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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

Quantitative analysis of Northern bobwhite (*Colinus virginianus*) cytokines and TLR expression to eyeworm (*Oxyspirura petrowi*) and caecal worm (*Aulonocephalus pennula*) glycoproteins

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Abstract

Helminth parasites have been a popular research topic due to their global prevalence and adverse effects on livestock and game species. The Northern bobwhite (*Colinus virginianus*), a popular game bird in the USA, is one species subject to helminth infection and has been experiencing a decline of > 4% annually over recent decades. In the Rolling Plains Ecoregion of Texas, the eyeworm (*Oxyspirura petrowi*) and caecal worm (*Aulonocephalus pennula*) helminths are found to be highly prevalent in bobwhite. While there have been increasing studies on the prevalence, pathology, and phylogeny of the eyeworm and caecal worm, there is still a need to investigate the bobwhite immune response to infection. This study utilizes previously sequenced bobwhite cytokines and toll-like receptors to develop and optimize qPCR primers and measure gene expression in bobwhite intramuscularly challenged with eyeworm and caecal worm glycoproteins. For the challenge experiments, separate treatments of eyeworm and caecal worm glycoproteins were administered to bobwhite on day 1 and day 21. Measurements of primary and secondary immune responses were taken at day 7 and day 28, respectively. Using the successfully optimized qPCR primers for TLR7, IL1β, IL6, IFNα, IFNγ, IL10, and β-actin, the gene expression analysis from the challenge experiments revealed that there was a measurable immune reaction in bobwhite in response to the intramuscular challenge of eyeworm and caecal worm glycoproteins.

Keywords Bobwhite · Caecal · Eyeworm · Cytokine · qPCR · TLR

Introduction

Helminth parasites have been a popular research area with their global prevalence (Kamal and Khalifa 2006; Albonico et al. 2008; Hotez et al. 2008; Yu and Blackburn 2019), negative effects on livestock and game species (Kellogg and Prestwood 1968; Hudson et al. 1992; Charlier et al. 2014; Greter et al. 2017), and ability to remain long-lived in their hosts (Maizels et al. 2004). They infect a wide variety of hosts and have evolved to invade numerous locations within their hosts (Maizels et al. 2004). Helminths have garnered increased attention to immunologists as well given their ability to suppress host immune defense mechanisms and regulate the immune system (Behnke et al. 1992; Maizels and Yazdanbakhsh 2003; Maizels et al. 2004).

Helminths have gained notoriety for their ability to modulate host immune responses, namely their ability to suppress T helper 1 (Th1) and induce a modified Th2 environment that enables helminths to increase their longevity within the host (Hewitson et al. 2009). A hypothesized route of this immune modulation is through the excretory/secretory (E/S) products released by helminths, specifically the glycans located on the glycoproteins and glycolipids that mimic the host glycans (Cummings and Nyame 1996, van Die and Cummings 2010). There are numerous studies demonstrating the role of helminth glycoproteins in other helminth parasites such as H-gal and H11 from *Haemonchus contortus*, TSL-1 (43 kDa antigen) from *Trichinella spiralis*, ES-62 from filarial nematodes, and TES-70 from *Toxocara canis* and their role in host immunomodulation (Munn et al. 1987, Appleton et al. 1991,

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Haslam et al. 1997, Whelan et al. 2000). However, many of these studies assessing host immune response to helminth infections have focused solely on mammals.

As a result, avian models are lacking largely due to low sequence homologies between birds and mammals in addition to a lack of information on avian cytokines (Wigley and Kaiser 2003; Umar et al. 2015). While poultry models have increased since the sequencing of the chicken genome, more avian models utilizing different species are needed to observe host immune function in response to a pathogen, like disease and parasites. These investigations are critical as these pathogens can have economic and social impacts on the poultry industry and wild avian populations, such as the Northern bobwhite quail (*Colinus virginianus*, Linneas 1758; hereafter, bobwhite).

The bobwhite, a popular game bird of economic significance to local communities in the Rolling Plains Ecoregion (Johnson et al. 2012) of Texas, has been experiencing a decline of > 4% annually over recent decades (Sauer et al. 2013). The decline has been attributed to changes in land use, habitat loss, and habitat fragmentation (Hernández et al. 2013). This species exhibits a 5-year "boom and bust" cycle, but the cause of these "booms" and "busts" is unknown (Guthery 2002; Hernández et al. 2002). However, the expected "boom" in the summer of 2010 did not occur despite stable habitat conditions (Dunham et al. 2017a). This sparked increasing interest from hunters, landowners, and researchers as to other possible causes. During a collaborative effort to investigate potential contributors to the bobwhite decline, eyeworm (*Oxyspirura petrowi* Skrjabin, 1929) and caecal worm (*Aulonocephalus pennula* Chandler, 1935) were found to be abundant and widespread in the Rolling Plains (Dunham et al. 2014; Bruno et al. 2018). Subsequent surveys continued to identify high prevalence of both parasites throughout the Rolling Plains, with some areas having 100% of infected bobwhite individuals (Dunham et al. 2017a; Henry et al. 2017; Brym et al. 2018).

The eyeworm, a heteroxenous nematode, has been identified in various avian species including Galliformes and Passeriformes (Saunders 1935; Cram 1937, McClure 1949; Pence 1972; Dunham et al. 2014). They are typically found underneath the eyelids and nictitating membrane (Saunders 1935; Jackson 1969; Dunham et al. 2014), in the orbital cavity (Addison and Anderson 1969), and within tissues surrounding the eye (Robel et al. 2003; Bruno et al. 2015). Pathological investigation of eyeworm-infected individuals by Bruno et al. (2015) and Dunham et al. (2016) found inflammation in the lacrimal duct and lesions on the Harderian gland in bobwhites harboring eyeworm infection. This may be of concern as these tissues are associated with tear production (Holly and Lemp 1977) and immune function (Payne 1994; Kaiser and Balic 2015), respectively. Furthermore, phylogenetic analyses performed by Kalyanasundaram et al. (2018) found the eyeworm to be related to the human eyeworm (*Loa loa*) and the human and carnivore eyeworm (*Thelazia callipaeda*). Both eyeworm species have been associated with vision impairment and irritation (Nayak et al. 2016; Barua et al. 2005) which may correlate with reports of bobwhite flying into stationary objects (Jackson 1969; Dunham et al. 2017a; Brym et al. 2018).

The caecal worm, also a heteroxenous nematode, is described as a free-floating parasite of the avian caecum (Chandler 1935). Dunham et al. (2017b) noted in pathological investigations that highly infected individuals had reduced digesta in the caecum which may be associated with nutrient deficiency in infected individuals. Rollins (1980) also reported hemorrhaging of the caecum in infected quail and suggests that high worm burdens (> 200) could impede caecum function. These hypotheses are further supported by the fact that relatives of the caecal worm, including *Toxascaris leonina*, have been associated with adverse effects on their host including lethargy, malnutrition, and death (Kalyanasundaram et al. 2017).

While there is increasing research relating to impacts of the eyeworm and caecal worm on wild bobwhite populations (Henry et al. 2017; Brym et al. 2018), there is a need to understand immunological influences between these parasites and bobwhite. Given that parasite infection and immune function can lead to life history trade-offs (Nordling et al. 1998), this could also have potential impacts on bobwhite populations. As critical components to both the innate and adaptive immune system, cytokines and toll-like receptors (TLRs) are vital in signaling immune responses in the presence of pathogens (Mogensen 2009). This allows them to serve as bioindicators of immune activity post-infection in challenge experiments (e.g., Lochmiller et al. 1993; Saino et al. 1997; Nordling et al. 1998; Christe et al. 1998). Both cytokines and toll-like receptors have been well documented in parasitic infections. For example, there has been observed downregulation of anti-inflammatory cytokines with oncoming Leishmania infections (Mosser and Karp 1999) and diminished expression of cytokines and TLRs in filarial-infected individuals (Babu and Nutman 2003).

To date, there are no studies documenting bobwhite immune response with cytokines and TLRs to infection of eyeworms and caecal worms. Therefore, in this study, our objectives include (i) development and optimization of qPCR primer sequences for bobwhite cytokines and TLRs; (ii) experimentally challenge captive bobwhite with glycoproteins of eyeworm and caecal worm separately; and (iii) assess resulting cytokine and TLR gene expression through qPCR to understand host-parasite dynamics between the bobwhite, eyeworm, and caecal worm.

Materials and methods

Sample collection

Wild bobwhite were collected during April 2018 from private ranches using trapping procedures as described in Commons et al. (2019) for glycoprotein collections of eyeworms and caecal worms. Adult eyeworms were extracted from the eyes and associated tissues as described in Dunham et al. (2014). However, instead of physiological saline solution at 37 °C, tissues were placed in petri dishes containing 0.01 M phosphate-buffered saline (PBS) pH 7.4 to remove all worms from the eyes and tissues around the eyes before worms were transferred to a 2-mL centrifuge tube. Adult caecal worms were collected from the caecum of bobwhites with procedures following Dunham et al. (2017b) and identified by morphological characteristics as described in Kalyanasundaram et al. (2017). All the samples were stored at − 80 °C prior to glycoprotein extraction.

Glycoprotein extraction

Frozen adult eyeworms and caecal worms were thawed and washed several times with tris-buffered saline (TBS) containing 20 mM Tris, 150 mM NaCl, 100 μM $CaCl_2$, and 10 μM $MnCl_2$ (pH 7.4). Adult worms were homogenized using a chilled mortar and pestle on ice in TBS (pH 7.4) with 1.0% v/v Triton X-100, and centrifuged at 2600×g for 30 min at 4 °C. The volume of the supernatants was recorded before and after it was filtered through a 0.45-μM filter. Concentration of crude whole-body protein extract of eyeworm and caecal worm was estimated at 280 nm using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) as per manufacturer's instructions. We used Concanavalin A (Con A), a plant metalloprotein with agarose beads (6%) for the purification of eyeworm and caecal worm glycoproteins, as it binds molecules containing α-D-mannopyranosyl, α-D-glucopyranosyl, and sterically related residues (Alves et al. 2012). Crude whole-body protein extracts were mixed with Concanavalin A (Con A) (Cat. #786-217, G-Biosciences, USA) and incubated for 1 h at 4 °C and mixed every 10 min. The mixtures of Con A and crude whole-body protein for both eyeworm and caecal worm were separately packed in Econo-Pac® columns (Cat. #732-1010, BioRad, USA). After packing, the columns were washed three times using column wash buffer with 0.25% v/v Triton X-100. Glycoproteins were eluted using column elution buffer containing TBS (pH 7.4) 0.25% v/v Triton X-100, 200 mM α-Methyl-D-mannopyranoside, and α-Methyl-D-glucopyranoside. Protein elute concentrations were estimated as described above. Elutes were run in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Elutes with similar protein profiles as determined by SDS-PAGE were pooled, elutes E1 and E2 for eyeworm and E1 and E2 for caecal worm, and used in challenge experiment. All elutes were stored in − 80 °C until use.

Total RNA extraction

For primer optimization, 1 mL of whole blood was collected from the jugular vein from wild bobwhite using 25 gauge needle and transferred into heparin-coated 2-mL BD Microtainer™ Plastic Capillary Blood Collectors (Fisher Scientific, USA). Total RNA was isolated with 100 μl of blood using the QIAamp RNA Blood Mini kit (Qiagen, USA) according to manufacturer's instructions with final elutions of 50 μl sterile UltraPure DEPC-treated water (Invitrogen, USA). Quantity and quality of total RNA was estimated by absorbance at 260–280 nm using Qubit 3.0 and stored at − 80 °C. cDNA was synthesized from total RNA using the QuantiTect Reverse Transcription kit (Qiagen, USA) according to manufacturer's instructions. Synthesized cDNA stored at − 40 °C for primer optimization.

Primer design

Specific primers for the cytokines (IL1β, IL6, IL10, IFNα, and IFNγ), TLR (TLR7), and β-actin of bobwhite were designed by IDT PrimerQuest using sequences retrieved from Northern bobwhite whole genome project (PRJNA188411) in NCBI (Halley et al., 2014; Oldeschulte et al. 2017). We designed primers for all of the above cytokines by multiple sequence alignments with other avian cytokines from Japanese quail (*Coturnix japonica*) and chicken (*Gallus gallus*). However, specific primers for cytokines (IL2, IL4, IL8, IL12α, IL12β, IL13, IL18, and IL25) and TLRs (TLR2 and TLR4) were unsuccessful due to lack of quality sequences from NCBI database and the primer standardization for qPCR failed because of non-specific amplification.

Newly synthesized primers (Table 1) were optimized with total RNA template extracted previously. PCR reactions performed with 5 μl of MyTaq™ Red Mix (Bioline, USA), 1 μl of 10 μM forward, 1 μl of 10 μM reverse primer, 1 μl of cDNA template, and 2 μl of nuclease free water were used for 10 μl reactions. PCR run conditions were as follows: 95°C for 3 min; 30 cycles of 95°C for 40 sec, 60°C for 1 min, and 72°C for 30 sec; and a final extension step of 72°C for 5 min. Purified PCR products of all the genes were sequenced.

Experimental study

Parasite-free, pen-raised bobwhite were individually housed indoors and acclimatized for a 2-week period before experimental study. Birds were provided food and water ad libitum. Prior to starting the experimental study, bird feces were collected and analyzed for eyeworm and caecal worm

Table 1 Sequences of the primers used in qRT-PCR.\

Primer	Oligonucleotide sequence	Melting temp™ (°C)	Accession number*	Product size (bp)
IFNαF	5′ CCTTGCTCCTTCAACCACACCCT 3′	61.5	AWGT02000063	100
IFNαR	5′ CTTTGGCGTTGACGGTCGATCCA 3′	61.8		
IFNγF	5′ TCACGTGCTCTGAAGGGCAC 3′	60.0	AWGU01322170	99
IFNγR	5′ CAAGCTACTGAAGCAGCCTC TGG 3′	59.8		
IL1βF	5′ GGAGGAGGTTTTTGAGCCTG TCACC 3′	61.6	AWGT02000201	93
IL1βR	5′ TCGAAGGACTGTGAGCGGGT GTA 3′	61.9		
IL6F	5′ AGTCGCTGTGCTACAGCACG AAG 3′	61.4	AWGT02000177	103
IL6R	5′ AGGGATTTCCGGGCAGCTGA 3′	61.5		
IL10F	5′ TCTACACGGATGAGGTCCTGCCC 3′	62.4	AWGT02000004	128
IL10R	5′ GGTGAAGAAGCGGTGACAGCG 3′	60.9		
TLR7F	5′ TCCTCTTCTGGCCACAGACGT 3′	60.4	AWGU01025717	101
TLR7R	5′ AGGATGTGTCCAGCTCACAGG 3′	59.0		
ACTBF	5′ TCACCACCACAGCTGAGAGAGA 3′	59.7	AWGU01047659	149
ACTBR	5′ GGTGATGACCTGACCATCAGGG 3′	59.5		

IFN interferon, *IL* interleukin, *TLR* toll-like receptor, *ACTB* β-Actin

*Accession numbers for the sequences from which primers are derived

presence by PCR using their specific primers as described in Kistler et al. (2016) and Kalyanasundaram et al. (2017). After acclimatization, birds were separated into three groups of ten including a group treated with eyeworm glycoproteins, a group treated with caecal worm glycoproteins, and an untreated control group. Each bird in the eyeworm and caecal worm groups received 200 μg of glycoprotein intramuscularly (IM) with a primary and secondary dose on days 1 and 21. This was based on 2 μg per gram of body weight that was used in other studies (Lung et al. 1996, Kopko et al. 2000, Killpack and Karasov 2012). The IM route is advantageous as it allows for rapid passage of protein antigen into the circulatory and lymphatic systems due to the high concentration of blood vessels (Turner et al. 2011). For gene expression analysis, 1 mL of whole blood was collected from the jugular vein from all birds at day 7 and day 28 as described earlier.

Gene expression analysis

Bobwhite cytokines (IL1β, IL6, IL10, IFNα, and IFNγ) and TLR (TLR7) were used to determine the relative expression, while β-actin was used as an endogenous control in this study. Total RNA isolation and cDNA synthesis were done as described above. Quantitative real-time PCR (qRT-PCR) was performed using PowerUp™ SYBR™ Green Master Mix (Applied Biosystem, USA). The qRT-PCR reaction volume follows with 5 μl of PowerUp™ SYBR™ Green Master Mix, 1 μl of 10 μM forward, 1 μl of 10 μM reverse primer, 1 μl of cDNA template, and 2 μl of nuclease free water were used for 10 μl reactions. Amplification and detection of specific products were performed in StepOnePlus real-time PCR detection system (Applied Biosystems, USA) with the following cycle profile: holding stage at 50°C for 2 min, 96°C for 15 sec; and 40 cycles of 96°C for 15 sec, 60°C for 1 min. Melting temperature (T_m) of the samples was determined by melt curve analysis following amplification. The samples were heated to 95°C for 15 sec and then cooled to 60°C for 5 sec before ramping back to 95°C in 0.5°C increments. The relative expression of each target gene was calculated using the methods described in Livak and Schmittgen (2001).

Statistical analysis

All statistical analyses were completed in Minitab (v18). The data for each gene was assessed for outliers, normality was assessed using the Ryan-Joiner normality test, and equal variance was analyzed with both the multiple comparison test and Levene's method. Data sets that were normally distributed with 95% confidence interval were analyzed with a two-sample *t* test and data sets that did not have a normal distribution were analyzed using the Mann-Whitney *U* test. Genes that have statistically significant ($P < 0.05$) expression changes compared with the control will be reported as such.

Results

The SDS-PAGE results of Con A–purified elutes exhibited the pattern of eyeworm and caecal worm glycoproteins with a molecular weight range from 20 to 260 kDa (Fig. 1). Elutes 1 and 2 for both eyeworm and caecal worms showed high intensity of prominent bands with concentrations of 234, 234, 456, and 457 μg/ml respectively.

Bobwhite cytokine and toll-like receptor sequence analysis

All the oligonucleotide primers were confirmed as species-specific by amplifying a single-gene product for each target sequence using PCR. Sequence results revealed the bobwhite cytokines IL1β, IL6, IL10, IFNα, and IFNγ, as well as TLR7 and β-actin genes. A BLASTX analysis confirmed the genetic identity to the bobwhite, where β-actin and IFNα, in particular, were found to have a 100% identity to bobwhite from Halley et al. (2014) and Oldeschulte et al. (2017) (Accession no.: AWGU00000000.2). The analysis also revealed the specified genes of bobwhite to have a close identity (90–100%) with the galliform species of chicken (*Gallus gallus*) and Japanese quail (*Coturnix japonica*).

Challenge experiments

Results comparing the gene expression between the control group and experimental groups dosed with glycoproteins of eyeworm and caecal worm are represented in Figs. 2 and 3, respectively.

Eyeworm

In the eyeworm experimental group, pro-inflammatory cytokines of IL1β, IL6, IFNα, and IFNγ exhibited upregulation at days 7 and 28 following primary and secondary challenge with its glycoproteins as related to the control group (Fig. 2). There was a statistically significant difference in IL1β at day 7 with upregulation greater (78.74-fold) than the other pro-inflammatory cytokines, IL6, IFNα, and IFNγ. However, at day 28, there was a statistically significant difference in IL6 expression with upregulation of 123.82-fold. In contrast, IL1β had a reduced fold change (27.47-fold) at day 28 as compared with day 7. There was no significant variation in IFNα expression between day 7 (5.25-fold) and day 28 (8.49-fold change) and little variation in IFNγ expression on either day. The regulatory cytokine, IL10, displayed increased expression at day 7 and day 28 as well. Further, IL10 gene expression was statistically significant as compared with the control birds on day 7 and day 28. TLR7 transcripts showed a statistically significant increase in expression level (73.8-fold change) compared with control at day 7 following primary challenge (Fig. 2). There was a decline (38.71-fold change) in TLR7 mRNA expression after secondary challenge at day 28.

Caecal worm

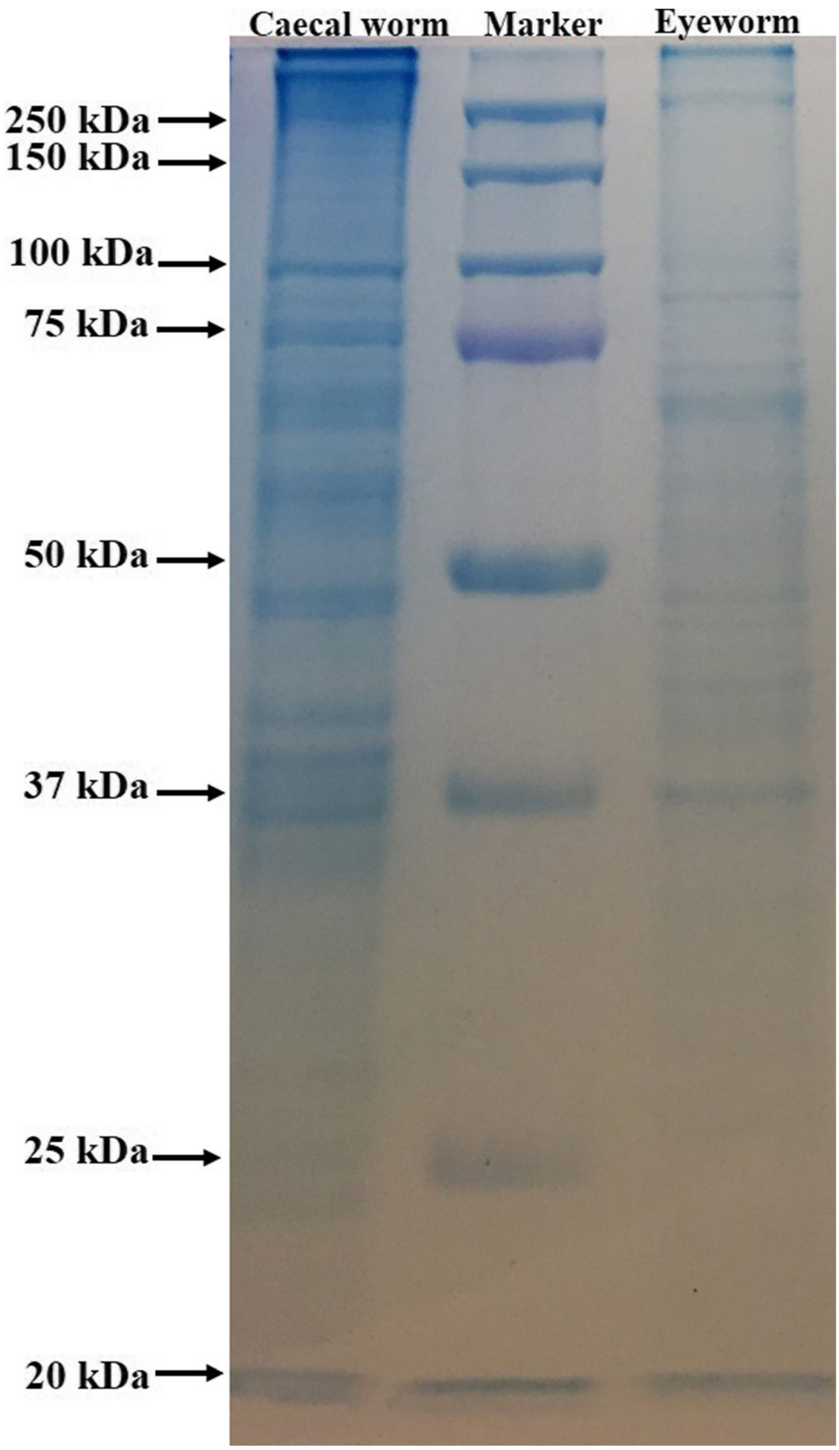
Results of gene expression between the experimental group treated with caecal worm and the control birds on days 7 and 28 are represented in Fig. 3. The pro-inflammatory cytokines all showed a low expression level after day 7, with increased expression at day 28. Among all pro-inflammatory cytokines, IL6 has the highest expression level (173.7-fold) following secondary challenge at day 28. Similarly, anti-inflammatory cytokine IL10 was upregulated on day 28. TLR7 gene showed a low expression at day 7 following primary challenge, whereas increased expression was seen by day 28 following secondary challenge. All the pro- and anti-inflammatory cytokine expressions were statistically significant except IFNγ at day 28.

Discussion

This preliminary study is the first to design and optimize qPCR primers for bobwhite cytokines and TLR7 from the sequenced bobwhite genome documented in Halley et al. (2014) and Oldeschulte et al. (2017). It is also the first study to experimentally challenge bobwhite with eyeworm and caecal worm glycoproteins where it was verified that changes in gene expression could be accomplished by qPCR with the designed primers. While a challenge experiment with glycoproteins may not exactly reflect the immune response of bobwhite infected with these parasites, these primers and qPCR methods could be utilized in future experiments with parasitized bobwhite in the laboratory. The following discussion will focus on the changes in gene expression observed and what these changes might mean if seen in parasitized bobwhite.

For eyeworms, gene expression at day 7 resulted in statistically significant upregulation of TLR7. In humans, TLR activation is typically related to strong inflammation and is responsible for signaling the presence of a pathogen and tissue damage to the immune system (Alzabin et al. 2012). TLR7 has also been related to detection of bacterial and viral nucleic acids in humans (Uematsu and Akira 2006) which is similar to the role of TLR7 in birds (Philibin et al., 2005; Abdul-Cader et al. 2016). Interestingly, Abdul-Cader et al. (2018) found that stimulation of avian macrophages at the TLR7 ligand induced an anti-viral response in the form of an increase in IL1β expression. IL1β activates the avian immune system in

Fig. 1 SDS-PAGE pattern of Con A–purified native glycoproteins from eyeworm and caecal worm used in experimental challenge. Lane 1: Caecal worm (pooled elutes 1 and 2); lane 2: Marker; lane 3: Eyeworm (pooled elutes 1 and 2)



an acute-phase response, typically leading to inflammation and fevers (Wigley and Kaiser 2003Kaiser & Stäheli, 2014). With the expressions of both TLR7 and IL1β, the combined reaction may signify an acute inflammatory response. These results may coincide with inflammation in lacrimal ducts, lesions on the Harderian gland, and keratitis observed in bobwhite infected with eyeworms (Bruno et al. 2015; Dunham et al. 2016).

The resulting upregulation of the anti-inflammatory cytokine, IL10 (Kaiser & Stäheli, 2014), at day 7 after challenge with eyeworm glycoproteins may signify a reaction to inflammation. IL10 expression is associated with the induction of an antibody or humoral immune response (Wigley and Kaiser 2003) which may also be occurring at day 7 with the expression of IL10. Lastly, the lack of significance in IFNα, an antiviral cytokine in chicken (Pei et al. 2001), but concurrent

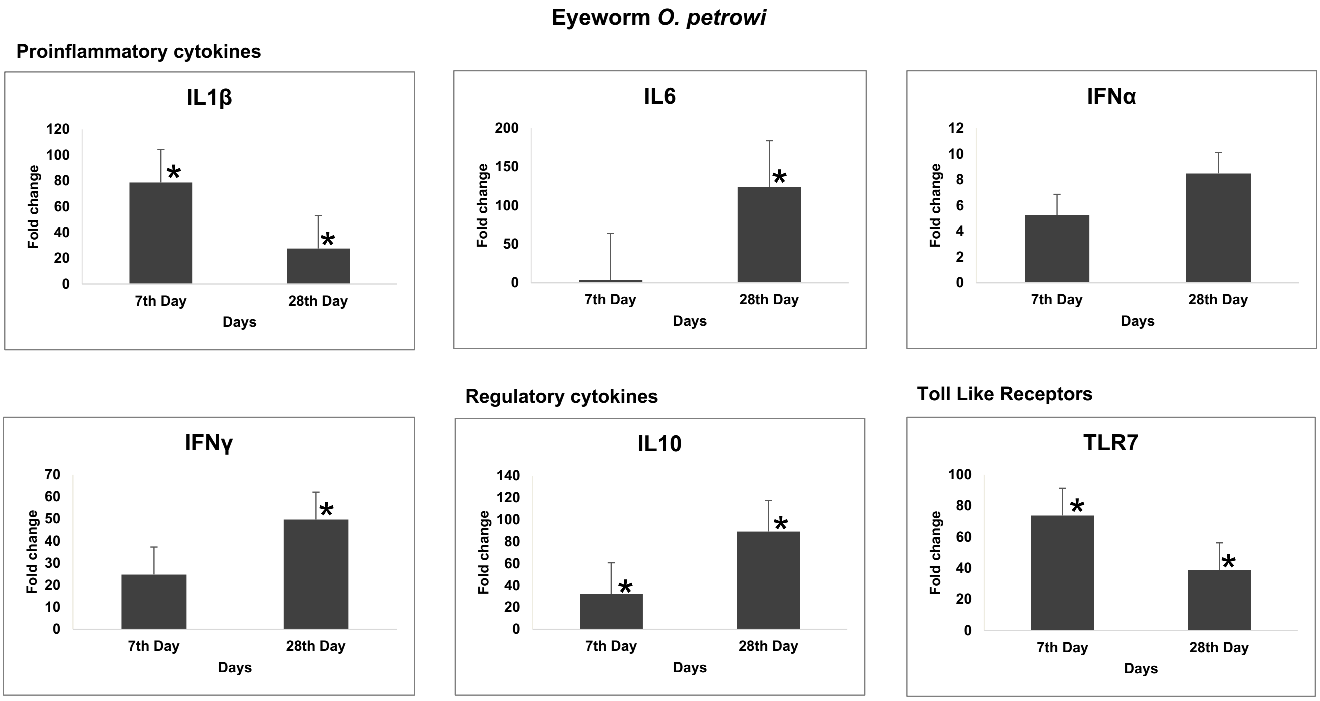


Fig. 2 Gene expression analysis of pro-inflammatory (IL1β, IL6, IFNα, IFNγ), regulatory cytokines (IL10), and toll-like receptor (TLR7) mRNA levels in lymphocytes of bobwhite quail after primary and secondary challenges with Con A–purified eyeworm glycoproteins. Statistical significance was assessed using Student *t* test and Mann-Whitney *U* test (*$P < 0.01$)

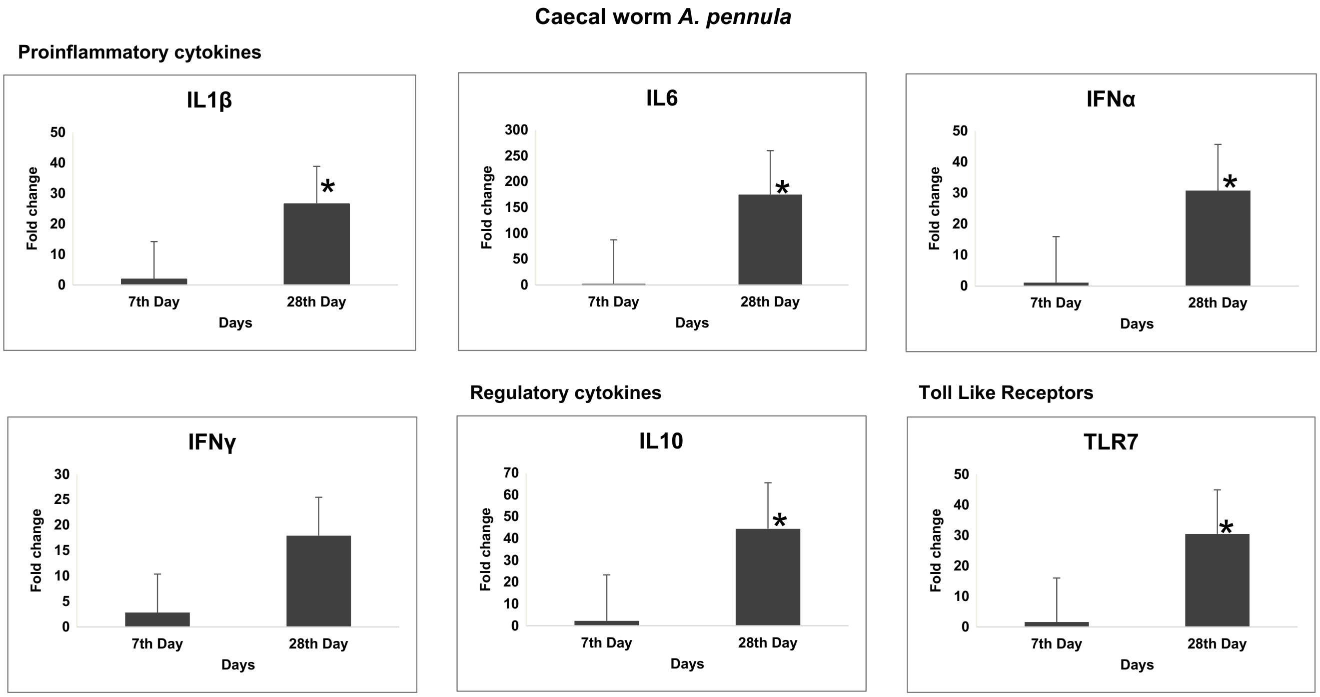


Fig. 3 Gene expression analysis of pro-inflammatory (IL1β, IL6, IFNα, and IFNγ), regulatory cytokine (IL10), and toll-like receptor (TLR7) mRNA levels in lymphocytes of bobwhite quail after primary and secondary challenges with Con A–purified caecal worm glycoproteins. Statistical significance was assessed using Student *t* test (*$P < 0.01$)

upregulation of TLR7 between the treated and untreated groups may suggest that eyeworm glycoproteins have few specifically viral properties to the bobwhite immune system.

At day 28, there is a statistically significant upregulation, albeit lower, in TLR7 and IL1β expressions. Based on these results of persistent upregulation in inflammatory cytokines, it is possible that this reaction further coincides with pathological investigations. However, the lower levels of upregulation in TLR7 and IL1β at day 28 may be linked to the resulting upregulation in IL6, IL10, and IFNγ. Like IL1β, IL6 is often released by the avian immune system in response to inflammatory stimuli (Amrani et al. 1986). IL6 is also a multi-functional cytokine in the avian immune system that is involved in acute-phase responses and immune regulation (Kishimoto et al. 1995; Wigley and Kaiser 2003). Given IL6's inflammatory properties, this may also be in response to a prolonged inflammatory reaction that was followed by an increase in anti-inflammatory expression from IL10. In the chicken, IL6 has also been associated with infectious diseases including *Eimeria* infections (Lynagh et al. 2000) and *Salmonella enterica* (Kaiser et al. 2000). Additionally, the expression of IL6 may be related to the properties of IL6 that have been linked to a susceptibility in nematode infection (Smith and Maizels 2014). For example, IL6-deficient mice were noted to have a significant increase in worm burdens of filarial nematodes (Muhsin et al. 2018) like those related to the eyeworm (Kalyanasundaram et al. 2018). As for IFNγ, this pro-inflammatory cytokine is involved with the induction of macrophages (Wigley and Kaiser 2003) and controlling infections with intracellular pathogens in the avian immune system (Kaiser & Stäheli, 2014). The upregulation of IFNγ seen at day 28 may be the bobwhite immune system's attempt to control the invasion of eyeworm glycoproteins via macrophages.

Interestingly, bobwhite treated with caecal worm glycoproteins had a reduced expression at day 7, whereas there was a significant upregulation in most all genes by day 28. On day 28, with the expression of TLR7, there was an accompanying upregulation of pro-inflammatory cytokines, except IFNγ, and IL10. Similar to the bobwhite response to eyeworm glycoproteins, bobwhite challenged with caecal worm glycoproteins may be associated with anti-inflammatory cytokine expression to reduce the cellular stress created by pro-inflammatory cytokines from helminth infections (Maizels and Yazdanbakhsh 2003). Considering the roles of each cytokine and TLR7 in the avian immune system, the response of bobwhite to the experimental challenge of eyeworm and caecal worm glycoproteins may suggest potential immune impacts. However, with the lack of expression at day 7 and subsequently increased expression at day 28, these results may suggest that cytokine and TLR response to caecal worm infection may best be observed in a chronic experimental infection.

In the wild, bobwhite of the Rolling Plains is likely subject to chronic infection due to the long-lived nature of helminths such as the eyeworm and caecal worm. Chronic infection of a parasite is related to the tolerance of the host to maintain infection at a level below damage (Schmid-Hempel 2009). However, chronic infection of helminth infections may be related to the parasite's ability to evade the immune system (Maizels et al. 2004; Schmid-Hempel 2009), and prolonged infection from helminths can lead to inflammatory disorders and delayed pathology in humans (Bethony et al. 2006; King 2007). Filarial parasites have also been linked to impaired pro-inflammatory cytokines and a strong response in regulatory cytokines (Hoerauf et al. 2001; King et al. 1993).

However, assessing the effects of chronic infection was out of the scope of this study; a long-term study would be valuable to understand how these parasites impact bobwhite. Future studies would also benefit if other genes that are commonly expressed in helminth infections, such as IL4 (Maizels et al. 2004), were designed and optimized. Nevertheless, this study is valuable as it gives insight into a new avian species with the optimization of qPCR primers for 6 new immune genes of bobwhite. Although the results presented here may not be conclusive, there was a measurable response of these genes to the intramuscular challenge of eyeworm and caecal worm glycoproteins showing the usefulness of these genes for future work. Lastly, future work should include experimentally infected or infected wild bird species to better understand the role of cytokines and TLRs in species besides poultry.

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Compliance with ethical standards

This study contains no conflicts of interest. This experiment was approved by Texas Tech University Animal Care and Use Committee under protocol number 18044-05 and 16071-08 for bobwhite collection. All bobwhites were trapped and handled according to Texas Parks and Wildlife permit SPR-0715-095.

Conflict of interest The authors declare that they have no conflicts of interest.

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