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Research paper

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

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ABSTRACT

Modern chickens have been genetically developed to perform high under optimal conditions. We hypothesized that high-performance is associated with a higher sensitivity to environmental challenges in laying hens. By using 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 1000 eggs of *Ascaridia galli* and *Heterakis gallinarum* at 24 weeks of age. Hen performance parameters, humoral immune responses in plasma and egg yolks and worm burdens were assessed at several occasions over a period of 18 weeks post infection (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 laying rate in infected LB hens decreased significantly ($P < 0.05$) in the early infection period (i.e. by 3 wpi), the decrease in LD hens appeared much later (i.e. by 14 wpi). Worm burdens resulting from the experimental infection were 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. 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 subsequent natural 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 poly-unsaturated fatty acids (PUFA), especially *n*-6-PUFA, was also lower in egg yolks of the infected hens ($P = 0.032$).

We conclude 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. The two genotypes exhibited similar levels of resistance after a fully controlled experimental infection, but the high performing hens were more susceptible to subsequent natural infections. Infections negatively affected economically important egg-quality traits, including egg weight, fat content and fatty acid profiles in egg yolks.

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

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(Zuidhof et al., 2014). As a consequence of selection for increased production efficiency, high performance in chickens is 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). An increased persistency in lay performance through extending the productive life of hens is a current breeding goal (Bain et al., 2016). Thus a higher physiological load, i.e., an ever greater performance associated physiological pressure, may be expected for hens bred in future. Animals being continuously under high physiological pressure often fail to mount effective immune responses against pathogenic stressors, likely due to a disturbed allocation of metabolic resources (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). In Germany alone, approximately 46 million day-old male birds are killed annually (Destatis, 2017). Globally, there are 7.5 billion laying hens (FAO, 2016). This implies the maximum number of male birds that might potentially be killed after hatch, assuming that hatch sex ratio is 1:1. 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 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 (Permin et al., 1999; Thapa et al., 2015). 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 (Das 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, particularly with *Ascaridia galli* and *Heterakis gallinarum,* 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 exhibit a lower performance level than high-performing genotypes (Mueller et al., 2018; Siekmann et al., 2018), high-performance associated health and welfare problems may be 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. Our objective was to use 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 into the 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 the remaining 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 × genotype × 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 subsequent natural infections to occur. 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) was 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). Eggs of both worm species were assessed several times to determine the percentage of fully embryonated eggs as described elsewhere (Rahimian et al., 2016). 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 1000 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

Randomly selected hens (n = ca. 30 per wpi) were necropsied at 2, 4, 6, 10, 14 and 18 wpi to quantify worm burdens (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 h 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-pincers 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 h at 40 °C). Thereafter the EDTA-solution was passed through a 20 µm sieve to collect the tissue larvae.

H. gallinarum were harvested from 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 at 40x magnification. 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 species.

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; i.e. 7–11 hens per genotype and wpi). 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 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 1 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 1). Blood (N = 677, i.e. 7–10 hens per genotype, infection status and wpi) 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 2500 *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). For ascarid specific IgY in both plasma and egg yolks, single measurements were performed. The laboratory-specific intra- and inter-assay coefficients of variability for the assay were 5.0% and 8.4%, respectively.

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 number of eggs collected per genotype and infection status ranged from 20 to 53 at 2 wpi, and decreased to 11 to 19 at 14 wpi. The analyses were performed at the University of Hohenheim as described by Simons (2017); Grashorn (2016, 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 N (N) and maximum force to break the shell was recorded, thereafter. Head speed was 5 mm/min. The eggs were broken on a glass plate. Albumen was removed from the shells, whereas, membranes were left. The 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 their weights by 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. Egg yolk (N = 163, i.e. 4–11 samples per genotype and infection status and wpi) was separated from the albumen of each egg. The egg yolk was carefully rolled on a filter paper to remove any albumen residues, ensuring a complete separation of both egg components. The egg yolks were stored at −20 °C until analyses.

After homogenization of frozen egg samples and the addition of 0.6 mg C19:0 as an internal standard, total egg lipids were extracted in duplicate using chloroform/methanol (2:1, v/v) and an Ultra Turrax T25 (IKA, Staufen, Germany) 3 × 15 s at 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% (w/v) $CaCl_2$ solution. After centrifugation (530 *g*, 5 min), the organic phase was dried with Na_2SO_4 and K_2CO_3 (10:1, w/w), 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 60 °C water bath for 10 min. Subsequently, 0.5 ml of 14% boron trifluoride (BF_3) in methanol was added to the mixture, which was then shaken for an additional 10 min at 60 °C. 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 °C 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 x0.25 mm, Agilent, Santa Clara, CA, United States) 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, held for 5 min; before subsequently 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 min. 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 using C19:0 as the internal standard. For the calibration procedure the reference standard mixture 'Sigma FAME' (Sigma-Aldrich, Deisenhofen, Germany), the methyl ester of C18:1*cis*-11, C22:5*n*-3 and C18:2*cis*-9,*trans*-11 (Matreya, PA, USA), C22:4*n*-6 (Sigma-Aldrich, Deisenhofen, Germany) and C18:4*n*-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-, mono-unsaturated- and poly-unsaturated 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 (mono-unsaturated 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 (poly-unsaturated 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 (i.e. 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 replicates 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 block effect of pens. 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 fixed effects of infection, genotype, wpi and their interactions, plus block effect of pens. 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 fixed 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 fixed effects of genotype, infection, wpi, all possible interactions among these three factors and plus block effect of pens. Egg yolk anti-ascarid-antibody levels, which were measured on randomly selected eggs, were analyzed using a linear-mixed model including the fixed effects of infection, genotype, wpi, their interactions and block effect of pens.

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)

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

	Genotype				Infection				wpi		Interactions, P-Value, ≤		
Item	LD	LB	SE	P, ≤	Con.	Inf.	SE	P, ≤	P, ≤	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, kg/hen	1.74	1.89	0.29	0.001	1.83	1.80	0.28	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).

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 correction for 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 dependent on wpi. 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 fewer eggs than their uninfected control counterparts in the advanced infection period (i.e. 14, 17 and 18 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 of infected LB hens 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 XL-eggs less frequently (0.9 vs. 3.5%) but more S-size eggs (13.3 vs. 10.7%, respectively). 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). Over time (i.e. wpi), infected LB hens laid a 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).

LB hens consumed a significantly higher amount of feed than LD hens ($P < 0.001$; Table 1). Average daily feed intake of infected hens

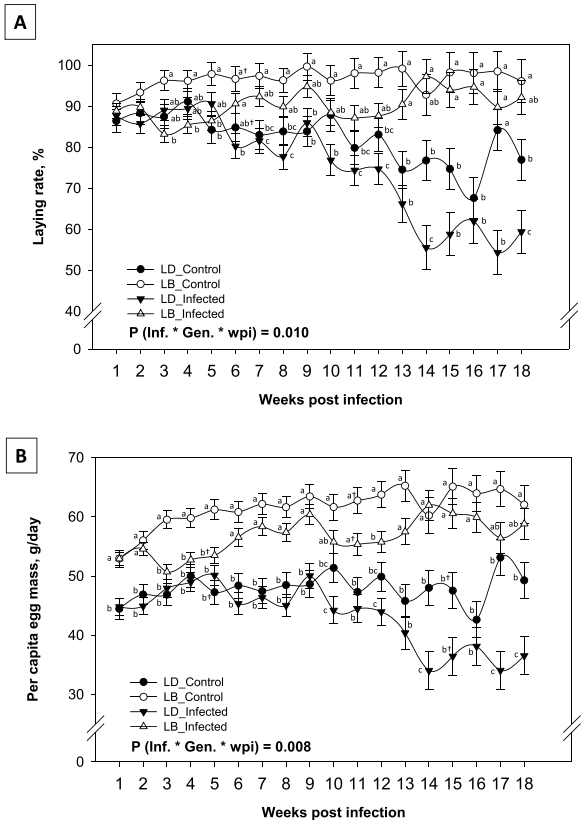

Fig. 1. Laying rate (**A**) and per capita egg mass production (**B**) in high (Lohmann Brown, LB) or lower (Lohmann Dual, LD) performing laying hens exposed to mixed nematode infections.
abc: indicate significant (Tukey, $P < 0.05$) differences between the four groups at the same time point. The values are LSMEANS with SE on the error bars.

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 entire 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

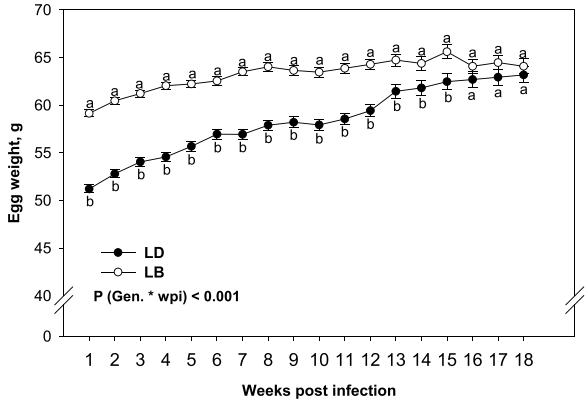

Fig. 2. Time-dependent alterations in egg weight in a high (Lohmann Brown, LB) or lower (Lohmann Dual, LD) performing host genotype.
ab: Indicates significant differences (Tukey, $p < 0.05$) between two genotypes at the same time point. The values are LSMEANS with SE on the error bars.

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).

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 = 0.110; 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).

The overall average total *H. gallinarum* burden was not different between the LD and LB hens (P = 0.453; 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 = 0.563), there was a significant difference in the number of larvae originating from re-infections (P < 0.001). The LB hens had increasingly higher numbers of second-generation larva than LD hens by wpi 14 (Fig. 3). Non-larva worm counts were not significantly different between the genotypes (P = 0.415).

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	576	545	103.8	0.058	0.001	0.066
EPD	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).
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.
*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.

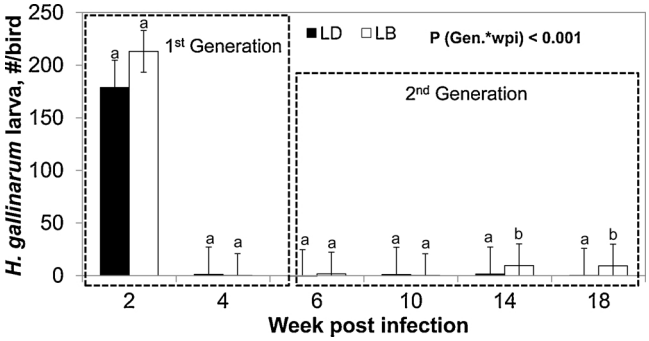

Fig. 3. *Heterakis gallinarum* larvae (total) in high or lower performing host-genotypes after experimentally induced and subsequent re-infections.
P (Gen.*wpi) describes the interaction effect between host genotype and weeks post infection in the entire study period.
ab: indicate significant (Tukey, P < 0.05) differences between the two genotypes at the same time point. The values are LSMEANS with SE on the error bars.

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

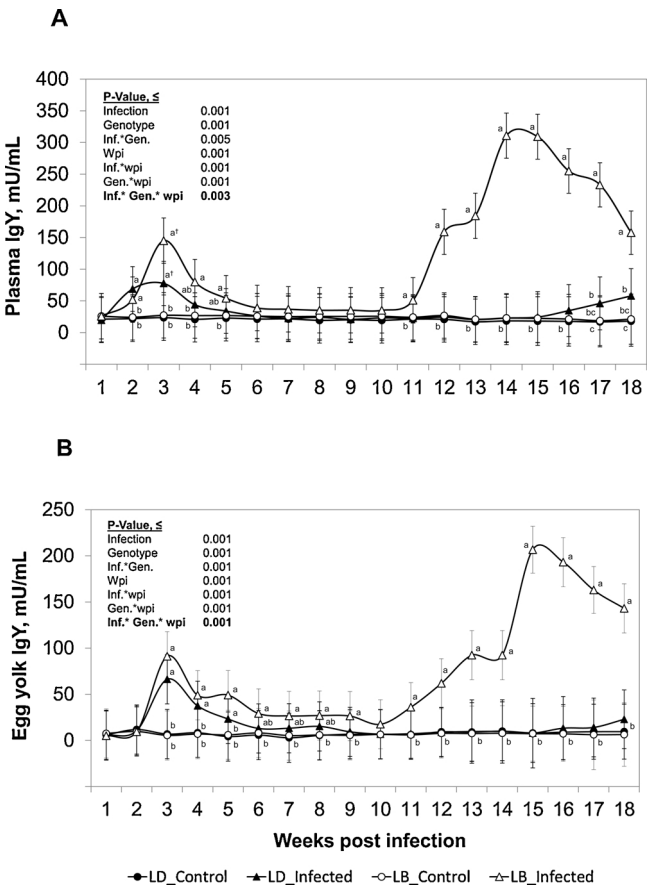

Fig. 4. Development of ascarid-specific IgY in plasma (A) and egg yolks (B) as response to mixed *Ascaridia galli* and *Heterakis gallinarum* infections in two chicken genotypes with different performance levels.
abc: indicate significant ($P < 0.05$) difference between the four groups at the same time point. The values are LSMEANS with SE on the error bars.

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 significantly 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 significantly different between the two genotypes up to wpi 8 (Fig. 4B; $P > 0.05$). By wpi 11, IgY levels in egg yolks were permanently higher ($P < 0.05$) in the infected LB hens than those of infected LD hens until end of the experiment, while IgY in egg yolks of infected LD hens remained at the level of controls ($P > 0.05$). Egg-yolk IgY levels of uninfected control animals of both genotypes did not differ at any time point ($P > 0.05$).

3.6. External and internal egg quality

With the exception of the elasticity ($P = 0.377$) and the egg shell proportion ($P = 0.752$), 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 mono-unsaturated FA (MUFA) than LD hens in the egg yolks, whereas percentage of poly-unsaturated 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

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

	Host genotype				Infection				wpi		Interactions, P-Value, ≤		
	LD	LB	SE	P, ≤	Con.	Inf.	SE	P, ≤	P, ≤	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).
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.
[1] Measured with the DSM Yolk Colour Fan (DSM-YCF) scale.

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. 5.7%; $P = 0.045$), 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.

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 the 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 subsequent natural infections were assessed using a 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 the 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 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

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

	Host genotype				Infection				wpi			Interactions, P-Value, ≤	
Item	LD	LB	SE	P, ≤	Con.	Inf.	SE	P, ≤	P, ≤	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, %	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.

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, i.e. subsequent natural infections. 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 for other chicken parasite diseases, e.g. coccidiosis (McDougald et al., 1990; Lensing et al., 2012), histomonosis (Liebhart et al., 2013) and ectoparasite infestations (Mullens et al., 2009), whereas most nematode-infection studies reported only negative effects on nutritional parameters (i.e., feed intake and feed conversion efficiency; Das 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 observed in this study may therefore be a consequence of the multi-species infection model, which in addition is clearly more representative of the conditions in the field. The infection with two worm species may therefore have developed a more severe physiological pressure on the hens than that by single worm species. 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 observed 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 both growth rate and feed intake were decreased in equal shares, feed intake was not affected by infections in either genotype used in the present study (Table 1). 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 et al., 2007), likely to compensate for an imbalanced nutrient in a specific diet (Das et al., 2010).

Laying performance in both genotypes was reduced at different time points (Fig. 1). 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 fewer 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 genetic programming, infected LD hens were not able to maintain their performance level at that of non-infected LD hens. Collectively, these results are indicative of 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 with both species, originating from the experimental infection, decreased over the study period (Table 2). The results are 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 subsequent natural 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 subsequent natural 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 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. (2019), 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 the shorter prepatent period of *H. gallinarum* than that of *A. galli* (Ramadan and Abou Znada, 1991; 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 altered 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, 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 health status of the birds (Sepp et al., 2011; Lucas et al., 2014), potentially indicating 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 consumers' perceptions (Grashorn, 2016), thus being of crucial importance for marketing of the eggs. Thus, strategies to reduce worm burdens benefit not only the animals, by increasing animal health and welfare, but may 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 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 eggs from 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 reduced feed conversion efficiency may indicate a diverted distribution of fat components 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., 2011; 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 an imbalance of fatty acid composition might be indicative of an impaired reproductive fitness, as they serve as a source of energy or are essential nutrients for the 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 conforms to the course of infections. As known from other recent nematode infection 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 have migrated back from the tissue to the lumen, IgY concentrations decreased again (by 4 wpi). During the primary immune response, LB hens exhibited higher IgY levels than LD did, even if not significant. However, different IgY levels agree with 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 induced by subsequent natural 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. The effect of 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 from those of controls during the entire study period (except wpi 10), indicating that IgY antibodies are more stable in egg yolks than in plasma. Because more than one follicle is formed in 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. Particularly relationships between IgY and worm burdens in different developmental stages should be investigated in detail.

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., experimental vs. subsequent natural infections). The two genotypes exhibited similar levels of resistance after a fully controlled experimental infection, but the high performing hens were more susceptible to subsequent natural infections. Infections negatively affected economically important egg-quality traits, including egg weight, fat content and fatty acid profiles in egg yolks.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.108925.

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