced laying period, when overall host performance (i.e. per capita egg mass production) increased. Nevertheless, the classical laying hens reduced the performance already in the beginning of lay, indicating a better tolerance against nematode infections for dual-purpose hens at least for a certain time period. However, as dual-purpose hens reduced their performance only at peak of lay, it might be assumed that the lower laying persistency by time result in a renewed tolerance ability in the advanced infection period. However, this needs to be studied in future studies. Both male and female birds of the dual purpose genotype were less susceptible to re-infections than the layer-type chickens, which are mainly used at least for egg production. In our opinion the lower susceptibility was expected to be in association with the less active behaviour if compared with the classical layer-type chickens (Malchow et al., 2018) that likely resulted in a lower ingestion of nematode eggs from the environment. As final worm burdens in the field are determined by the continuous intake of nematode eggs (i.e., naturally occurring re-infections), the less active behaviour of dual-purpose chickens may result in lower final worm burdens. Thus, direct (i.e., reduced host performance) and indirect effects (i.e., co-infections with bacteria, increased mortality) of the worms on the host animal may occur in lesser degrees. Concerning meat-type chickens, a further advantage of dual-purpose chickens yields from the lower incidence of foot-pad dermatitis and skeletal disorders (i.e. splayed legs) that are current major health problems in meat-type chickens (Knowles et al., 2008; Shepherd and Fairchild, 2010). Additionally, mortality was lower in the dual-purpose than meat-type chickens, likely a consequence of the high cardiovascular problems of meat-type chickens (Olkowski, 2007) and a comparable stronger heart for the dual-purpose chicken based on a greater number of heart capillaries and beneficial aortic mechanical properties (Harash et al., 2019). Infection did not affect mortality. In laying hens the less active behaviour of the dual-purpose genotype was associated with the lower feather pecking behaviour and cannibalism as compared with classical laying hens finally resulting in better plumage conditions and lower mortality rates (Giersberg et al., 2017). Considering the prohibition of beak trimming in classical laying hens in Germany since 2017 (Anonymous, 2014) this might have important implications for animal health in the field.

The higher tolerance, demonstrated with the male birds and at least partly with the female birds, and the lower susceptibility to nematode infections as well as the obvious better fitness prospects of the dual-purpose chickens imply a lower necessity to use costly anthelmintics and antibiotics. In addition, it cannot be ruled out that resistance and tolerance against chicken pathogens others than nematodes occurs similarly. Fundamentally seen, dual-purpose genotypes have crucial benefits in relation to animal health and welfare if compared to classical meat- and layer-type chickens. But are dual-purpose chickens the best alternative to avoid the culling of male layer chicks if compared with other alternatives? The main benefit of using dual-purpose chickens is that two problems will be solved with one approach: the culling of male layer chicks and the several health and behavioural problems of classical meat- and layer-type chickens, which cannot be avoided when in-ovo sex determination is applied. However, as dual-purpose chickens were considered to replace the classical laying hens but not the broiler chickens completely, improvement in animal health related traits may mainly appear for the layer lines then. The two alternatives, the in-ovo sex determination (Weissmann et al., 2013, 2014; Galli et al., 2016, 2017, 2018) and the fattening of the male layers, will solve the culling problem as well but neither will have positive effects on the health of the meat- and layer-type chickens. The fattening of male layers anyway is not considered as the best approach to avoid the culling. The extremely low growth rates and the high feed requirement compared to the classical meat-type chicken (Damme and Ristic, 2003; Gerken et. al., 2003; Murawska and Bochno, 2007; Koenig et al., 2012; Kaufmann and Andersson, 2013) cause high production costs (Kaufmann and Andersson, 2013). However, not only the fattening of male layers but also that of dual-purpose chicken is associated with higher feed requirements, although the fattening of dual-purpose chickens is much more efficient due to a higher feed conversion and faster growth rate than that of male layers as observed by our own. Nevertheless, in face of an increasing world population (Dunwell, 2013; Alexandratos and Bruinsma, 2012; FAO, 2017), the increasing demand for poultry products (FAO, 2006; Alexandratos and Bruinsma, 2012; Preisinger, 2018) and the growing scarcity of resources for the nutrition of humans and animals then (Balatsky et al., 2015), dual-purpose genotypes may waste important natural resources. In this context, the in-ovo sex determination is a resource friendly approach that further on is currently expected to be the cheapest alternative. However the in-ovo sex determination does not really solve the ethical problem by sorting out male layers as an embryo has already been started to develop when sex is determined (Krautwald-Junghans et al., 2018). The use of the in-ovo sex determination in layer hatcheries is further on not yet possible at this time, whereas dual-purpose genotypes are already available. Further improvement in performance of the dual-purpose genotypes may increase their chance to get used widely in practice. Which alternative will be used in the future to avoid the culling is currently not known and will probably be dependent on the consumers demand and political decisions and probably the countries.

5.6 General conclusions

Meat-type chickens with high growth rate are less tolerant to nematode infections than the lower performing male birds of dual-purpose and layer-type chickens as shown by an impaired growth in the high performing meat-type chickens only. The lower tolerance of this genotype is likely associated with the lower nutrient intake induced by a reduction in feed intake, resulting in a nutrient deficiency. Resistance to the nematodes is host and worm species dependent and is presumably a consequence of differences in the intestinal environment more favourable in the high performing meat-type chickens at least for *A. galli.*

The tolerance of laying hens against nematode infections is clearly associated with the performance level of the host genotype, yet it does not mean that tolerance is automatically higher in lower performing genotypes as hens of both genotypes are vulnerable at their individual production peaks. Resistance to both worm species is not different between the genotypes. Infections impair economically and market relevant egg quality traits as well as yolk fatty acids, probably with disadvantages for the offspring. In both males and females naturally occurring re-infections occur more pronounced in the layer-type chickens than dual-purpose and meat-type chickens, likely because of behavioural differences. In all genotypes, independent of host sex, the majority of worms were expelled in a short time, a well-known mechanism of host resistance called worm expulsion. Juvenile worms are specifically targeted. Only a small population of worms survive the expulsion mechanisms, reach maturity and induced re-infections. Humoral and local immune responses are associated with worm expulsion. It is concluded that the ability to cope with nematode infections in both male (growth rate) and female chickens (laying performance) is host performance dependent.

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ZUSAMMENFASSUNG

Das Ziel der vorliegenden Arbeit war es zu untersuchen, ob die Zucht auf hohe Leistungen (i.e.S., Wachstum und Legeleistung) beim Nutzgeflügel zu Einschränkungen in der Resistenz und Toleranz gegenüber Nematoden-Infektionen geführt hat. Weiterhin wurde das Phänomen der Wurmausscheidung analysiert sowie die relevanten Mechanismen untersucht. Anhand der gewonnenen Ergebnisse sollte abgeleitet werden, ob Zweinutzungshühner Vorteile gegenüber konventionell verwendeten Mast- und Legegenotypen haben. Die Arbeit wurde am Institut für Ernährungsphysiologie ‚Oskar Kellner' des Leibniz Instituts für Nutztierbiologie (FBN) in Dummerstorf bei Rostock im Zeitraum von 2015 bis 2018 durchgeführt. Die Untersuchungen wurden in zwei Versuchen getrennt voneinander durchgeführt.

Im ersten Versuch wurden Hähne dreier Genotypen mit unterschiedlichem Wachstumspotential verwendet, welche für die Erzeugung von Fleisch (Masttiere, Ross-308, R), Eiern (Legetiere, Lohmann Brown Plus, LB) oder beidem (Zweinutzungshuhn, Lohmann Dual, LD) gezüchtet wurden. Die Tiere wurden als Eintagsküken eingestallt und über 10 Wochen gehalten. Am 7. Lebenstag wurde eine definierte Anzahl an Tieren je Genotyp experimentell mit Eiern der Nematoden *Ascaridia galli* und *Heterakis gallinarum* infiziert (250 Eier je Wurmspezies), während nicht-infizierte Tiere als Kontrollen dienten. Mit Beginn der Infektion wurde eine definierte Anzahl an Tieren im wöchentlichen Intervall geschlachtet, um Infektionsparameter (z.B. Wurmzahlen) zu erheben. Leistungsdaten (Futteraufnahme, Körpergewicht) wurden wöchentlich ermittelt. Im zweiten Versuch wurden konventionelle Legehybriden (Lohmann Brown Plus) und Hennen der Zweinutzungslinie Lohmann Dual miteinander verglichen. Die Hennen wurden im Alter von 24 Wochen mit insgesamt 1000 Eiern von *A. galli* und *H. gallinarum* infiziert (500 Eier je Wurmspezies). Eine vergleichbare Zahl an Hennen diente als Kontrollen. Die Hennen wurden über einen 18-wöchigen Zeitraum untersucht, in denen beide Genotypen ihr maximales Leistungsniveau erreichten. Eine bestimmte Anzahl an Hennen wurde zu definierten Zeitpunkten (2, 4, 6, 10, 14 und 18 Wochen nach der Infektion) geschlachtet, um Wurmparameter zu erheben. Die Legeleistung (Eianzahl, Eigewicht) wurde täglich erhoben. Die Futteraufnahme und das Körpergewicht sowie Blut- und Eigelbproben wurden wöchentlich zur Antikörperanalyse ermittelt.

Sowohl im Versuch mit den männlichen als auch weiblichen Tieren wurde eine starke Reduktion der Wurmbelastung mit beiden Wurmspezies über die Zeit beobachtet, welche unabhängig vom Geschlecht des Tieres und der Leistungsrichtung war. Daher entschieden wir uns dafür, das Ausmaß und die Dauer der Wurmausscheidung mit beiden Wurmspezies zu untersuchen. Humorale und zell-vermittelte Immunantworten sowie damit verbundene

Populationsdynamiken wurden in diesem Kontext ebenfalls untersucht **(Chapter Two)**. Es zeigte sich, dass Hühner in der Lage sind einen Großteil der Wurmbelastung mit *A. galli* und *H. gallinarum* in kurzer Zeit zu eliminieren. Die Ausscheidung der Würmer erfolgte dabei in drei strikten Phasen. Die erste Phase der Wurmausscheidung beschränkt sich dabei alleinig auf den Tag der Infektion, an dem die Larven im Darm schlüpfen und sich der Peristaltik des Darms entgegensetzen müssen. Die Passagezeit des Darminhaltes ist vermutlich die größte Herausforderung für die Larven um sich im Darm zu etablieren. Die zweite Phase der Ausscheidung, welche gleichzeitig am effizientesten ist, wird vermutlich durch lokale Immunantworten im Darm gesteuert. Wir nehmen an, dass die erhöhte Expression von Th2-Zytokinen und Muzin-regulierenden Genen zu einer verstärkten Motilität des Darms führt und damit die Ausscheidung der Würmer stimuliert. Die Ausscheidung der Würmer stand grundsätzlich im Zusammenhang mit dem Entwicklungsstadium, da hauptsächlich junge Wurmstadien eliminiert wurden. Da insbesondere Larven negative Effekte auf den Wirt haben, ist die Eliminierung der Larven eine präventive Strategie des Wirtstieres. Nur eine kleine Anzahl an Würmern war in der Lage den Ausscheidungsmechanismen zu trotzen. Diese Würmer erreichten die sexuelle Reife und induzierten Re-Infektionen (dritte Phase). Dass nur eine kleine Anzahl an Würmern sich erfolgreich im Tier etablierte steht vermutlich mit dem genetischen Hintergrund der Würmer im Zusammenhang.

Im Versuch mit den männlichen Tieren **(Chapter Three)** induzierte die Infektion eine Reduktion der Futteraufnahme in allen drei Genotypen, während die Futterverwertung trotz infektionsbedingter Veränderungen im Darmgewebe nicht beeinflusst wurde. Die Infektion reduzierte nur die Wachstumsleistung der hochleistenden Masthähnchen (R), wohingegen die Infektion bei den langsam-wachsenden LB und LD Hähnen keine Auswirkungen auf die Wachstumsleistung hatte. Dies wies daraufhin, dass die Toleranz gegenüber Wurminfektionen abhängig vom Leistungsniveau des Genotyps ist. Die beeinträchtigte Toleranz der R Hähne war assoziiert mit dem relativen Mangel an Nährstoffen, welcher durch die geringere Futteraufnahme zustande kam. Basierend auf dem unterschiedlichen Nährstoffbedarf der Genotypen, führte das nährstoffreiche Masthähnchen-Futter bei den langsamer wachsenden LB und LD Hähne zu einer Überversorgung mit Nährstoffen, die vermutlich zu einer besseren Toleranz gegenüber den Nematoden führte. Die *A. galli* Belastung war höher in den R als LB Hähnen, während sich die Wurmbelastung der LD Hähne von keinem der beiden Unterschied. Die höhere *A. galli*-Belastung in R war vermutlich mit den Gegebenheiten der Darmumwelt assoziiert. Der größere Darm, die höhere Nährstoffdichte aufgrund der Futteraufnahme sowie die höhere Konzentration an Milchsäure im Darm mögen vorteilhaft für die Etablierung und

Überlebensfähigkeit der Würmer sein. Die Wurmbelastung mit *H. gallinarum* nach der experimentellen Infektion war hingegen nicht unterschiedlich zwischen den Genotypen. Die Anfälligkeit gegenüber Re-Infektionen mit *H. gallinarum* hingegen war höher für die LB als LD Hähne, während R Hähne kaum Re-Infektionen zeigten. Die unterschiedliche Anfälligkeit gegenüber Re-Infektionen war vermutlich mit der unterschiedlichen Aktivität der Tiere bzw. dem unterschiedlichen Verhalten assoziiert.

In dem Versuch mit den weiblichen Tieren **(Chapter Four)** hatten die Infektionen keinen Effekt auf die Futteraufnahme und das Körpergewicht, wohingegen die Verwertung des Futters in Eimasse beeinträchtigt wurde. Die Legeleistung wurde infektionsbedingt in beiden Genotypen reduziert, wenn auch zu unterschiedlichen Zeitpunkten. Infizierte LB Hennen reduzierten die Legeleistung bereits in der frühen Infektionsphase (i.e.S., ab 3. Woche p.i.), während die Legeleistung der LD Hennen erst Wochen später zurück ging (i.e.S., ab der 14. Woche p.i.). Die Reduktion der Legeleistung war assoziiert mit dem Zeitpunkt der maximalen Legeleistung, welcher bei den LD Hennen im Vergleich zu den LB Hennen verspätet auftrat. Nichtsdestotrotz zeigen die Ergebnisse, dass die Toleranz gegenüber Wurminfektionen abhängig vom Leistungsniveau ist. Im Gegensatz zu den männlichen Tieren war die Wurmbelastung mit beiden Wurmspezies nicht unterschiedlich zwischen den zwei Genotypen. Die Anfälligkeit gegenüber Re-Infektionen hingegen war auch hier höher für die LB als LD Hennen. Die Infektion reduzierte die Frequenz größerer Eier und führte damit zu einer Änderung der Eigrößenverteilung. Weiterhin führte die Infektion zu einem geringeren Eigewicht und reduzierte die Dotterfarbe und den Fettanteil im Eidotter. Der Anteil mehrfach-ungesättigter Fettsäuren (PUFA), insbesondere n-6-PUFA, war ebenso geringer im Eidotter infizierter Tiere. Hiermit wurde erstmalig gezeigt, dass Wurminfektionen in Legehennen vermarktungsrelevante Eiqualitätsparameter negativ beeinflusst. Änderungen in der Eidotter-Zusammensetzung könnten weiterhin negative Auswirkungen auf den Nachwuchs haben.

Zusammenfassend lässt sich sagen, dass die Toleranz gegenüber Nematoden-Infektionen abhängig ist von dem Wachstumspotential bzw. der Legeleistung männlicher bzw. weiblicher Hühner. Die zeitabhängige Beeinträchtigung der Legeleistung begründet sich vermutlich durch die unterschiedliche genetische Programmierung der Legeleistung und Legepersistenz der zwei Hennen-Genotypen. Die unterschiedliche Toleranz der männlichen Tiere war vermutlich assoziiert mit der Disbalance zwischen dem Genotyp-spezifischen Nährstoffbedarf und der Nährstoffversorgung während des Infektionsgeschehens. Daraus lässt sich ableiten, dass das individuelle Leistungsniveau der Tiere sowie die Nährstoffversorgung der Tiere während Infektionen eine bedeutsame Rolle in der Toleranzfähigkeit einnehmen.

Die Resistenz gegenüber Nematoden-Infektionen jedoch ist abhängig vom Typ der Infektion (i.e.S., experimentell vs. natürliche Re-Infektion). Während die allgemeine Resistenz gegenüber Nematoden-Infektionen abhängig ist vom Immunstatus sowie den Bedingungen der Darmumwelt, scheint die Anfälligkeit gegenüber Re-Infektionen im Zusammenhang mit dem Verhalten der Tiere zu stehen.

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