Selection for pro-inflammatory mediators produces chickens more resistant to *Eimeria tenella*

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ABSTRACT We recently developed a novel selection method based on identification and selection of chickens with an inherently high and low phenotype of pro-inflammatory mediators, including interleukin (IL)-6, CXCLi2, and CCLi2. The resultant high line of chickens is more resistant to *Salmonella enterica* serovar Enteritidis (*Salmonella* Enteritidis) compared to the low line. In the current study, we sought to determine if the high line birds were also more resistant to the protozoan parasite *Eimeria tenella*. In three separate experiments, 14-day-old chickens from the high and low lines were challenged orally with 10 $\times$ 10^3 to 45 $\times$ 10^3 *E. tenella* oocysts. Birds were sacrificed 6 d postchallenge and the caeca was removed and scored for lesions and body weight gain compared to mock-infected controls. The high line birds were more resistant to intestinal pathology as demonstrated by lower lesion scores ($P \leq 0.04$) compared to the low line. There were no differences in body weight gain between the lines. The results from this study showed that in addition to enhanced resistance against *Salmonella* Enteritidis, high line chickens are also more resistant to the pathology associated with coccidial infections compared to the low line birds. Taken together with our initial study utilizing the high and low lines, selection based on increased pro-inflammatory mediator expression produces chickens that are more resistant to both foodborne and poultry pathogens, including cecal pathology associated with costly coccidial infections.

Key words: broiler, coccidiosis, *Eimeria tenella*, pro-inflammatory, selection for resistance

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INTRODUCTION

Coccidiosis is a parasitic disease caused by apicomplexan protozoa that are members of the genus *Eimeria* and is an important disease in all animal livestock production, especially the poultry industry. Coccidiosis is one of the most widely reported and economically important diseases affecting commercial poultry production, with an estimated cost over $800 million USD annually in the United States and in excess of $3 billion USD worldwide (De Gussem, 2007; Sharman et al., 2010; Blake and Tomley, 2014). At least 70% of that cost is a result of subclinical coccidiosis characterized by substandard flock performance, including decreased weight gain and increased feed conversion. The disease outcome can range from mild enteritis to death and is dependent on numerous factors, including the *Eimeria* species involved, the severity of the infection, and the site of infection. The seven species of *Eimeria* known to infect chickens are *E. tenella*, *E. acervulina*, *E. maxima*, *E. brunetti*, *E. necatrix*, *E. praecox*, and *E. mitis*. Each species is pathogenic to chickens in varying degrees; however, *E. acervulina*, *E. maxima*, and *E. tenella* are the most pathogenic and prevalent within the broiler industry (McDougald, 1998). Each species targets a distinct region of the gut: *E. acervulina* pathology is characterized by white lesions in the upper small intestine, *E. maxima* results in petechiae in the midgut, while *E. tenella* targets the cecal pouches and results in bloody feces (Hammond and Long, 1973).

Current prevention of coccidiosis in poultry flocks is achieved by strict husbandry practices combined with the addition of anticoccidial drugs to the feed, and by vaccination with live parasites (De Gussem, 2007; Sharman et al., 2010; Blake and Tomley, 2014). Over the past 60 to 70 years, virtually every poultry producer has extensively used anticoccidials to control coccidiosis in their flocks (Chapman, 2009). Programs have been implemented that include rotating the drugs or using them in combination with another drug with a different mode of action. However, some of the drugs are under heavy use pressures and are still losing their efficacy (Abbas et al., 2011). Additionally, there is public outcry for removal of all drugs from poultry feed, further pressuring the poultry industry to find suitable alternative control and preventative measures (McDougald, 1998), including the use of natural compounds (Lee et al., 2009; Cox

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et al., 2010; Abbas et al., 2011; Zaman et al., 2012; Shanmugasundaram et al., 2013).

There is little to no cross-protection between the different *Eimeria* spp., therefore making the disease costly and difficult to control (Rose and Long, 1962). Furthermore, there may not be protection following infection with a different strain of the same species (Martin et al., 1997; Chapman, 2008). From a practical vaccine perspective, this immunological variation means a single vaccine may not convey protection against all strains of a species of *Eimeria* in the field; therefore, employing an effective and efficient vaccination protocol is difficult (Chapman, 2014). Additionally, vaccinations with live parasites can be effective but they are costly to produce, and identifying immunogenic and immunoprotective molecules for use in a subunit vaccine has also proven difficult (Blake and Tomley, 2014). Chapman (2014) notes, "The resilience of the oocysts and its ubiquitous presence wherever poultry are reared provides a continuing threat to the health of poultry, and therefore there will be a continued need for basic and applied research into all aspects of the biology of these organisms." Consequently, identifying a line of chickens with increased natural resistance to coccidiosis would be extremely valuable to the poultry industry in combating this very costly disease.

We recently developed a novel selection method based on identification and selection of chickens with an inherently high and low phenotype of pro-inflammatory mediators, including interleukin (IL)-6, CXCLi2, and CCLi2. We have shown the resultant high line of chickens is more resistant to the foodborne pathogen *Salmonella enterica* serovar Enteritidis compared to the low line (Swaggerty et al., 2014). If the high line of birds were more resistant to both a foodborne pathogen and a poultry pathogen, such as *Eimeria*, it would provide additional validation of selection based on pro-inflammatory mediators, and therefore be more appealing and valuable to the poultry industry. Therefore, the objective of this study was to determine whether the same trend of enhanced resistance (decreased incidence of cecal pathology) in the high line of birds was observed following challenge with 2 different field isolates of *E. tenella*.

MATERIALS AND METHODS

Experimental Chickens

The high and low lines of broilers were produced following selection parameters previously described (Swaggerty et al., 2014). Briefly, sires that have inherently high levels of pro-inflammatory mediators (IL-6, CXCLi2, and CCLi2) were mated to randomly selected high dams, and the resultant progeny are the high line. The low line is a product of sires with low levels of the pro-inflammatory mediators being mated to randomly selected low dams. Fertilized eggs from the high and low lines were obtained from the commercial company, and were incubated and hatched under standard conditions (Stromberg, 1975). Each line was coded to conceal their identity. On the day of hatch, straight-run chicks from each line were placed in separate floor pens (3 × 3 m) within the same room. Each pen contained wood shavings, supplemental heat, water, and a balanced, unmedicated corn and soybean meal based chick starter diet *ad libitum*. The feed contained 23% protein and 3,200 kcal metabolizable energy/kg of diet, and all other nutrient levels met or exceeded established requirements (National Research Council, 1994). Experiments were conducted under the supervision of the USDA animal care committee, which operates in accordance with established principles and specific guidelines (National Research Council, 1996).

Parasite Preparation and Experimental Design

E. tenella was propagated and prepared as previously described (Hammond and Long, 1973). Briefly, *E. tenella* oocysts were collected from the caeca of 21-day-old broiler chickens 7 d postinfection, sporulated, and sterilized by 5% sodium hypochlorite, washed 3 times with PBS, and enumerated using a hemocytometer prior to experimental infections. All reagents were obtained from Sigma–Aldrich Corporation (St. Louis, MO). Oocysts were kindly provided by Dr. Mark Jenkins, USDA–Agricultural Research Service, Beltsville, MD (Beltsville strain), and Dr. Lorraine Fuller, University of Georgia, Athens, GA [Pennsylvania State University (PSU) strain]. At 13 d of age, birds were leg banded, weighed, and randomly assigned to control or challenge groups. The following day, chickens from high and low lines were challenged orally with 10×10^3 to 45×10^3 oocysts, and killed by cervical dislocation 6 d postchallenge. The PSU strain was used in 2 separate experiments and birds were given 0, 15×10^3, 30×10^3, or 45×10^3 oocysts (n = 19 to 26 per dose per experiment). One additional experiment was conducted using the Beltsville strain of *E. tenella* and birds (n = 24 to 26 per dose) were administered 0, 10×10^3, 20×10^3, or 40×10^3 oocysts. On the last day of the experiment, the birds were weighed, and the caeca were removed and scored for lesions by a standard method (Johnson and Reid, 1970). The experimental challenges were conducted a total of 3 times with chickens from separate hatches (2 biological replicates with the PSU strain and one with the Beltsville strain). All birds administered the same dose were housed in the same room, but the lines were maintained in different pens. The birds were not vaccinated at any time during the experiment nor did they receive any medications.

Lesion Scoring

To eliminate bias, one person blindly scored all tissues for lesions. Lesions were scored on a scale of 0 to 4

with 0 being normal healthy tissue; a score of 1 is characterized by a few scattered petechiae, no thickening of the cecal wall, and normal brownish colored content; a score of 2 is characterized by increased numbers of petechiae, some bleeding at the mucosal surface, relatively normal cecal content, and slight thickening of the cecal wall; a score of 3 is defined by increased bleeding with clotting at the distal end of the pouch that becomes hardened as the sloughed mucosal surface unites with bloody cecal content, and there is marked thickening of the cecal wall; and a score of 4 is accompanied by severe bleeding, severe thickening of the wall, and eroding of the mucosal surface featuring a hard core of material inside the cecal pouch (Johnson and Reid, 1970).

Statistical Analyses

Three separate challenges were conducted using chickens from a different flock for each trial, and 19 to 26 birds per dose were used for each experiment. A total of 282 and 274 low and high birds, respectively, were used in the 3 experimental challenges. The 2 challenge trials using the PSU strain were combined for statistical analyses. The challenge experiment using the Beltsville strain was analyzed separately to support the consistency of the results. All analyses were performed in SigmaPlot Version 12 (Systat Software, San Jose, CA). The mean and SEM for lesion scores and body weight gain (grams) were determined and statistical analyses were performed (Student's t test); comparisons were made for comparable doses. For all analyses, significance was considered if $P \leq 0.05$.

RESULTS

E. tenella Challenges Using the PSU Strain

Chickens from the high (n = 175) and low (n = 182) lines were administered varying doses of the PSU strain of *E. tenella*, over 2 biological replicate experiments and the high line birds were more resistant compared to the low line chickens as indicated by reduced cecal lesion scores. Each line had a total of 42 to 47 birds per dose (0, 15 $\times$ 10^3, 30 $\times$ 10^3, and 45 $\times$ 10^3 oocysts). The average lesion score following administration of 15 $\times$ 10^3 oocysts was significantly lower ($P = 0.04$) for the high line (1.4) compared to the low line (1.9) (Table 1). This is a result of a disparity in the severity of the observed lesions; only 5 birds in the high line had lesion scores of 3 or 4, compared to the low line where 13 birds had lesion scores of 3 or 4. A similar pattern was observed in the 45 $\times$ 10^3 dose, where the average high and low line lesion scores were 2.1 and 2.8, respectively (Table 1). Similarly, 14 high line birds had lesion scores of 3 or 4, compared to 31 for the low line. Following administration of 30 $\times$ 10^3 oocysts, the average high line lesion score was 2.2 compared to 2.6 for the low line. Despite the numerical difference there was no significant difference at this dose ($P = 0.07$). No lesions were observed in either line of control birds.

Table 1. Distribution (no. chickens) and average lesion scores challenged with the Penn State University strain of *Eimeria tenella*.

	Lesion score[1]						
Line-dose ($\times$ 10^3)	0	1	2	3	4	Ave. $\pm$ SEM	P value
Low-0	47[2]	0	0	0	0	0 $\pm$ 0	
High-0	44	0	0	0	0	0 $\pm$ 0	
Low-15	4	12	16	9	4	1.9 $\pm$ 0.16	0.04
High-15	7	16	16	5	0	1.4 $\pm$ 0.14	
Low-30	1	4	15	19	6	2.6 $\pm$ 0.13	0.07
High-30	3	9	13	17	3	2.2 $\pm$ 0.16	
Low-45	0	4	10	20	11	2.8 $\pm$ 0.13	0.00005
High-45	0	11	17	14	0	2.1 $\pm$ 0.12	

[1]Lesions were scored on a scale of 0 to 4, with 0 being normal healthy tissue; a score of 1 is characterized by a few scattered petechiae, no thickening of the cecal wall, and normal brownish colored content; a score of 2 is characterized by increased numbers of petechiae, some bleeding at the mucosal surface, relatively normal cecal content, and slight thickening of the cecal wall; a score of 3 is defined by increased bleeding with clotting at the distal end of the pouch that becomes hardened as the sloughed mucosal surface unites with bloody cecal content, and there is marked thickening of the cecal wall; and a score of 4 is accompanied by severe bleeding, severe thickening of the wall, and eroding of the mucosal surface featuring a hard core of material inside the cecal pouch (Johnson and Reid, 1970).

[2]Actual number of chickens in the experiment with a specific lesion score.

Table 2. Body weight gain following challenge with the Penn State University strain of *Eimeria tenella*.

Line-dose ($\times$ 10^3)	No. animals	Ave. $\pm$ SEM (g)	P value
Low-0	47	326.7 $\pm$ 13.0	0.74
High-0	44	332.3 $\pm$ 10.7	
Low-15	45	308.5 $\pm$ 15.3	0.55
High-15	44	304.7 $\pm$ 11.5	
Low-30	45	294.1 $\pm$ 16.3	0.77
High-30	45	296.0 $\pm$ 15.0	
Low-45	45	287.1 $\pm$ 17.5	0.97
High-45	42	287.9 $\pm$ 15.2	

In addition to lesion scores, body weight gain was also monitored. There were no differences between the initial starting body weights between the high and low lines (data not shown). At the conclusion of the study, there were no differences between the lines administered comparable doses of the PSU strain of *E. tenella* ($P \geq 0.55$). The average body weight gain in the low line challenged birds ranged from 287.1 to 308.5 g, while the high line challenged birds ranged from 287.9 to 304.7 g (Table 2).

E. tenella Challenge Using the Beltsville Strain

To demonstrate that the high line of chickens was generally more resistant than low line chickens, we utilized a different field isolate of *E. tenella* for the third experiment (Beltsville strain). High (n = 99) and low (n = 100) chickens were administered 0, 10 $\times$ 10^3, 20 $\times$ 10^3, or 40 $\times$ 10^3 oocysts (24 to 26 birds per dose for each line). The distribution of lesion scores is

Table 3. Distribution (no. chickens) and average lesion scores challenged with the Beltsville strain of *Eimeria tenella*.

	Lesion score[1]						
Line-dose ($\times 10^3$)	0	1	2	3	4	Ave. ± SEM	*P* value
Low-0	26[2]	0	0	0	0	0 ± 0	
High-0	25	0	0	0	0	0 ± 0	
Low-10	5	12	5	1	1	1.21 ± 0.2	0.003
High-10	16	8	2	0	0	0.46 ± 0.12	
Low-20	2	6	11	5	1	1.88 ± 0.2	0.0003
High-20	9	10	4	1	0	0.88 ± 0.18	
Low-40	4	6	7	6	2	1.84 ± 0.24	0.0001
High-40	14	8	1	1	0	0.54 ± 0.16	

[1]Lesions were scored on a scale of 0 to 4, with 0 being normal healthy tissue; a score of 1 is characterized by a few scattered petechiae, no thickening of the cecal wall, and normal brownish colored content; a score of 2 is characterized by increased numbers of petechiae, some bleeding at the mucosal surface, relatively normal cecal content, and slight thickening of the cecal wall; a score of 3 is defined by increased bleeding with clotting at the distal end of the pouch that becomes hardened as the sloughed mucosal surface unites with bloody cecal content, and there is marked thickening of the cecal wall; and a score of 4 is accompanied by severe bleeding, severe thickening of the wall, and eroding of the mucosal surface featuring a hard core of material inside the cecal pouch (Johnson and Reid, 1970).

[2]Actual number of chickens in the experiment with a specific lesion score.

Table 4. Body weight gain following challenge with the Beltsville strain of *Eimeria tenella*.

Line-dose ($\times 10^3$)	No. animals	Ave. ± SEM (g)	*P* value
Low-0	26	316.2 ± 6.9	0.35
High-0	25	307.9 ± 5.3	
Low-10	24	317.9 ± 11.4	0.31
High-10	26	331.6 ± 6.8	
Low-20	25	317.3 ± 15.1	0.38
High-20	24	327.3 ± 7.3	
Low-40	25	319.9 ± 10.2	0.40
High-40	24	329.8 ± 5.8	

summarized in Table 3. As observed with the PSU strain, high line birds showed enhanced resistance against cecal pathology. The average lesion score of high line birds dosed with 10 $\times$ 10^3 oocysts was 0.46, compared to 1.21 for the low line ($P = 0.003$). Following administration of 20 $\times$ 10^3 oocysts, the average lesion scores, for the high and low line, were 0.88 and 1.88, respectively ($P = 0.0003$). Similar differences between the high (0.54) and low (1.84) line were observed following challenge with 40 $\times$ 10^3 oocysts ($P = 0.0001$). No lesions were observed in either line of control birds.

Body weight gain was also determined following challenge with the Beltsville strain of *E. tenella*. There were no differences between the initial starting body weights between the high and low lines (data not shown). At the end of the study there were no differences between the lines administered comparable doses of the Beltsville strain of *E. tenella* ($P \geq 0.31$) (Table 4). The average body weight gain in the low line challenged birds ranged from 317.3 to 319.9 g, while the high line challenged birds ranged from 327.3 to 331.6 g.

DISCUSSION

As poultry breeding companies keep up with global consumption, consumer demands, and increasing regulations, yet all the while improving robustness and livability, addressing animal welfare concerns, and reducing costs, new methods to select birds with natural resistance to poultry and foodborne pathogens will become increasingly important. The high and low line chickens used in the present study are selected for an inherently high and low phenotype of IL-6, CXCLi2, and CCLi2, respectively. The resultant high line birds from this novel selection strategy have increased resistance against the foodborne pathogen *Salmonella enterica* serovar Enteritidis (*Salmonella* Enteritidis) compared to the low line (Swaggerty et al., 2014). Preliminary studies indicate the high line is more resistant to *Campylobacter jejuni* (C. L. Swaggerty, unpublished data) and *Pseudomonas* (I. Y. Pevzner, unpublished data), and the high line had a lower incidence of tibial head necrosis (Robert F. Wideman Jr., University of Arkansas, Fayetteville, personal communication), following evaluation in an established model (Wideman et al., 2012). The current study demonstrated the high line birds were also more resistant to *E. tenella*, one of the most costly species of *Eimeria* associated with coccidiosis in poultry, thereby suggesting this novel selection method could be used to produce chickens with increased resistance against important foodborne and poultry pathogens.

Observing differences in susceptibility to *Eimeria* infections in chickens is not a novel concept. Early pioneering studies showed it was possible to select for resistance by exposing chickens to a heavy dose of *E. tenella*, then using survivors to produce subsequent generations (Rosenberg et al., 1954); those studies were then further characterized (Johnson and Edgar, 1982). In addition to selection pressures to identify individuals with increased resistance to *Eimeria*, existing experimental (Bumstead and Millard, 1987; Lillehoj et al., 1989; Pinard-Van Der Laan et al., 1998) and commercial (Swaggerty et al., 2011) lines of chickens show varying degrees of resistance that are readily detectable. However, the aforementioned experimental and commercial lines of birds were not subjected to special selection pressures for enhanced resistance; they were merely evaluated in challenge models, thus distinguishing them from the high and low lines evaluated in the present study. To the authors' knowledge, there are no other experimental or commercial lines of poultry that have undergone selection pressures for pro-inflammatory mediators, or for that matter any other innate immune-based phenotype.

Even though there are no comparable studies in the literature to which we can directly compare the present study, there are numerous references to support the key role that cytokines and chemokines have in determining resistance (or susceptibility) of chickens and turkeys to various species of *Eimeria*. Chickens more resistant

to *E. maxima* have increased levels of interferon-γ and several pro-inflammatory cytokines and chemokines, including IL-1β, IL-6, and IL-8 (now referred to as CXCLi2), while more susceptible lines have increased levels of IL-10 and IFN-α (Rothwell et al., 2004; Kim et al., 2008). Although not addressing the role of resistance, there are additional studies that profile the cytokine and chemokine responses following exposure of chickens to *E. tenella*, which demonstrate the birds mount strong pro-inflammatory responses characterized by increases in IL-1β, IL-6, IL-17, and MIP-1β (Laurent et al., 2001; Hong et al., 2006), and similarly in turkeys following challenge with *E. adenoeides* (Gadde et al., 2011). Future studies to evaluate the local and systemic cytokine and chemokine profile of high and low lines should be determined. Increases in inflammatory cytokines are also associated with reduced fecal oocyst counts and improved performance in coccidial-challenged birds, following dietary supplementation to enhance resistance against coccidiosis (Shanmugasundaram et al., 2013). Oocyst counts were not evaluated in the current study, but should be considered in later experiments as well as challenges using other relevant *Eimeria* spp., including *E. maxima* or *E. acervulina*, to demonstrate enhanced resistance to diverse pathogens.

There was variability in the pathogenicity of the two *E. tenella* strains used in the current studies; higher lesion scores were observed with the PSU strain compared to the Beltsville strain. The differences are likely due to naturally occurring variation between distinct strains of *E. tenella*. It is also possible the variation is due to a reduction in pathogenicity of the Beltsville strain stock due to storage and passage (or both), but this would seem less likely as fresh oocysts were used in the study. Similar reductions in pathogenesis have been observed between experiments (Swaggerty et al., 2011). Regardless of the cause of the variation either between the experiments and/or strains tested, the overall trend observed for the lines remained consistent with the high line being more resistant than the low line.

Poultry coccidiosis is associated with large-scale production losses due, in part, to weight loss, poor feed conversion ratio, and a general failure to thrive (Blake and Tomley, 2014; Shivaramaiah et al., 2014). Body weight gain following challenge with *E. tenella* was determined in the present study, but no differences were observed at any point (Tables 2 and 4). Previously, performance data on our selected lines showed the live weight of low line birds is significantly heavier at 6 wk age compared to high line birds (Swaggerty et al., 2014). It is possible that body weight differences were not detected because of the short duration of the study (20 d). Because of less intestinal pathology and lower cecal lesion scores following exposure to *E. tenella*, it would be reasonable to expect that the infected high line birds would have overall better performance than the low line. A true account of performance including live weight at 6 wk age, feed conversion ratio, and breast meat yield following challenge would be important data for the industry, and should be incorporated into future experiments.

Collectively, these studies would support the hypothesis that an effective innate immune response accompanied with a strong and efficient pro-inflammatory response could limit the pathology, and subsequent loss of productivity, associated with coccidial infections. Therefore, chickens with an inherently higher phenotypic profile of pro-inflammatory mediators like the high line produced and described herein would have the potential to be very important for the poultry industry. The findings from this study, and a previous report showing increased resistance against *Salmonella* Enteritidis (Swaggerty et al., 2014), provide additional validation for a breeding program and selection strategy that also considers the pro-inflammatory mediator profile of the sires.

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