**Differential gene response to coccidiosis in modern fast growing and slow growing broiler genotypes**

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**Abstract**

We analysed intestinal tissues from groups of fast growing (Ross 308) broilers with natural or experimental coccidiosis, by genomic microarray. We identified genes that were differentially expressed (DE) in all groups and analysed expression of a panel of these, by qPCR, in Ross 308 and slow growing (Ranger classic) broilers, infected with 2500 or 7000 oocysts of *Eimeria maxima* for 6 or 13 days post-infection (dpi). Four genes (ADD3, MLLT10, NAV2 and PLXNA2) were upregulated ($P < 0.05$) in Ross 308 but were not DE in Ranger Classic at 6 dpi with 2500 oocysts. Six genes (PTPRF, NCOR1, CSF3, SGK1, CROR and CD1B) were upregulated ($P < 0.05$) in both Ross 308 and Ranger Classic infected with 2500 oocysts at 6 dpi but were not DE at 6 dpi with 7000 oocysts. At 13 dpi with 7000 oocysts, NAV2 and NCOR1 were upregulated in Ross 308 ($P < 0.05$) and PTPRF was upregulated in both genotypes ($P < 0.05$). DE of immune genes within the biomarker panel also occurred, with CSF3 upregulated in both genotypes infected with 2500 oocysts at 6 dpi and in Ranger Classic infected with 7000 oocysts, at 6 and 13 dpi ($P < 0.05$). IL-22 was down-regulated in Ranger Classic infected with 2500 or 7000 oocysts at 6 dpi ($P < 0.05$) but upregulated in both genotypes at 13 dpi ($P < 0.05$). CD72 was down-regulated in Ranger Classic infected with 2500 oocysts at 6 dpi and with 7000 oocysts at 6 and 13 dpi ($P < 0.05$). CD72 was upregulated in Ross 308 infected with 2500 oocysts at 6 dpi but was down-regulated following infection with 7000 oocysts at 13 dpi ($P < 0.05$). In conclusion, differential gene expression occurs in fast and slow growing broiler genotypes with coccidiosis. In addition, we highlight a potential genetic biomarker panel for early diagnosis of coccidiosis.

## 1.0 Introduction

Seven species of *Eimeria* can infect chickens (Allen et al., 2002) of which three species; *E. acervulina*, *E. maxima* and *E. tenella* are most commonly isolated (Cornelissen et al., 2009). Coccidiosis is one of the most economically important diseases of chickens and significantly impacts on attempts to increase global poultry production (Blake et al., 2014). Economic loss in the broiler industry results from reduced feed intake, feed conversion and the cost of treatment (Voeten et al., 1988; Williams et al., 1999; Dalloul et al., 2005). Control measures routinely include the use of in-feed coccidiocidal and coccidiostatic drugs and by vaccination (Chapman et al., 2016) but significant resistance to these drugs has been reported (Chapman, 1993; Bafundo et al., 2008; Chapman et al., 2016) and current concerns over antimicrobial resistance in the human food chain is leading to reduction in metaphylactic antibiotic usage. To compound this issue, although vaccines are available (Marugan-Hernandez et al., 2016) their cost and the requirement for multiple parasite lines in each vaccine have been significant obstacles to widespread use (Shirley et al., 2005; Clark et al., 2017).

Coccidiosis is known to be a predisposing factor for Salmonellosis (Arakawa et al., 1981; Qin et al., 1995) and necrotic enteritis (Moore, 2016) and different strains of the same *Eimeria* species can have varying pathogenicity (De Gussem, 2007). Diagnosis of coccidiosis can, therefore, be difficult and usually relies upon the recognition of site specific enteric lesions at post-mortem (Shirley et al., 2005). Although PCR diagnosis of oocysts in faecal samples has recently been reported there were significant differences between the sensitivity of the test in detecting different *Eimeria* spp. (Peek et al., 2017) and it remains to be elucidated how this diagnostic test performs against multiple field strains of the same species.

One possible diagnostic technique would be the use of genomic biomarkers, whereby early changes in expression of chicken genes could be used to detect a shift from healthy to disease status.

In addition, little is known about differential gene expression in fast and slow growing broiler genotypes infected with *Eimera* spp. Fast growing genotypes have been artificially selected for increased efficiency and faster growth rates, which may have had consequences on functional traits (Zuidhof et al., 2014; Hill, 2016; Tallentire et al., 2016). Slower growing genotypes have emerged in the broiler industry (Tallentire et al., 2018) partly due to animal welfare concerns (Clark et al., 2016; Clark et al., 2017).

The aim of this study was to investigate whole genomic change in fast (Ross 308) and slow (Ranger Classic) growing broilers infected with coccidiosis and from these to select key diagnostic genes which can be used in rapid multiplex PCR platforms.

## 2.0 Materials and methods

### 2.1 Broiler chickens

Fast growing (Ross 308) and slow growing (Ranger Classic) broilers from the Aviagen Rowan Range® were investigated in this study. Fast growing Ross 308 broiler chickens were routinely monitored on a Belgian farm with a history of coccidiosis. Eight birds with clinical signs of disease were euthanized prior to post-mortem examination and tissue preservation for molecular analysis. Chickens with clinical signs of disease had intestinal lesions consistent with *Eimeria maxima* and *Eimeria acervulina* infection (causing coccidiosis). Intestinal tissues were also supplied from five Ross 308 chickens with a presumptive diagnosis of coccidiosis in Galicia, Spain. At Newcastle University, 72 male day-old chicks of a fast growing line (Ross 308), and an equal number of a slow growing line (Ranger Classic), originating from a breeding station,

were housed in a windowless, thermostatically controlled room in 24 circular pens with a diameter of 1.2m (1.13$m^2$). There was *ad libitum* access to feed and water throughout the trial. Diets were starter (d1-10) and grower (d11-26) commercial type, wheat-based diets with soybean as a protein source. Birds were orally inoculated at 13 days of age (experimental day 0) with a single dose of $H_2O$ (0.5 ml) (control group), 2,500 (low-dose group), or 7,000 (high-dose group) sporulated *E. maxima* oocysts of the Weybridge laboratory reference strain. Bird weight and pen feed intake were measured at 1 and 13 days of age and from days 0-13 post-infection. On day 6 and day 13 post-infection, a randomly selected bird from each pen was culled and necropsied.

**2.2 Tissue preparation**

Comparative sampling was established by a standard operating procedure (SOP) provided by the University of Nottingham (available on request) adopted by Ghent University, Newcastle University and Coren. Following necropsy, samples were immediately placed in 5 ml of RNAlater® (Life Technologies, Carlsbad, California, USA). Samples were then dispatched to the University of Nottingham and stored at -80°C until further use. Approximately 30 mg of tissue was then homogenised using 5mm steel beads with a TissueLyserII (Qiagen GmbH, Hilden, Germany). Total RNA extraction was then performed with the RNeasy Fibrous Tissue Mini Kit on a QIAcube (Qiagen) following the manufacturer's recommendations. Total RNA was then quantified using a Nanodrop 8000 spectrophotometer (Thermo scientific, Waltham, Massachusetts, USA).

**2.3 Microarray hybridisation**

We examined the intestinal tissue samples by whole genomic microarray. Custom Agilent 4x44K microarrays based on the V2 Chicken Gene Expression Array (Agilent Technologies, Santa Clara, California, USA), similar to those previously reported (Ranaware et al., 2016; Liu et

al., 2017) were used for the microarray hybridisation. Five samples each of healthy and infected birds were processed for hybridisation using the Agilent Low Input Quick Amp Labelling protocol. Briefly an Agilent RNA Spike-In Kit, One Colour was used to spike each sample with RNA. The Spike Mix was diluted with Dilution Buffer prior to spiking samples. 10-200 ng of each sample were added to a final volume of 1.5 µl in a 1.5ml microcentrifuge tube, which was spiked with 2µl of the RNA spike mix. 0.8 µl of T7 primer and 1.0 µl nuclease-free water were added to each sample for a final volume of 5.3 µl. The primer and template were then denatured by incubating the reaction at 65° C in a Bioshake iQ (Quantifoil Instruments GmbH, Jena, Germany) for 10 minutes. The samples were then placed on ice, during which the cDNA master mix comprising 2 µl First Strand Buffer, 1µl 0.1 M DTT, 0.5 µl 10 mM dNTP and 1.2 µl Affinity Script RNase Block Mix was made. 4.7 µl of the cDNA master mix was then added to each sample so that each tube contained 10 µl. The samples were then incubated at 40° C for 2 hours after which they were incubated at 70° C for 15 minutes. Samples were then placed on ice, during which the transcription master mix comprising 0.75 µl nuclease-free water, 3.2 µl 5x transcription buffer, 0.6 µl 0.1 M DTT, 1 µl NTP Mix, 0.21 µl T7 RNA Polymerase Blend and 0.24 µl cyanine 3-CTP was made. 6 µl of the transcription master mix was then added to each sample which was then incubated in the Bioshake iQ at 40° C for 2 hours. Following the labelling process, the mRNA samples were purified with a Qiagen RNeasy Mini Kit following the manufacturer's recommendations.

After purification, the protocol One-Colour Microarray-Based Gene Expression Analysis (Quick Amp Labelling) with Tecan HS Pro Hybridisation was used. Briefly, 1.6 µg of cyanine 3-labelled cRNA up to a total volume of 22.8 µl was added to 6µl 10x blocking agent and 1.2 µl fragmentation buffer, nuclease-free water can be added if necessary if the cRNA is concentrated.

This was incubated at 60° C for 30 minutes to fragment the RNA. After incubation, 30 µl of 2x GE Hybridisation Buffer HI-RPM was added to stop the fragmentation reaction. The Tecan HS Pro hybridisation station (Tecan, Männedorf, Switzerland) was then set up with Pre-hybridisation buffer, wash buffers 1 and 2 and a bottle of water for rinsing. The Gene Expression programme was followed which includes 6 steps beginning with a wash with Pre-hybridisation Buffer at 65° C, followed by sample injection of 55 µl. Hybridisation occurs over a 17 hour period at 65° C. The arrays were then washed to remove unbound sample with Wash buffer 1 at room temperature then were washed again with Wash buffer 2 at 37° C. The arrays were then dried for 2 minutes at 30° C. The arrays were removed from the Tecan HS Pro hybridisation station and scanned using a GenePix 4000B Microarray scanner. GeneSpring GX13 software (Agilent Technologies) was used to qualify and normalise image analysis data and to determine the fold changes in gene expression. An asymptotic t-test analysis with p-value $<0.05$ was performed to analyse the significance between the infected and uninfected groups. To generate a signal ratio, the signal values from chickens with coccidiosis were divided by values from uninfected negative controls.

**2.4 Quantitative PCR (qPCR).**

Based on differential expression levels and biological function, we chose a panel of genes from our microarray study to further investigate in experimental infections with *E. maxima* in Ross 308 and Ranger Classic chickens. To do this, we used qPCR which would validate our microarray results and enable us to analyse expression of our gene panel in more chickens within the experimental groups. Primer and probe design was based upon sequences available from public databases (Shown in Table S1). To determine the fold change in these genes of interest a reference gene was used to act as a baseline from which a ratio of infected/control could be

obtained. The reference gene used in this study was the 18srRNA gene. Eight biological replicates of healthy and infected chickens were used for qPCR, and each sample was run in triplicate. RNA samples were reverse transcribed using the Applied Biosystems High Capacity cDNA Reverse Transcriptase Kit. A master mix composed of 2.0 µl 10xRT buffer, 0.8 µl 25x dNTP Mix (100mM), 2.0 µl 10x RT random primers, 1.0 µl MultiScribe™ Reverse Transcriptase and 4.2 µl nuclease-free $H_20$ was added to 10 µl of RNA sample. An Applied Biosystems 2720 thermal cycler (Applied Biosystems, Foster City, California, USA) was then programmed for the following conditions; 10 minutes at 25° C, 120 minutes as 37° C, 5 minutes at 85° C followed by a holding temperature of 4°C. The resulting cDNA concentration was then quantified using a nanodrop. A qPCR reaction was then performed using the Applied Biosystems™ TaqMan™ Fast Advanced Master Mix. Briefly 5.0 µl of TaqMan® Fast Advanced Master Mix (2x), 0.25 µl forward primer (10 mM), 0.25 µl reverse primer (10 mM), 0.1 µl probe (10 mM), 3.4 µl nuclease-free water and 1.0 µl of cDNA (1 pg-100 ng) were mixed in a final volume of 10 µl. For qPCR, a Roche LightCycler® 480 was used with the following protocol; 2 minutes at 50° C, 20 seconds at 95° C, then 40 cycles of 3 seconds at 95° C, and 30 seconds at 60° C. Analysis of the fold change between genes was performed using the Pfaffl's method (Pfaffl et al., 2001) and the standard deviation (SD) was calculated using the comparative Ct method.

**2.5 Statistical analysis**

Students t-tests were performed to statistically analyse differences between mean Ct values during qPCR, from infected and control chickens, using Graph Pad Prism software licensed to the University of Nottingham.

**3.0 Results**

Genomic mircroarray analysis detected differential expression of 3148 genes > 2 fold ($P$ <0.05) in Ross 308 chickens naturally infected with *E. acervulina* and *E. maxima* (field monitoring samples from Belgium) and 1081 genes > 2 fold ($P$ <0.05) in Ross 308 chickens experimentally infected at 6 dpi with 2500 *E. maxima* oocysts (experimental samples from Newcastle) (Table 1). In samples from Ross 308 chickens in which a presumptive diagnosis of coccidiosis was made (field samples from Spain), 3639 genes were differentially expressed > 2 fold ($P$ <0.05) (Table 1). Heat maps showing differential gene expression indicated that in field samples from Belgium and Spain, flock mates which were not diagnosed with or suspected of harbouring coccidial infection may have been sub-clinically infected. In both Belgium (Fig 1 B) and Spanish (Fig 1C) field samples, differential gene expression occurred in undiagnosed flock mates, whereas in experimental samples from the UK (uninfected/ non-exposed Ross 308) this was not the case (Fig 1A).

Seventy three genes were differentially expressed in all three sample groups, ranging from genes involved with cell cycle control and cell junction integrity to immune response genes (Table 2). From these we chose sixteen genes to further test as a biomarker of coccidiosis in Ross 308 and Ranger Classic broilers. The genes chosen had a broad profile of biological activity including immune response genes, such as IL-22, CD1B, CSF3, and genes involved with cell integrity and cell cycle, such as ADD3, NAV2 and SGK1 (Table 3).

Overall, differential expression of these genes changed according to dose and dpi, with greatest differential expression of genes occurring at 6 dpi with 2500 oocysts. This corresponded with greatest clinical signs of disease using the two infectious doses and sampling at the two time points (6 and 13 dpi) that we did. However, it is possible that peak infection in broilers infected

with 7000 oocysts may have occurred before day 6 and that similar gene expression to that measured at 6 dpi with 2500 oocysts may also have occurred then. Similarly, increased expression of some genes, such as PTPRF, NAV2 and NCOR1 at 13 dpi with 7000 oocysts, may have occurred after 13 dpi with 2500 oocysts. Nevertheless, there was a different gene expression profile in Ross 308 and Ranger Classic broilers infected with E. maxima oocysts.

In Ross 308 broilers infected with 2500 oocysts of *E. maxima*, the expression of ADD3, MLLT10, NAV2, PLXNA2 and CD72 was significantly up-regulated ($P < 0.05$) after 6 dpi (Fig 1A). In Ranger Classic, CD72 expression was significantly down-regulated ($P < 0.05$) and the other four genes were not differentially expressed at 6 dpi (Fig 1A). IL-22 was also significantly down-regulated ($P < 0.05$) in Ranger Classic infected with 2500 oocysts at 6d pi but was not differentially expressed in Ross 308 (Fig 1A). Six genes (PTPRF, NCOR1, CSF3, SGK1. CROR and CD1B) were significantly up-regulated ($P < 0.05$) in both Ross 308 and Ranger Classic broilers (Fig 1A).

In Ranger Classic infected with 7000 oocysts, IL-22 and CD72 were significantly down-regulated and CSF3 was significantly up-regulated at 6 dpi ($P < 0.05$) but these genes were not differentially expressed in Ross 308 (Fig 1B). In contrast, SGK1 was significantly up-regulated and IGLL1 was significantly down-regulated ($P < 0.05$) in Ross 308 but were not differentially expressed in Ranger Classic (Fig 1B). No other genes in the biomarker panel were differentially expressed.

After 13 dpi with 2500 oocysts, IL-22 was significantly up-regulated ($P < 0.05$) in both Ross 308 and Ranger Classic but none of the other genes in the biomarker panel were differentially expressed (Fig 2A). In chickens infected with 7000 oocysts, expression of NAV2 and NCOR1 was significantly increased in Ross 308 ($P < 0.05$) but not differentially expressed in Ranger

Classic (Fig 2B). IGLL1 was significantly decreased in Ranger Classic but was not differentially expressed in Ross 308 while CD72 was significantly decreased in both Ross 308 and Ranger Classic ($P < 0.05$) (Fig 2B). Expression of PTPRF and IL-22 was significantly up-regulated ($P < 0.05$) in both Ross 308 and Ranger Classic (Fig 2B).

**4.0 Discussion**

Fast growing broiler genotypes have been artificially selected to produce broilers with maximum energy efficiency that reach market weight faster than ever before (Zuidhof et al., 2014), thus, lessening the environmental impact (feed requirement) and increasing the sustainability of broiler production. However, in the EU there has been movement towards slower growing genotypes, in part, resulting from consumer concerns over animal welfare (Clark et al., 2016; Clark et al., 2017). It is therefore important to elucidate whether fast and slow growing broilers respond differently to disease and, in the post-antibiotic era, whether gene biomarker profiles, which could be used for diagnosis of disease, may differ in these different genotypes.

We report that fast growing (Ross 308) and slow growing (Ranger Classic) broilers differ in their genetic response to coccidiosis as evidenced in longitudinal and dose response experiments with *E. maxima*. Our initial genomic microarray study showed that seventy three genes were differentially expressed in Ross 308 broilers experimentally infected with *E. maxima* oocysts, in Ross 308 naturally infected with *E. maxima* and *E. acervulina* and in Ross 308 with a presumptive diagnosis of coccidiosis. However, a much greater total number of genes were differentially expressed in naturally infected chickens compared to experimentally infected chickens. This may reflect co-infections with more than one species of *Eimera* (as was the case

in samples from Belgium in which lesions consistent with both *E. maxima* and *E. acervulina* were found post-mortem), multiple strains of the same species or co-infections involving *Eimeria* and other pathogenic species.

The genes chosen to study further, in experimental *E. maxima* infections by qPCR, were highly differentially expressed gave a broad biological profile of the response to *E. maxima*. ADD3, MLLT10, NAV2 an PLXNA2 were significantly upregulated in the intestine of Ross 308 but were not differentially expressed in Ranger Classic after 6 dpi with 2500 oocysts. Increased expression of ADD3, which encodes γ-Adducin required for junctional integrity in the intestine (Naydenov and Ivanov, 2010) and NAV2 which is required for neuronal growth (Marzinke et al., 2013) may suggest that tissue repair following infection occurs more quickly in Ross 308 than Ranger Classic. MLLT10, which encodes a chimeric transcription factor (protein AF-10) is associated with human cancer (Chaplin et al., 1995; de Bruijn et al., 2001). However, a study by Mahmoudi et al., (2010) reported a function for this protein in intestinal homeostasis. Therefore, early up-regulation of MLLT10 expression in the intestine of Ross 308 but not Ranger Classic may also highlight a more rapid tissue repair mechanism in faster growing broilers. PLXNA2 encodes a semaphorin type 3 protein (Plexin A2) which induces F-actin re-organisation and migration of human dendritic cells (DC) (Curreli et al., 2016) which may indicate increased DC migration into the intestine and a more active immune response in Ross 308. However, semaphorins are best known for their role in axonal guidance in the nervous system (de Wit and Verhaagen, 2003) and the increase in PLXNA2 signal may correlate in some way with increased NAV2 and neuronal regeneration.

Immune genes included in the biomarker panel were also expressed differently according to genotype, oocyst dose and dpi. This may have reflected different cellular migration patterns in

response to infection in the different broiler genotypes. In Ross 308 chickens, *E. maxima* infection is associated with increased migration of CD4+ lymphocytes and macrophages into the intestine and increased expression of IFN-γ which is associated with inflammatory CD4+ (T helper cell type 1) lymphocytes (Cornelissen et al., 2009). However, our data shows no differential expression of genes which code for some critical intracellular molecules such as lymphocyte specific protein tyrosine kinase (LCK) (Rudd et al., 1988) or plasma membrane myosin protein (MYO1G) (Patino-Lopez et al., 2010). This may indicate a lack of T cell migration into the intestine following infection or lack of lymphocyte signalling occurring within migrated lymphocytes at 6 and 13 dpi.

CSF3, which was previously named myelomonocytic growth factor (MGF) (Gibson et al., 2009) was reported to be similarly (and very significantly) increased in the intestines of White Leghorn (PA12) layer chickens infected with *E. maxima* or *E. tenella* (Laurent et al., 2001) but a lower CSF3 response to LPS was observed in broilers compared to layers (Leshchinsky and Klasing, 2001). Here we show that CSF3 was up-regulated in Ross 308 and Ranger Classic in response to *E. maxima* at 6 dpi with 2500 oocysts but, following infection with 7000 oocysts, up-regulation of CSF3 was only observed in Ranger Classic chickens and this was maintained at 13 dpi. Very little is known about CSF3 in chickens but in mammals the importance of CSF3 in granulocytopoiesis has been known for a number of years (reviewed by Tay et al., 2016). Our study may indicate that slower growing Ranger Classic can prolong granuolocyte recruitment in response to *E. maxima* or that *E. maxima* are cleared more efficiently in faster growing Ross 308.

In contrast, IL-22 was down-regulated in Ranger Classic chickens infected with either 2500 or 7000 oocysts at 6 dpi but was not differentially expressed in Ross 308. Since IL-22 is known to induce production of antimicrobial peptides from chicken epithelial cells (Kim et al., 2012) this

may indicate an initial suppression of antimicrobial peptides by *E. maxima* in Ranger Classic broilers. However, IL-22 suppression was not maintained and by 13 dpi, IL-22 was significantly upregulated in the intestine of both Ranger Classic and Ross 308 infected with either 2500 or 7000 oocysts. In humans, IL-22 is produced by CD4+ Th cells and Th17 cells (Liang et al., 2006; Zheng et al., 2007). However, the study by Kim et al., (2012) reported high levels of IL-22 expression in chicken intestine but stimulation of chicken lymphocytes with Con A did not induce differential expression of IL-22 transcript and IL-22 transcript was reduced by 87% in lymphocytes following culture with LPS. Therefore, the source and function of IL-22 has yet to be fully elucidated in chickens.

Reduced B cell migration into the intestine of Ranger Classic broilers, following *E. maxima* infection, was indicated by down-regulation of CD72 expression at 6 dpi with either 2500 or 7000 oocysts at 6 dpi. In contrast, CD72 was up-regulated at 6 dpi in Ross 308 broilers infected with 2500 oocysts at 6 dpi and was not differentially expressed at d6 pi in Ross 308 infected with 7000 oocysts. Ligation of CD72 induces B lymphocyte proliferation and maturation (Wu and Bondada, 2009) and is down-regulated in chickens with infectious bursal disease (Ruby et al.,2006; Smith et al., 2015; Dulwich et al., 2017) which is known to induce immunosuppression. However, at 13 dpi with 2500 oocysts CD72 was not differentially expressed in either Ross 308 or Ranger Classic broilers but was down-regulated at 13 dpi in both lines infected with 7000 oocysts.

In conclusion, our study reports differential gene expression in fast growing (Ross 308) and slow growing (Ranger Classic) broilers. These differences may indicate that Ross 308 repair intestinal tissue faster than Ranger Classic, following *E. maxima* infection, and that some degree of immunosuppression may occur in Ranger Classic, early in infection. Our study also reports a

potential gene biomarker panel for coccidiosis in Ross 308 and Ranger Classic broilers. This will be used as the basis for future work to test the biomarkers specificity, sensitivity and performance in natural infections.

## Conflict of interests

None to declare.


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**Figure Legends**

**Figure 1. Heat maps comparing differential gene expression in Ross 308 experimentally infected with *E. maxima* and Ross 308 with diagnosed or suspected coccidiosis.**

Genomic microarray heat maps were constructed from intestinal tissues excised from three Ross 308 chickens experimentally infected at 6 dpi with 2500 oocysts *E. maxima* (A); Diagnosed with coccidiosis (B); or suspected of having coccidiosis (C). Colour bar denotes fold changes in gene expression and direction (-/+) with fold changes >2 significant at $P = 0.05$. Hierarchical tree comparing E. maxima infected with uninfected (A); coccidiosis diagnosed with non-diagnosed (B) and coccidiosis suspected with not suspected (C).

**Figure 2. Differential expression of a gene biomarker panel in Ross 308 and Ranger Classic broilers infected with 2500 or 7000 oocysts of *E. maxima* after 6 days post-infection (6 dpi).**

Ross 308 and Ranger Classic broilers were infected with either 2500 (A) or 7000 (B) E. maxima oocysts. Following euthanisation at 6 dpi, expression of biomarker genes was determined by qPCR analysis. Histograms show mean fold changes in gene expression calculated from eight samples per group performed in duplicate. Fold changes are calculated from Ct ratios obtained from infected and control samples in each group. Asterisk (*) denotes a significant difference between these values at P =0.05. Error bars indicate standard deviation from the mean. is shown by bars above each value. ☐ = Ross 308; ■ = Ranger Classic.

**Figure 3. Differential expression of a gene biomarker panel in Ross 308 and Ranger Classic broilers infected with 2500 or 7000 oocysts of *E. maxima* after 13 days post-infection (13 dpi).**

Ross 308 and Ranger Classic broilers were infected with either 2500 (A) or 7000 (B) E. maxima oocysts. Following euthanisation at 13 dpi, expression of biomarker genes was determined by qPCR analysis. Histograms show mean fold changes in gene expression calculated from eight samples per group performed in duplicate. Fold changes are calculated from Ct ratios obtained from infected and control samples in each group. Asterisk (*) denotes a significant difference between these values at P =0.05. Error bars indicate standard deviation from the mean. is shown by bars above each value. ☐ = Ross 308; ■ = Ranger Classic.