UNRAVELING THE PATHOGENESIS AND MOLECULAR DEVELOPMENT OF *EIMERIA* SPECIES UNDER HEAT STRESS AND THERMONEUTRAL CONDITIONS

by

GUSTAVO HENRIQUE SCHNEIDERS

(Under the Direction of Samuel E. Aggrey)

ABSTRACT

Host responses to parasitic infections are complex and involve many facets of cellular and humoral immune mechanisms. Many intestinal infections, such as coccidiosis represent economically important diseases for the poultry industry. Coccidiosis is caused by intracellular protozoan parasites belonging to several different species of the genus *Eimeria* (*E*.) and leads to impaired the growth, higher feed conversion and mortality in poultry. Heat stress (HS) is one of the major environmental stresses in the poultry industry and results in direct losses to production. HS results in reduced oocyst output in chickens. The general objective of this work is to understand the interplay between HS and *Eimeria* spp. pathogenesis in meat-type chickens. We herein combine classical methods with molecular biology tools to elucidate the pathogenicity of the infection with focus on heat stress of the host. The results of this work demonstrate that heat stress significantly reduces development of asexual stages of *E. tenella*. We also show that the reduced outcome of *E. maxima* infection in poultry is result of the reduced sexual reproduction of this parasite in the intestine, and that infection with *E. maxima* leads to disruption of the intestinal

barrier by differential regulation of tight junction genes, resulting in increased intestinal permeability and cellular influx. Lastly, our results indicate a second peak of replication of *E. maxima* without reinfection, not previously reported in the literature. Understanding the nature of the diminished outcome of infection in heat stressed chickens provides basis for future research seeking new methods of preventing and controlling the disease.

INDEX WORDS: *Eimeria*; Coccidiosis; Host-parasite interactions; Heat stress; Pathogenicity; Intestinal barrier.

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GUSTAVO HENRIQUE SCHNEIDERS

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GUSTAVO HENRIQUE SCHNEIDERS

Major Professor:	Samuel E. Aggrey
Committee:	A. Lorraine Fuller
	Susan M. Williams
	David S. Peterson
	Dennis Kyle

Electronic Version Approved:

Ron Walcott
Dean of the Graduate School
The University of Georgia
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CHAPTER 1

INTRODUCTION

Host responses to parasitic infections are complex and involve many facets of cellular as well as humoral immune mechanisms. Many intestinal infections, such as coccidiosis represent economically important diseases for the poultry industry. Coccidiosis is caused by intracellular protozoan parasites belonging to several different species of the genus *Eimeria* (*E.*) and leads to impaired growth, higher feed conversion and mortality in poultry [1], culminating in severe economic losses for this industry. Although the economic impact of coccidiosis is unclear, it has been estimated in more than US$3 billion worldwide [2].

Eimeria, the largest genus of the Coccidia family, presents a self-limiting intestinal infection based on the number of ingested oocysts [3]. Seven species of *Eimeria* can infect the chicken, each one developing and causing disease in different sections of the intestinal tract: *Eimeria acervulina* and *E. praecox* develop mostly in the duodenum, with the possibility of extending to the mid-intestine in heavy infections. *Eimeria mitis*, *E. maxima* and *E. necatrix* develop in the mid-intestine and can extend to the posterior intestine. *Eimeria tenella* develops in the ceca [4]. *Eimeria brunetti* develops in the lower intestine and rectum [5]. The site of reproduction remains unaltered despite concomitant infection in the host [6].

The life cycle of *Eimeria* spp. goes through a series of stages with a specific number of schizogenous generations. When sporulated *Eimeria* oocysts are ingested, the mechanical movement of the gizzard ruptures the oocyst wall, releasing sporocysts. Trypsin and bile in the

duodenum promote excystation of the sporozoites from the sporocysts, followed by infection of intestinal epithelial cells by sporozoites. Depending on the species, sporozoites penetrate in site-specific host mucosal cells in different segments of the intestinal tract, passing through the basal membrane into the lamina propria, where they are engulfed and carried by macrophages to the glands of Lieberkühn, then leaving the macrophages and entering the epithelial cells of the glands, where they come to lie beneath the host cell nucleus (away from the lumen). They round up and turn into the first generation meronts. Each meront forms several first generation merozoites by multiple fission (schizonogony or merogony). The first generation merozoites invade new host cells and forms the second generation of merozoites. Some of them enter a new host cell to form a third generation meronts. Others, however, enter the new host cell and begin the sexual phase of the life cycle, known as gamogony or gametogony, when the meronts develop into female gametes (macrogametes) or male gamonts (microgamonts). The microgamonts fertilize the microgametes to form zygotes, later developing into an unsporulated oocyst that will be eliminated in the feces [7,8]. Some sporozoites of *Eimeria* (*E. brunetti* and *E. praecox*) develop within the cells of the surface epithelium, in which they initially penetrate [7] Other species' sporozoites (*E. acervulina, E. maxima, E. necatrix* and *E. tenella*) develop into trophozoites in other sites after being transported via the crypt epithelium [9,10].

Development within the host's intestinal tissue results in several clinical signs. *Eimeria tenella* produces hemorrhage in both caeca, which is accompanied by the presence of white dots (schizonts and oocysts) that can be seen from the serosal surface. *Eimeria tenella* also penetrates deep in the intestinal tissue, producing heavy damage to the mucosa and mucosal layer. The cecal lumen becomes filled with coagulated blood and necrotic mucosal debris. Microscopically, the epithelium appears enlarged and denuded with hemorrhages. The cecal wall is often greatly

thickened because of edema, cellular infiltration and later scar tissue [11,12]. *E. maxima* causes congestion and edema, cellular infiltration, thickening of the mucosa, with the later generations of schizonts and sexual stages developing deeper in the tissues, causing considerable disruption of the mucosa [12]. Coccidiosis caused by *E. maxima* causes emaciation, pallor, roughening of feathers and anorexia, abundance of yellow-orange mucus and fluid in the distal portion of the jejunum and proximal portion of the ileum, edema, thickening and disruption of the mucosa and sometimes presence of blood in the intestinal lumen are observed at necropsy [12].

Heat stress (HS) is one of the major environmental stresses in the poultry industry and results in direct losses to production in the form of decreased body weight [13,14], protein and fat retention, feed consumption [15], lower meat quantity and quality [16], decreased egg quality and production [17], higher mortality rate and feed conversion [18]. Stress is a predisposing factor of immunosuppression in broilers, offering a good opportunity for normal commensals to induce infection and disease [19-21]. Heat stress has been reported to alter microbial (*Lactobacillus* sp.) composition in chickens [22], and predispose them to enteritis [23], to enhance *Salmonella enterica* serovar Enteritidis attachment and colonization to intestinal tissue and reduce intestinal crypt depth [21]. Heat stress has a negative effect on oocysts counts during primary coccidial infections in poultry, as noted by the reduced oocyst output when compared with uninfected chickens under HS, however, it does not exert an effect on a second exposure to *Eimeria* infection in chickens older than 27 days [24]. Recently, a study observed that chickens infected with mixed *Eimeria* spp. and raised under HS did not shed any oocysts [25], indicating that the interplay between oocyst shedding and heat stress might be related to other factors such as species of *Eimeria* involved and infectious dose.

Heat stress has also been linked to a decrease in intestinal immune activity and to compromised intestinal morphology following pathogenic bacteria invasion of the body through intestinal epithelium [26-29], these are attributed to the diminished macrophage activity, as evidenced by a decreased oxidative burst [30], allowing intracellular bacteria to survive in the macrophages of heat-stressed chickens [28], and also the loss of integrity of the intestinal barrier, leading to increased intestinal permeability and intestinal inflammation [28,31]. Conversely, heat-stressed broiler chickens present a trend towards a decrease in gross lesion scores and significantly lower microscopic scores of necrotic enteritis, as noted by less congestion and villi fusion with milder bacterial colonization in the villi [32]. These enhanced clinical conditions in heat-stressed chickens are believed to be related to the stress-induced regulation of intestinal inflammation, specifically, the decrease in polymorphonuclear cell infiltration, resulting in the prevention of development of *C. perfringens* type A-mediated necrotic enteritis [32].

The detrimental effect of HS on primary *Eimeria* infections is believed to be attributed to changes in immunity under heat stress conditions. For instance, HS reduces the expression of IL-12 and plasma levels of IFN-γ, important pro-inflammatory cytokines [33]. In addition, it is common knowledge that coccidiosis tends to be less severe in the summer [34,35], presumably due to effect of the heat on host and parasites. The relationship between HS and coccidiosis is not yet well elucidated.

Due to the complex life cycle of *Eimeria* spp., it is not surprising that several components of the immune system play a role in mitigating the infection. While specific mechanisms such as cellular immunity, especially $CD8^+$, are the most effective against coccidiosis [36-38], non-specific barriers are of fundamental importance, especially in the early days in the course of infection and in the development of cellular immunity [39,40]. Non-specific factors include physical barriers,

phagocytes, leukocytes and the complement system. Intestinal forms of the parasite such as meronts, gamonts and oocysts, cause severe morphological alterations to the host`s gastrointestinal tract (GIT), marked by the disruption of the intestinal barrier, distortion, rupture, segregation of adjacent cells, edema, cellular infiltration, sloughing of intestinal epithelia, resulting in the clinical signs observed in the disease [12,38,41].

Tight junctions (TJ) are multi-protein complexes responsible for the regulation of permeability in the intestine via the modulation of its proteins [42], by sealing the paracellular space between adjacent epithelial cells [43,44]. The main clinical sign of *E. bovis* infection in cattle is diarrhea, linked to the downregulation of TJ gene expression [45]. Fluorescein isothiocyanate dextran (FITC-d) is a 3000-5000 Da molecular weight and does not cross intact intestinal blood barrier in high quantities unless the intestinal barrier is compromised [46]. Oral administration of FITC-d has been broadly used to assess the disruption of the intestinal barrier in chickens by measuring intestinal permeability [38,47]. Similarly, the differential expression of TJ proteins such as zona occludens-1 protein (ZO-1), occludin, claudin 1 and junction adhesion molecule 2 (JAM-2) have been used to assess intestinal permeability in chickens [48,49], producing contrasting results.

The general objective of this work was to elucidate the interplay between HS and *Eimeria* spp. pathogenesis in meat-type chickens. The first specific objective of this study is to investigate the effect of temperature on viability, infectivity and development of *E. tenella* sporozoites in vitro, as well as the development of merozoites and fecal oocyst shedding in chickens infected with *E. tenella* and exposed to HS. The second specific objective is to investigate the effect of HS on the pathogenesis of *E. maxima* infection in broilers, as well as differential expression of host cytokines that might affect the curtailed development of the parasite. The third specific objective is to elucidate the dynamics of the intestinal barrier and parasite development, analyzing intestinal

permeability elicited by *E*. *maxima* infection and the ontogeny of lesion development throughout the first 10 days of infection, as well as investigating possible biomarkers for intestinal lesion in coccidiosis poultry models.

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CHAPTER 2

LITERATURE REVIEW

SPECIES, GENOME AND STRUCTURE

Commonly found in backyard and industrial production of chickens, *Eimeria*, the largest genus of the Coccidia family, presents a self-limiting intestinal infection based on the number of ingested oocysts [1]. Seven species of *Eimeria* can infect the chicken, each one developing and causing disease in different sections of the intestinal tract. *E. acervulina* and *E. praecox* develop mostly in the duodenum, with the possibility of extending to the mid-intestine in heavy infections. *E. mitis*, *E. maxima* and *E. necatrix* develop in the mid-intestine and can extend to the posterior intestine. *E. tenella* develops in the ceca [2]. *E brunetti* develops in the lower intestine and rectum [3]. The site of reproduction remains unaltered despite concomitant infection in the host [4].

The *E. tenella* genome comprises 50 to 60 Mb DNA [5] with predicted 8,786 proteins and gene density of 160 per Mb^{-1} [6]. Repetitive DNA sequences in *Eimeria*`s genes have been demonstrated in the past [7] and more recently the chromosome of *E. tenella* was shown to have an unusual segmented structure with three repeat-rich segments containing a large number of simple sequence repeats [8]. There are also indications that large part of the genome displays copy-number variation between *E. tenella* strains, what is believed to be related to the constant need of adaptations to emerging resistance in the host [8].

Structure

Apical complex

Many of the distinct characteristics of the apicomplexans constitute a collection of unique organelles named the apical complex. These include the rhoptries, the micronemes, the apical polar ring and the conoid.

Rhoptries and micronemes

Rhoptries and micronemes are unique secretory organelles that contain products required for motility, adhesion and invasion to host cells and the establishment of the parasitophorous vacuole [9,10]. Proteins derived from the rhoptry secretory organelles are crucial for the invasion and survival of apicomplexan parasites within the host cells. The rhoptry organelle contain two populations of proteins that are localized in two different segments of the organelle: Rhoptry neck proteins (RON) and Rhoptry proteins (ROP). RON are secreted early in the invasion and are necessary for the formation and function of the moving junction between parasite and host cell membranes [11,12]. ROP proteins, released later, into the nascent parasitophorous vacuole, where they exert their role in modifying the vacuolar environment, and into the host cell, where they act as determinants of virulence through their ability to interact with host cell signaling pathways, causing an array of downstream effects, such as "confusion" of the immune system in detecting and destroying infected cells [13].

In the process of invasion, microneme proteins are secreted from the microneme organelle and play a key role in parasite adhesion and cell tropism by supporting parasite gliding motility, active cell invasion and migration through cells. Microneme proteins bind to sialylated glycans and are considered to be major determinants of the host tissue tropism of *E. tenella* [14-16]. The

microneme is located at the apical end of the zoite and varies between the different genus, species and developmental stages. In general, zoites displaying more gliding or migration activity have more micronemes [15]. For example, merozoites of *Plasmodium*, which neither glide or migrate but rapidly invade erythrocytes have a few micronemes [17], while sporozoites and merozoites of *Eimeria* spp. which glide, migrate through the intestinal contents and actively invade erythrocytes have many [18]. In *E. tenella*, microneme secretion of a temperature-dependent regulated process. *In vitro*, this process is mimicked by temperature and the addition of fetal calf serum or purified albumin to extracellular sporozoites. Microneme proteins are released onto the parasite surface at the apical end and are then redistributed backwards in an actin-dependent capping reaction [16].

Conoid

This structure is a small hollow truncated cone-shaped structure composed of a spiral of filaments believed to play a mechanical role in the invasion process and present only in some apicomplexans. Ducts of rhoptries and some microtubules are within the conoid [19-21]. The conoid is believed to aid in the penetration of host cells, together with the secretions of rhoptry and microneme proteins [22].

Apicoplast (plastid)

Besides the apical complex, the apicoplast is another unique structural feature of apicomplexans. The apicoplast is an essential chloroplast-like organelle, result of a secondary endosymbiosis by ancient apicomplexan parasites [23], prevenient from green algae [24]. It contains and ensemble of bacteria-like pathways to replicate and express its genome plus an anabolic capacity of generating fatty acids, heme and isoprenal precursors. The apicoplast is essential and

perturbing it usually result in parasite death, which makes this structure an interesting target for drug development [25].

In a summary, McFadden [25] describes the apicoplast function as a precursor of isoprenoids, ubiquinone side chains, dolichols and as an essential component for the modification of tRNAs. This suite of anabolic pathways is fueled with phosphorylated three carbon sugars imported from the parasite`s cytosolic glycolytic pathway using plastid-like transporters located in the membrane of the apicoplast. These metabolic pathways work in a manner identical to a non-photosynthetic plant of algal plastid. However, the exact importance of the organelle and why these phylum of parasites retained this structure long after they relinquished photosynthesis remains unclear.

Mitochondria

The mitochondria of apicomplexans in general is a single tubular organellar structure with notable variability in its appearance and the form and size of its cristae, depending on the species and life-stages in question [26]. It is believed that mitochondria plays key roles in the energy metabolism of apicomplexan parasites. Most of the members of the phylum, with the exception of the genus *Cryptosporidium* spp., have both apicoplast and mitochondrial genomes. But due to the intimate relationship of mitochondria and apicoplast, it is difficult to determine the exact functions of each one of the organelles individually. Some studies have shown that the mitochondria contributes in the iron-sulfur cluster biosynthesis [27] and, in *Plasmodium* [28] and *Toxoplasma* [29], the mitochondria has also importance in heme and pyrimidine biosynthesis.

The complete mitochondrial genome sequence of *Eimeria innocua* has been determined to contain three protein-coding genes (*cox1*, *cox3* and *cytb*) and a large number of fragmented rDNAs (19 fragments encoding large subunit (LSU) rRNA and 14 fragments encoding small subunit

(SSU) rRNA). Fragmented rDNA is typical of apicomplexan mitochondrial genomes [30]. Similarly, the mitochondrial genome of *Eimeria mitis* has been show to consist of the same three protein-coding genes described for *E. innocua*, but with 12 gene fragments encoding LSU rRNA and 7 gene fragments for the SSU rRNA [31]. A barcoding study in *Eimeria* spp. has demonstrated that partial mitochondrial cytochrome c oxidase subunit I (*cox1*) sequences are more reliable species-specific than complete nucleic SSU rRNA sequences, and that they provide more synapomorphic characters. Additionally, multiple copies of *cox1* are found within the mitochondria of coccidia, making it a good PCR target [32].

Other structural organelles

Apicomplexans are haploid for the majority of their lives, with complex life cycles that involves differentiation to forms that invade distinct tissues and hosts. This differentiation can include the fertilization of the female gametes (macrogametes) by the male gametes (microgametes) to form the zygote, the diploid form. This is known as gamogony. The zygote undergoes meiosis to re-establish haploid organisms. In some cases, this differentiation also permits the infection of other organisms (vectors), such as ticks and mosquitoes [33,34]. There are three types of multiple fission in Apicomplexa: Sporogony (formation of sporozoites), Merogony (formation of merozoites) and Gamogony (formation of gametes). These are reviewed in detail by Chobotar and Scholtyseck [22]:

Sporozoites and merozoites are elongated, fusiform motile stages capable of selecting and penetrating host cells and initiating intracellular development. They are enclosed with a tri-membranous pellicle that contain one or more micropores. The anterior of the body is packed with a series of organelles, including micronemes, rhoptries, often at least one Golgi complex, one or

two polar rings and a conoid preceded by two pre-conoidal rings. Other cytoplasmic organelles include lipid bodies, amylopectin granules, mitochondria, endoplasmic reticulum, ribosomes, and a nucleus, as depicted in Figure 2.1 [22].

After entering host cells, sporozoites gradually increase in width and have a gradual disappearance of the inner pellicular complex, subpellicular microtubules and organelles of the apical complex, transforming into the spheroid stages. During this transformation, the refractile bodies undergo change in shape, size and location. The anterior refractile body usually becomes smaller and disappears, whereas the posterior body undergoes fragmentation and gradually disappears or becomes incorporated into the merozoites. Additional ultrastructural changes include an increase in the size of the nucleus and nucleolus, an increment in the amount of endoplasmic reticulum and ribosomes, a raise in the size and number of mitochondria, and depletion of amylopectin granules. The nuclear division preceding the merozoite formation is a mitotic process associated with the appearance of kinetic centers (centrioles) and an eccentric or centrally placed intranuclear spindle. The spindle apparatus, consisting of an undetermined number of microtubules and originate at two dense poles names centrocones. As development continues, the centrocones and the nucleus become elongated and division is completed by an invagination of the nuclear membrane at the midregion. Merozoites of the various species of coccidia vary somewhat in their size, presence or absence of refractile bodies, and number of subpellicular microtubules and of micronemes [22].

The process of gamogony is similar to merogony. Merozoites from the final asexual generation penetrate host cells, where they reside in the parasitophorous vacuole and undergo transformation from a fusiform to a spheroid shape with the subsequent loss of the organelles of the apical complex. Microgamete formation begins when the gamonts have peripheral nuclei over

which develops a surface protrusion containing two centrioles. Also present is an intranuclear spindle apparatus with centroconed directed to the centrioles, a mitochondrion in close association with the nucleus. The centrioles develop into basal bodies and give rise to the flagella, which grow from the protrusion in the parasitophorous vacuole. Maturation of the microgramete is marked by elongation of the mitochondria, nucleus and flagella, followed by separation of the residual cytoplasm. In the macrogamete formation, cytosolic changes occur soon after cell entry. After assuming an ellipsoid or spheroid shape, young macrogamonts can be differentiated from the microgamonts because the former have and enlarged nucleus and a prominent osmiophilic nucleolus. Portions of the inner pellicular complex, some micronemes, and subpellicular microtubules may persist for a time as remnants of the former merozoite. There is usually a marked increase in the rough endoplasmic reticulum, ribosomes, mitochondria, and Golgi complexes. Young macrogamonts have little to no lipid bodies or amylopectin granules, but these become increasingly numerous as development continues. The most distinctive characteristic in macrogamonts is the presence of wall forming bodies, which aid in the formation of the oocyst, as explained later on this review. At maturity, the macrogamont is considered a macrogamete and is bounded by one to three membranes, has wall forming bodies usually near the periphery, with lipid bodies and amylopectin granules in the interior of the wall forming bodies and a large nucleus and nucleolus. During fertilization, microgametes seek macrogametes and adhere to their surface, and fertilization occurs by the fusion of the plasmalemmas of the two gametes, resulting in the formation of a narrow stalk through which the nucleoplasm of the microgamete passes into the macrogamete cytoplasm [22]. Following fertilization, there is the development of the oocyst, which will be explained in more details further.

Oocyst structure

Perhaps one of the most important features of the genus *Eimeria* spp. is their resilience to the external environment. The oocyst wall, developed from material synthesized during the maturation of the macrogamete, is the primary barrier between the harsh environment and the eight infectious sporozoites contained inside, conferring resistance to both chemical and physical insults, including several disinfectants. Additionally, every pair of sporozoites inside the oocyst is protected by another barrier, the sporocyst [35].

There are two types of oocyst wall forming bodies (WFB), WFB1 and WFB2, that are synthesized during maturation of the macrogamete, which appears to give rise to the two distinct layers that form the oocyst wall. Complimentary information also indicates the presence of another structure, a veil, that encloses the developing intracellular oocyst, and appears to be lost during excretion with the feces. The structure of the veil is conserved across coccidia [36].

Several oocyst wall proteins have been identified and characterized to different degrees, including EtOWP6, a cysteine-rich protein, found in the outer layer of the oocyst wall and related to other proteins found in apicomplexans. Other proteins identified include a histidine-rich protein localized in the inner part of the oocyst wall, and two proteins found in the wall forming bodies type 2 of macrogametocytes, GAM56 and GAM82, that contain tyrosine-rich regions and are processed into smaller proteins, which in turn are incorporated in the inner layer of the oocyst wall [37]. Similar to the oocyst wall, the sporocyst wall also contains cysteine-rich proteins, such as EtSWP1, indicating that the biochemistry of sporocyst wall assembly is analogous to that of oocyst walls [38].

The process of oocyst formation is described in details by Frolich, et al. [39]. In brief, as the macrogametocytes mature, the veil forming bodies (VFB) and WFBs are synthesized as small

vesicles that enlarge and spread out in the cytoplasm during development. Once the developing parasite has reached its final size, the veil, composed mostly of proteins and glycoproteins, is laid down, surrounding the parasite membrane. In mid and late-stage macrogametocytes, the neutral lipids containing WFB1 align themselves at the periphery of the parasite, forming links and chains, and their contents are then exocytosed as lipid patches, called rafts, into the space between the two parasite membranes, in an organized patchwork pattern. During the outer wall formation, the WFB2 proteins, which are richly composed of glycoproteins GAM56, GAM82 and GAM230, begin to coalesce in the gametocyte cytoplasm. Then, these tyrosine-rich glycoproteins are enzymatically cleaved into smaller peptides that are cross linked and inserted into the inner wall. At last, there are some leftover WFB that remain in the cytoplasm of the newly formed oocyst. Therefore, the outer layer is constituted of mainly lipids, whereas the inner layer is composed of mainly glycoproteins [40]. The structure of the oocyst is depicted on Figure 2.2.

LIFE CYCLE AND PATHOGENESIS

The proliferation of apicomplexan parasites is intracellular. It occurs by invasion of host cell and is followed by parasite growth and cell division until the host cell lyses by the parasite replication [10]. The majority of the apicomplexan parasites grow and replicate within the parasitophorous vacuole, a non-phagosomal structure that is segregated from most cellular trafficking pathways [10,41,42].

Apicomplexan parasites do not grow or replicate in the extracellular environment, therefore, after release of the parasites from the lysing infected host cell, these must rapidly invade other host cells in order to survive [43,44]. This causes repeated cycles of reinfection within the host,

resulting in a rapid increase in the number of parasites, increase in lysing of host cells and parasite re-invasion of new cells, potentially resulting in extensive tissue damage [44].

The self-limiting life cycle of *Eimeria* spp. goes through a series of stages with a specific number of schizongenous generations. When sporulated *Eimeria* oocysts are ingested, the mechanical movement of the gizzard ruptures the oocyst wall, releasing sporocysts. Trypsin and bile in the duodenum promote excystation of the sporozoites from the sporocysts, followed by infection of intestinal epithelial cells by sporozoites. Each one of the seven *Eimeria* species infects one segment of the intestinal epithelium of the domestic chicken, what is attributed to the specificity of binding to the different types of mucins through the gastro intestinal tract (GIT) [45]. Besides mucin, site specificity for invasion by each species has also been associated with unique conditions in the intestinal lumen, such as pH, enzyme makeup, cell metabolites, among others [46]. Depending on the species, sporozoites penetrate in site-specific host mucosal cells in different segments of the intestinal tract, passing through the basal membrane into the lamina propria, where they are engulfed and carried by macrophages to the glands of Lieberkühn, then leaving the macrophages and entering the epithelial cells of the glands, where they come to lie beneath the host cell nucleus (away from the lumen). They round up and turn into the first generation meronts. Each meront forms about 900 first generation merozoites (about 2-4µm long) by multiple fission (schizonogony, also knonw as merogony). Different species and strains of *Eimeria* produce different numbers of merozoites. The first generation merozoites enter the lumen of the intestinal segment infected about 2.5-3 days after inoculation. Each one enters a new host cell, rounds up and becomes a second generation meront, which lies above the host cell nucleus. It forms 200 to 350 second generation merozoites (16µm long) in about 5 days after inoculation. Some of them enter a new host cell to form a third generation meronts. Others, however, enter the new host cell

and begun the sexual phase of the life cycle, known as gamogony or gametogony. Most of these, round up and develop into female gametes (macrogametes). Others become male gamonts (microgamonts). The microgamonts fertilize the microgametes to form zygotes. These have eosinophilic granules (wall-forming bodies) around their periphery, which flatten out, fuse and form a three-layered wall around the zygote, resulting in the formation of an oocyst [47,48]. Some sporozoites of *Eimeria* (*E. brunetti* and *E. praecox*) develop within the cells of the surface epithelium, in which they initially penetrate [47] Other species' sporozoites (*E. acervulina, E. maxima, E. necatrix* and *E. tenella*) develop into trophozoites in other sites after being transported via the crypt epithelium [49,50]. The general life cycle of *Eimeria* spp. is depicted on Figure 2.3.

The reproductive index varies among *Eimeria* species, for example, *E. brunetti* has much higher reproductive potential as compared to *E. maxima* [51]. The reproductive potential may also be affected by the degree of infection of the host, a phenomenon called crowding effect. Generally, the reproductive potential of *Eimeria* spp. decreases as the number of oocysts given to the host increases [52]. The crowding effect can be a combination of factors such as initial availability of epithelial cells for parasitism, the sloughing of epithelium during the development of an infection and in the case of *Eimeria tenella*, the formation of cecal cores that prevent the discharge of oocysts [52,53]. Additionally, at the same infective doses, the oocyst production tend to be higher in older chickens, a factor attributed to the increased availability of epithelial cells for parasitism [54]. The availability of epithelial cells is believed to be a more important factor than immunity in the crowding effect, although both factors are undoubtedly connected [51]. In a comparison among the seven species of *Eimeria*, Williams [51] estimated that the crowding thresholds were 903, ≤16, 39, ≤14, ≤16, ≤16 or 72 sporulated oocysts, respectively, for *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* or *E. tenella*.

Clinical signs during *E. tenella* and *E. necatrix* infections, such as bloody feces, are associated with the schizont maturation, resulting in tissue damage due to cell lysis. The outcome of infection is the same for all *Eimeria* species, the release of the unsporulated oocyst in the feces, diploid containing a single cell and not infectious [48]. In order to become infectious, the oocyst must go through a sporulation process that includes many steps. The first one is a meiotic process, in which the DNA in each daughter nucleolus becomes half of that of the parent nucleus. The second division is a mitosis process, thus no apparent reduction in nuclear DNA content is observed in the newly formed nuclei. The third step is characterized by cytokinesis, which leads to the formation of four sporoblasts and eventual sporocyst maturation [55]. During sporulation, this cell undergoes reduction division, sporulating by sporogony, becoming haploid. Thereafter, the sporont divides into four sporoblasts and each turn into a sporocyst containing two sporozoites. Sporulation is induced by several factors, but the most important ones are exposure to an environment containing free access to oxygen, at the temperature range of 10˚C to 30˚C [56]. Under ideal conditions, sporulation occurs between 24 and 60 hours post release of the oocyst in the environment [57,58]. The sporulated oocyst is ready to be ingested and restart the infection cycle [48]. The level of parasitism might not be directly correlated with the degree of clinical signs observed, as infection levels vary substantially from hundreds to millions of oocysts per gram of feces, without clinical signs of coccidiosis being reported [59].

Site specificity for invasion by each species may be associated with unique conditions in the intestinal lumen, such as pH, enzyme makeup, mucous, cell metabolites, among others [46]. It is believed that host cell plays little role in the physical propulsion of the parasite into the host cell, however, its importance in the infection process might reside in the presence of surface molecules that might serve as receptors, or of metabolic products that attract or activate the parasite [46]. Several

studies were conducted where specific cell receptors were blocked by monoclonal antibodies [60,61]. And other studies have shown that the modification of the host cell with a variety of compounds significantly altered the ability of apicomplexan sporozoites to invade the treated cells, suggesting the presence of a host cell surface receptor or recognition site [62,63].

Lectins have been reported as part of the biochemical composition of *E. tenella*, *E. acervulina* and *E. maxima*. The lectin from each species has different sugar affinity and is found almost exclusively in the sporozoites. There was also significant correlation among the sporozoite lectin, the carbohydrate moieties of the intestinal cell surface, the pH of the lectin-carbohydrate interaction, and the area of the intestine that was invaded by each species [64]. Further evidence shows that several lectins bound to the surface of cultured primary turkey cells, with the exception of one (wheat germ agglutinin), failed to inhibit invasion by several species of *Eimeria* spp. [65].

Apicomplexans move by gliding motility. Rather than having a flagellum, as bacteria does, in apicomplexans this movement is substrate dependent and consists of three movements: circular gliding, upright twirling and helical gliding [66]. Sporozoite motility is a primary requisite for cell invasion. *Eimeria* spp. sporozoites are able to recognize, contact and enter the host cell through a circular gliding motion produced by the capping of the surface membrane proteins toward the end of the rear of the parasite [67,68].

Actin-disrupting or actin-stabilizing drugs (such as cytochalasin D and jasplakinolide), as well as myosin inhibitors (butanedione monoxime) have been shown to disrupt Toxoplasma motility and invasion [69,70]. Suggesting that actomyosin-based mechanism is the responsible for motility and invasion for other apicomplexans as well. Similarly, extracellular stages of *E. tenella* display actinmyosin-dependent gliding motility [67].

During invasion, apicomplexan parasites attach to host cells via their apical ends and form an intimate junction with the host cell membrane. This junction moves backwards on the parasite as the infection progresses and is eventually pinched off at its rear end [71]. Motility and invasion are accompanied by the secretion of molecules from specialized organelles located at the apical end of the parasite, such as rhoptrie and microneme proteins [9,10]. Microneme secretion starts even before the parasite enters the host cell [13].

In the specific case of *Eimeria* spp. this parasite forcefully invades the host cell using the actinmyosin-dependent glideosome motility specialized proteins from the rhoptry and microneme secretory organelles as mentioned earlier on this review. Microneme secretion *per se* is not dependent on parasite motility [16]. Host cell recognition is required for invasion, followed by apical attachment to the host cell surface, formation of tight moving junctions between the infective stage of the parasite and the host cell membranes, followed by forward movement of the parasite through this junction into a newly created intracellular, fusion-resistant, membrane bound parasitophorous vacuole, in which the parasite will grow and divide [10]. As mentioned above, microneme proteins are important for the invasion of host cells. Studies in *Eimeria nieschulzi* concluded that albumin, fetuin, cholic acid and trypsin enhanced motility, and that albumin and fetuin also enhanced cell invasion [72].

Host cells have their own regulatory mechanism to cope with changes occurred during invasion. The expression of indoleamine 2,3-dioxygenase1 (IDO1) in epithelial host cells has been shown to increase post infection with *E. falciformis* in mice, with the augmented expression lasting throughout the course of infection [73]. Chicken toll-like receptors 4 and 15 (TLR4 and TLR15, respectively) have also been shown to be upregulated and involved in the recognition of *E. tenella* post infection [74]. A more recent analysis of differentially expressed proteins found that a total of

195 proteins were significantly changed in baby hamster kidney (BHK-21) cells infected with *E. tenella*, most of them involved in the host signaling pathway, such as SOS1, that promotes *Ras* pathway activation, evidencing that this pathway is important for *E. tenella* invasion. Other differentially expressed proteins in the same study were involved in the phophatidylinositide-3-kinase-protein kinase B (PI3K-Akt), chemokine and retinoic acid-inducible gene I (RIG-I) receptor-mediated phagocytosis and in the *Wnt* and *p53* signaling pathways [75]. These results show that *Eimeria* spp. have the ability to reprogram host cell responses for their survival and reproduction. Other apicomplexan, such as *Cryptosporidium parvum*, *Theileria parva*, *Toxoplasma gondii* and *Neospora caninum* have also been shown to modulate host cell apoptosis to guarantee successful intracellular development [76-79].

Successful parasite replication is also dependent on the parasite-host interactions at the cellular membrane level. Zhao, et al. [75] also found that the differentially expressed proteins in BHK 21 cells infected with *E. tenella* also included amino acid transporters (SLC1A1), mitochondrial membrane ATP synthase (ATP51) and cytochrome C oxidase polypeptide 7A1 (COX7A1). Other differentially expressed proteins in the aforementioned study were found to be related to host metabolic and stress and defense response, such as apoptosis antagonizing transcription factor (AATF), cyl-CoA, lysophosphatidyl-glycerol acyltranferase 1 (Lpgat1), platelet-activating factor acetylhydrolase IB subunit beta (Pafah1b2), and acid ceramidase (Asah1), indicating that infected BHK21 cell produced several antimicrobial peptides as part of the innate immune response to defend against *E. tenella* infection.

Research has shown that embryo cecal epithelial cells infected with *E. tenella* had decreased apoptosis during the early developmental stages of the parasite, and inversely increase apoptosis during the middle and late developmental stages of infection [80]. It has been also proposed

that blocking the mitochondrial apoptotic pathway might be one of the methods used by *Eimeria* spp. to inhibit apoptosis of infected host cells [81]. Xu, et al. [82] demonstrated that *Eimeria tenella* modulates cell host apoptosis by interfering with host`s mitochondrial permeability transition pore (MPTP). Moreover, by inhibiting MPTP opening in infected cells causes an increase in mitochondrial transmembrane potential and decreased apoptosis. Through life cycle, *Eimeria* spp. expresses from 6000 to 9000 proteins, presenting the host with a wide portfolio of antigens [83].

HEAT STRESS, ANOREXIA AND PATHOGEN INTERACTIONS

Production environments for poultry invariably result in the exposure to immunosuppressive stressors and infectious diseases. An understanding of the presence and pathogenesis of immunosuppressive risk factors is essential for the management of optimal health and welfare, resulting in efficient production. The purpose of this review is to merge research findings on stressor factors such as high temperatures, feed restriction, other immunosuppressive factors and the interaction of an immunosuppressed host with *Eimeria* spp.

Behavioral and physiological responses to heat stress

During heat stress, birds tend to change their behavior to help maintain body temperature within the normal limits. This can occur very rapidly and at less cost to the bird than most physiological adjustments. These changes, however, are preceded by the molecular response to heat stress, that, in turn, is mediated by heat shock proteins. Chickens housed under heat stress conditions devote less time to walking and standing, consuming less feed and more water to compensate for water lost through evaporative cooling [84]. However, when heat stress occurs concomitantly to other stressors, a reduction in drinking time can be observed. Heat stressed birds

also spend less time engaging in social behavior. When housed in cages, heat stressed chickens tend to distance themselves from each other, pant, and often stand with their wings drooped and lifted slightly from the body, maximizing heat loss [84].

The physiological responses to heat stress in birds involve the functional integration of several organs to meet the metabolic needs of birds that are trying to dissipate heat and maintain homeostasis. Exposure of chickens to high environmental temperatures produces an initial increase in the temperature of the peripheral tissues and subsequently in core body temperature. The responses to heat also depend on the time of adaptation to the new temperature. The body temperature of chickens starts to increase when the ambient temperature is of 30˚C or above when the raise of ambient temperature is fast. On the other hand, if the ambient temperature raises more slowly, the birds maintain their normal body temperature until the ambient temperature reaches to 33˚C [84]. In general adult chickens require 3 to 5 days to acclimatize to both hot and cold environments [85].

Several responses that increase the dissipation of heat are implemented once heat accumulates in the tissues. For instance, there is an increase in water consumption and simultaneous decrease in feed consumption. The increase in water consumption occurs immediately and is necessary to meet the requirements of evaporative cooling from respiratory surfaces, whereas the drop in feed consumption is usually delayed until several hours after the chickens have been exposed to high temperatures, and reduces the contribution of metabolic heat to the total heat load that requires dispersion, as reviewed by Etches, et al. [84]. Fasting for 1 to 3 days has shown to increase the survival rates of heat-stressed chickens (McCormick 1979).

Information compiled by Quinteiro-Filho, et al. [86] describes the effects of heat stress in avian species as: 1) reduction in feed consumption, body weight gain, total white blood cell count

and antibody production; 2) decrease in the number of peripheral blood lymphocytes and induction of an electrolyte imbalance; 3) decrease in blood lymphocytes and spleen weight; 4) reduction in the feed conversion and intake, and macrophage activity; 5) decrease in $CD4^+$ and $CD8^+$ lymphocytes and antibody production; 6) decrease in growth rates, intestinal villi height, and wet and dry weights of the jejunum. Apart from those, heat stress appears to negatively affect mortality rates and intestinal development, especially the integrity of the intestinal epithelium [86-88]. Moreover, induced heat stress of 31˚C and 36˚C applied for 10 hours per day from the 35 - 42 days of age, in broilers, decreased performance parameters and peritoneal macrophage activity, increasing corticosterone serum levels and inducing minor changes in the intestinal mucosa, indicating an inflammatory process in broiler chickens [86].

The relationship of stressors and involution of lymphatic organs has been previously reported [89]. In a more detailed study, the decrease in the relative weights of the bursa of Fabricius, spleen and thymus, is suggested due to the high levels of stress, hypothalamus-pituitary-adrenal (HPA) axis activation and corticosterone serum levels [86].

Lesions in the intestinal tract have been reported as some of the earliest manifestations of stress. It has been extensively documented that a normal intestinal morphology is necessary to prevent bacteria translocation from the intestinal tract to the body, and for digestion and absorption of nutrients. A short exposure of chickens to heat stress conditions has been shown to reduce ileum`s crypt depth, villus height and in the villus:crypt ratio [87,90]. These morphological changes, however, are still controversial as some studies have reported unaltered intestinal morphology under heat stress conditions [86]. Interestingly, the intestinal epithelial structure is replaced in less than 36 hours after a stressful situation [90].

The sole administration of corticosterone in broiler chickens, as a model to mimic stress, has been shown to decrease daily gain and increase feed conversion. Moreover, the administration of corticosterone reduced feed movement in the intestines, which in turn reduces feed intake. Corticosterone also alters intestinal morphology, reducing villus hight in the small intestine and, consecutively, absorptive capability of the intestine. These effects are accompanied by increased activity of trypsin, amylase and lipase, and decreased digestibility of proteins [91].

Other physiological changes following heat stress in chickens include changes in the vasculatory system, increasing the transport of cool venous blood in proximity to arterial blood to dissipate the maximum heat to the environment [92], and increasing cardiac output with a reduction in peripheral resistance [93]. Besides the lack of feathers in the neck region, naked neck chickens also lack 30-40% of the feather coverage in the body. This characteristic is not interesting for cold weathers, but it is an advantage to hot weather systems as these chickens have a better ability to dissipate heat, presenting superior growth rate, viability, egg weight and female reproductive performance [84,94]. Panting is one of the most visible responses to heat observed in poultry. This specialized form of respiration dissipates heat by evaporative cooling at the surfaces of the mouth and respiratory passageways [84]. Panting increases the loss of carbon dioxide from the lungs, reducing the partial pressure of carbon dioxide and thus bicarbonate in the blood plasma. This lowered concentration of hydrogen ions causes a rise in plasma pH (alkalosis) [95-97].

Heat shock proteins (*hsp*)

As a common response of all organisms to elevated temperature is the increased synthesis of a group of proteins known as heat-shock proteins. This group of proteins is one of the most conserved ones in all the species, an indication of the important role they exert in restoring normal

functions of cells and organisms that are exposed to potentially damaging stimuli, such as heat. As reviewed by Etches, et al. [84], heat shock proteins play an essential role by associating with a variety of proteins and affecting their conformation and location. As example, in heat-sensitive cells, heat-shock proteins might bind to heat-sensitive proteins and protect them from degradation or may prevent damaged proteins from immediately precipitating and permanently affecting cell viability. In general, organisms recovered from a mild stressful condition, such as heat, usually present elevated expression of heat-shock proteins, that cause the organism to exhibit a certain level of tolerance to the stressor, that would normally cause the development of abnormalities or even death.

A further feature of heat-shock proteins is that they might be involved in immunity and immunopathology. Although excessive heat can have many deleterious effects on the structure and physiology of a cell, including impairment of transcription, RNA processing, translation, post-translational processing, oxidative metabolism, membrane structure and function, cytoskeletal structure and function, among others, it is not yet clear which of these is the most harmful or which one is alleviated by any particular heat-shock protein [84,98].

Heat shock proteins in *Eimeria* spp.

Two heat shock proteins have been described in *Eimeria* spp.: *hsp*70 and *hsp*90. Heat shock protein 70 is a chaperone protein associated with cell motility and ability of parasites in adapting to new environments within the host [99,100] by facilitating the production T helper cell type 1 (Th1) inhibitors, such as IL-17 and IFN-γ in the host [101]. Heat shock protein 70 is important for cell invasion, cooperating with *hsp90* to facilitate the stabilization of transmembrane proteins and cell adhesions [102]. Heat shock protein 70 is also linked to virulence, as more attenuated strains of *E.*

tenella express less *hsp*70 [103]. Heat shock protein 90 is essential for invasion of host cell and schizont growth [102]. While expression of *hsp*90 can be observed throughout all the life stages of *E. tenella*, it is not observed during sporulating and sporulated oocysts of *E. acervulina* [104]. Heat shock protein 90 has been suggested as a target against human protozoan infections [105]. Diclazuril, commonly used in the treatment of coccidiosis in chickens, downregulates the expression of *hsp90* in *E. tenella* merozoites [106]. The specific mechanisms of *hsp*90 in the coccidia life cycle still remain to be investigated.

Effects of anorexia on intestines and immunity

Anorexia or fasting is a common occurrence in wild populations of birds, such as penguins and geese, and it is usually associated with breeding, incubation, migration and molting, even though food is often readily available. This fasting can occur regularly in their life cycle and may vary from a very limited food intake to no food intake at all [107]. During fasting periods, a bird undergoes a series of adjustments to resolve the underlying conflict between energy-sparing and the metabolic requirements necessary for maintaining body homeostasis. Larger birds, such as geese, show a decrease in metabolic rate while fasting. Larger birds also tend to reduce their locomotion rate during fasting periods [108].

Chickens respond to starvation by reducing circulating levels of triiodothyronine (T_3), whereas thyroxine (T_4) concentration increases. This reduction in T_3 is an adaptive response to reduce metabolic rate and preserving nutritional stores [109]. Additionally, food deprivation generally increases plasma concentration of corticosterone in chickens [110], hence, the application of corticosterone has been used as a model to mimic stress in chickens.

Feed restriction also alters the morphology of digestive organs in chickens. In a study were broiler chicks had feed restriction from 7 to 14 days of age, it was observed that the densities (weight/length) of the small intestine segments were significantly decreased at 14 days of age when compared to the groups fed ad libitum. The researchers also observed that the intestinal mucosa of feed-restricted chickens was thinner than that of controls [111]. Other reports have also shown some atrophy of the small intestine of chickens during feed restriction, with shorter and thinner villi in feed-restricted birds [91,112].

The effects of feed restriction are also dependent on the genetic background of the chickens studied. In a study comparing two divergent genetic lines of chickens able to produce high (H) and low (L) antibody titers, it was demonstrated that forced feed restriction does not alter antibody production to immunization, however, the levels of antibody binding are reduced under feed restriction conditions. The study also reveals that periods of more that 4 weeks of feed restriction result in suppression of mitogenic responses of lymphocytes, suggesting that these birds cannot maintain their T cell proliferative capacity. Interestingly, feed restriction seems to increase some innate immune responses, such as reactive oxygen intermediates (ROI) production. Only severe feed restriction, between 3 and 5 weeks, seem to increase plasmatic cortisol levels, however this is related to the genetic background of the birds (H or L). Perhaps the most interesting finding in the described study is that environmental stress does modulate the magnitude of the immune and endocrine responses, indicating additive relationships between genotype and environment. Moreover, feed restriction does not exert a significant effect on specific antibody response to either Th_1 or Th_2 types of antigens, although suppressing parts of cell mediated immunity and enhancing innate immunity, suggesting that innate and cellular immune components are more sensitive to stress and more energy demanding as compared to humoral immune components [113].

Besides some negative effects, feed restriction is commonly used in the rearing of chickens, such as broiler breeders. According to review done by Tsiouris, et al. [114], feed restriction increases the serum concentration levels of corticosteroids, which lead to the detachment of heterophils from the endothelium of the blood vessels and migration to the tissues. Moreover, feed restriction in poultry increases the heterophil:lymphocyte ratio, so the number of migrant heterophils is even higher. The lower growth rate of the birds, the reduction of the hematocrit value and the beneficial effect of feed restriction on the cardiovascular system facilitate the migration of heterophils to the tissues, including the gastrointestinal tract. These effects may favor blood circulation and innate immune response of the intestinal mucosa against pathogens, such as *Clostridium perfringens*.

Other causes of Immunosuppression in chickens

Several factors can cause immunosuppression in chickens, including infectious and non-infectious agents (Figure 2.4). As general response to immunosuppression, there is a generic reduction in genetic and nutritional potential for efficient production. For the purpose of this review, immunosuppression will be divided in the following categories: viruses, husbandry, fungal-origin.

Virus-induced immunosuppression:

Several viruses can cause immunosuppression in chickens, among them infectious bursal disease virus (IBDV), chicken infectious anemia virus (CIAV), Marek disease virus (MDV), retroviruses causing tumor diseases, such as reticuloendotheliosis virus (REV) and avian leukosis virus (ALV), avian reovirus (ARV), and Newcastle disease virus (NDV).

Infectious bursal disease (IBDV)

The virus is horizontally transmitted and infects young chickens. IgM B lymphocytes in the bursa of Fabricius are targeted, causing a rapid depletion of lymphocytes from the bursal follicles, causing an influx of T-follicles that help to limit viral replication, but in doing so, promote bursal cell destruction. Birds that survive acute infection repopulate the bursa B lymphocytes. However, young chickens that survive the acute infection by IBDV are immunosuppressed despite the repopulation of the bursa with B cells, that is because infection of chickens that are 14 days of age or younger renders the B lymphocyte seeding of secondary lymphoid centers curtailed, resulting in a permanent defective humoral immunity. In chickens older than 14 days old, the virus causes transient immunosuppression and diminished mucosal immunity [115].

Chicken infectious anemia virus (CIAV)

The virus has vertical transmission, and, at hatch, it targets the chick`s hemocytoblasts in the bone marrow and lymphocytes in the thymus, resulting in aplasic anemia, thrombocytopenia, leukopenia and thymus depletion in chickens from 7 to14 days of age. The virus can also spread horizontally through digestive tract and feather dander [116,117]. Any effect on the bursa appear to result from an indirect effect of CIAV infection mediated by disruption of cytokine networks resulting from destruction of other effector populations, or enhanced pathogenicity of other agents which adversely affect the bursa by direct means [118].

Marek's disease virus (MDV)

Initial impairment of the immune response is the result of the lytic infection of lymphocytes during the first cytolytic infection. Permanent immunosuppression coincides with the second phase

of the cytolytic infection and tends to correlate with eventual tumor development. The virus can stablish latency. Reactivation of the lytic infection will cause loss of additional B and T cells, thus compounding the situation, and resulting in the bursal and thymic atrophy seen in chickens. The humoral and cell-mediated immunosuppression caused by the virus leads to reduced antibody response to a variety of antigens and alterations in T cell functions, such as mitogen stimulation of lymphocytes, delayed hypersensitivity, reduced NK cell activity, primary and secondary infections with coccidia [119].

Retroviral tumoral diseases

Avian leukosis virus (ALV) and reticuloendotheliosis virus (REV) cause tumors, reduced productivity, immunosuppression and other problems. REV causes bursal atrophy, resulting in immunosuppression, that, in turn, contributes to the development of acute leukemia by inhibiting the proliferation of cytotoxic cells directed against the tumor cells antigens. A myeloblastosis strain of ALV capable of inducing osteoporosis caused atrophy and bacterial clearance. Another strain of ALV, causing erythroblastosis, results in thymus atrophy and reduced T cell competence [115].

Avian reovirus (ARV)

Virulent strains of this virus cause atrophy of lymphoid tissues and interfere with humoral immunity. Reovirus can replicate in monocytes but not in lymphocytes, thus the lymphoid atrophy is not caused by reovirus tropism for lymphocytes, in contrast to MDV, CAV and IBDV [115]. Among other outcomes if infection, ARV can cause immunosuppression due to the lesions in the

bone marrow, spleen and bursa. In enteric reovirus infection, virus can persist for a long period in ileum, cecal tonsil and rectum [120].

Newcastle disease virus (NDV)

The virus is known to cause lymphocyte necrosis and depletion from lymphoid organs, causing apoptosis of peripheral blood lymphocytes and mononuclear cells, leading to the increase in susceptibility to secondary bacterial infection [115].

Husbandry-induced immunosuppression

Several husbandry practices can induce stress in chickens, such as inadequate temperature, high density of chickens in the house, starvation, transportation, among others. These are all known to increase corticosterone levels, leading to immunosuppression, as previously addressed on this review.

Fungus- related immunosuppression

Products of the fungus metabolism, the mycotoxins, are known to target components of the innate and acquired immune systems, therefore contributing to an increased severity of concurrent infectious diseases. Hoerr [115] briefly reviews the interactions of the most common mycotoxins in poultry.

Aflatoxin is broadly immunosuppressive in chickens and other birds. Among the effects reported are depletion of lymphoid tissues, impaired lymphocyte function, reduction in serum complement, resulting in increased rate of concomitant infectious diseases and vaccination failures. Trichothecenes directly damage protective barriers of mucosal membranes and feathers.

They also cause a depletion of lymphoid organs, reduce lymphocyte mitogenic response, and are directly cytolytic to macrophages. Fumonisins cause lymphoid depletion, reduced antibody formation, and are directly toxic to macrophages and lymphocyte functions. Ochratoxins induce generalized atrophy of lymphoid organs, impairment of cells mediated and humoral immunity, reduced vaccination response and phagocytic activity, resulting in an overall increased severity of concurrent disease. A diverse set of other less common mycotoxins, such as citrinin, cyclopiazonic acid, sterigmatocystin and rubratoxin have also been reported to cause generalized lymphocyte depletion and immune disfunction in various avian species [115].

Host-pathogen interaction during heat stress

Heat stress (HS) is one of the major environmental problems of broiler farming in tropical and subtropical regions. Stress is a predisposing factor of immunosuppression in broilers, offering a good opportunity to normal commensals to induce infection and disease [90,121,122]. Heat stress has been reported to enhance pathogen attachment, colonization, shedding, reduce intestinal crypt depth and also impact on food safety risks [90,123-125]. The increase in pathogen colonization in heat stressed chickens is believed to be related to the disturbances in microbiota composition in heat stressed chickens, thereby leading to a loss of protection against pathogenic microorganisms [125].

Research has associated HS to decreased IgA and IFN-γ plasmatic levels; decreased mRNA expression of IL-6, IL-12 in the spleen and IL-1betta, IL-10 and TGF-β in cecal tonsils; decreased mRNA expression of β-defensin 4 (BD4) and 6 (BD6) in cecal tonsils; and reduced mRNA expression of TLR2 in spleen and cecal tonsils of chickens infected with *Salmonella* Enteritidis. IgA is responsible for mucosal protection against bacterial, viral and parasitic pathogens, and the decrease in IgA levels could impair the first barrier mechanism used by the immune system in attempt to respond to pathogens [126].

Heat stress has also been linked to a decrease in intestinal immune activity and to compromised intestinal morphology following enteritis and pathogenic bacteria invasion of the body through intestinal epithelium [90,125,127,128], these are attributed to the diminished macrophage activity, as evidenced by a decreased oxidative burst [86], allowing intracellular bacteria such as *Salmonella* spp. to survive in the macrophages of heat-stressed chickens [125], and also the loss of integrity of the intestinal barrier, leading to increased intestinal permeability and intestinal inflammation [125,129]. Furthermore, the intestinal immune disfunction and the disruption in the intestinal barrier are responsible for the migration of commensal bacteria to lymphoid organs and liver in stressed mice [130]. Supporting this data, chickens exposed to HS conditions have an increase in the inflammation in the intestines, marked by the presence of heterophils in the lamina propria [125].

Controversial results have been published regarding the effect of probiotic supplementation while chickens are on heat stress. Song, et al. [131] reported that the supplementation with probiotics in the diet significantly enhanced lactic acid producing bacteria counts, another study [132] did not observe effects of HS or supplementation with probiotics on the GIT microflora. Other studies show that the utilization of organic acids in the feed of chickens exposed to heat stress inhibit the growth of intestinal pathogenic bacteria and coccidia, and favors the growth of beneficial microflora [133].

There is a bidirectional signaling between the gastrointestinal tract and the brain, and this is regulated by the central nervous system, the neuroendocrine and neuroimmune systems, the sympathetic and parasympathetic arms of the autonomic nervous system, in conjunction with the intestinal microbiota [134], and when of these factors breaks down, such as in heat stress conditions

or during bacterial, viral or parasitic infections, there is an imbalance in the protective immune response, interfering with the expulsion of noxious agents from the intestine [125].

Heat-stressed broiler chickens present a trend towards a decrease in gross lesion scores and significantly lower microscopic scores of necrotic enteritis, as noted by less congestion and villi fusion with milder bacterial colonization in the villi. These enhanced clinical conditions in heat-stressed chickens are believed to be related to the stress-induced regulation of intestinal inflammation, specifically, the decrease in polymorphonuclear cell infiltration, resulting in the prevention of development of *C. perfringens* type A-mediated necrotic enteritis [135].

Although HS seems to render chickens susceptible to most intestinal infections, we have previously observed the significant reduction in *Eimeria* spp. oocyst shedding in broiler chickens under HS conditions. The underlying factors behind this interaction are yet to be elucidated, but preliminary data suggests that the sole increase in temperature plays an important factor in the reduction of *Eimeria* spp. replication in the intestine of chickens [136]. As HS reduces the expression of inflammatory chemokines and cytokines, besides the reduction in IgA levels, it remains to be verified if heat stresses chickens infected with *Eimeria* spp. are able to mount an effective immune response besides presenting drastically low levels of infection.

Heat stress has a very significant effect in oocysts counts during primary coccidial infections in poultry, as noted by the diminished oocyst output in chickens infected with *Eimeria* spp. for the first time as compared to non-infected chickens. Conversely, HS does not exert effects on second exposure to *Eimeria* infections in chickens older than 27 days. This positive effect of heat stress on primary infections is attributed to a brief enhancement of cell mediated immunity under heat stress conditions [137].

The poultry industry has focused on rapid growth and development of poultry due to economic advantages. However, rapidly increasing body weight gain has a burden on bird health, leading to increase in metabolic, musculoskeletal and cardiovascular disorders in broilers with the highest productivity [138]. The impact of rapid bird development in infectious diseases is, on the other hand, less well described. *Clostridium perfringens* is one pathogen that is tightly linked to the intensive rearing methods. High feed intake has been documented to be closely linked to disease caused by *C. perfringens* in broilers [139,140].

When comparing breeds of chickens developed for fast and slow goth, Dierick, et al. [141] suggest that the higher prevalence of necrotic enteritis in faster growing chickens is due to their increased feed intake, that reduces the diversity and richness of the microbiota composition in the ileum, therefore allowing for the prevalence of *C. perfringens*, resulting in disease. The authors still suggest that slow growing chickens tend to present a better functioning intestinal barrier function and less intestinal inflammation, which could contribute for the lessened prevalence of necrotic enteritis on this type of bird. Taken the findings in consideration, a feed restriction is proposed to alleviate the clinical signs of necrotic enteritis in broiler chickens.

Different from the description of the intestinal status reported in Dierick, et al. [141] study, however, other authors have reported changes in the intestinal mucosa and augmented intestinal inflammation [86,125], integrity of the intestinal epithelium [86,87,142], ileum crypt depth and villus height [87,90] and enterocyte replication [90] during heat stress in chickens. Studies with *Eimeria* spp. reveal that the initial phase of parasite replication in the intestinal tract is marked by the suppression of inflammation due to the induction of host chemokines and cytokines such as IL-10, IL-1β, TNF-α and IL-6 [143-145]. Besides the results from our previous study describing the reduction in *E. tenella* replication *in vivo* and *in vitro* following the sole increase in 2˚C in the temperature of incubation

(*in vitro*) and in heat stressed birds, it is also plausible that the another factor in the nature of the interaction of *Eimeria* spp. and heat stressed chickens must reside in the inflammation and enterocyte replication in the intestine, but this remains to be properly tested.

It is also important to note that protozoan parasites spread in the host by inducing or inhibiting host-cell apoptosis [146,147]. Yan, et al. [80] showed that *E. tenella* protects host cells from apoptosis at early stages of infection but promotes apoptosis during the middle to late stages of parasite development. Moreover, the authors also demonstrate that the inhibition of host cell apoptosis is counterintuitively problematic to the host, as it promotes *E. tenella* intracellular development, leading to higher infection rates. The apoptosis process is dependent on several factors, among them are the inhibition of NF-κβ activation [148] and activation of caspase-9 [149]. *E. tenella* and *E. maxima* inhibit apoptosis of parasitized cells by activation of NF-κβ during early stages of parasitemia, and induce apoptosis of the same cells during later stages of infection, after complete schizont maturation [150]. *E. tenella* also regulates host cell apoptosis by inhibiting caspase-9 activity, therefore blocking mitochondrial apoptotic pathway. Consequently, when host cell apoptosis is lower, more cells can be invaded by merozoites, the intracellular growth and development of *E. tenella* is enhanced, increasing infection rates [80]. The faster enterocyte turnover during heat stress could also be inhibiting proper *Eimeria* spp. life cycle, curtailing the infection and asexual replication, resulting in the reduced oocyst shedding. However this hypothesis also remain to be tested.

IMMUNITY

The detailed avian protective mechanism for coccidiosis is not well understood. Although resistance to *Eimeria* infection includes many elements of both, humoral and cell-mediated

immune responses, considerable evidence suggests that the cell-mediated immunity plays a predominant role in host defense [4,50,151,152]. The primary target tissue for coccidia is the epithelium [153], thus, understanding the immune system-parasite interactions in the intestine, leading to parasite elimination is imperative for the development of new approaches to control the disease.

The first line of defense against intestinal parasites is composed of the non-specific barriers. The main factors playing a role on the non-specific defense of the gastro-intestinal tract (GIT) are the gastric secretion, bile salts, intestinal mucus and the expression of antimicrobial proteins [154]. The second line of defense against *Eimeria* is composed by the gut-associated lymphoid tissue (GALT) and represents a component of the mucosal associated lymphoid tissues (MALT) [155], a larger group of tissues responsible for conferring immunity across mucosal surfaces in different areas of the body. The MALT has evolved with specialized features that reflect their role as the first line of defense on mucosal surfaces. These features include the presence of antigen presenting cells (APCs), immunoregulatory cells, and effector cell types that are distinct from their counterparts in the systemic immune system. Due to the unique location of the MALT and its constant exposure to environmental antigens, the investigation of the mucosal immune system is crucial to our understanding of food allergy, tolerance to ingested antigens and also immune response to intestinal infections [156].

GALT are localized within the lining of the intestine and are composed of a multilayered tissue comprised of an outer epithelial layer and a row of lymphocytes above the basement membrane, and immediately below the basement membrane is located the lamina propria, housing lymphocytes, followed by the submucosa. GALT are responsible for providing the most effective line of defense against intestinal pathogens such as *Eimeria* spp. [156,157].

In the avian species, the GALT has evolved in different specialized immune complexes such as Peyer's patches, cecal tonsils, bursa of Fabricius, Meckel's diverticulum and lymphocyte aggregates, hosting a variety of immune cells such as natural killer (NK) and dendritic cells. The development of the GALT begins at the lamina propria of the villi and may extend to the submucosa of the intestine [158,159]. The immune response is highly coordinated within the GALT and include antigen processing and presentation, lymphocyte stimulation, cytokine secretion and activation of resident immune cells and co-stimulation of cell mediated immunity [160,161].

The bursa of Fabricius is a hollow oval sac located dorsally to the cloaca and is the central lymphoid organ for B cell lymphopoiesis and lymphocyte maturation, where antibody diversity is generated [162], therefore being the primary humoral immune organ unique to birds [163]. The bursa is found in the GALT pathway for B cell development in birds. This pathway uses the bone marrow in mammals [164]. Studies have shown that the bursa is a major channel through which the environmental antigens stimulate the immune system and induce the formation of specific antibodies [165].

Peyer`s patches have a morphologically distinct lympho-epithelium constituted of microfold cells (M cells), follicles, B cell-dependent subepithelial zone, a T cell-dependent central zone and an absence of goblet cells [166,167]. The Peyer's patches are the major inductive site for immunoglobulin A (IgA) responses to pathogenic microorganisms in the GIT, and contain around 40% B lymphocytes in mammals, from which a large percentage bears surface IgA [168]. Another 40% of the cells in the Peyer's patches are regulatory T cells, and a significant percentage of these cells bear Fc receptors for IgA and are important on the regulation of the IgA isotype responses. Another cell subpopulation in the Peyer's patches include macrophages, varying between 5 to 9%, dendritic cells and M cells [169]. There is limited knowledge in regarding the cellular composition

of Peyer's patches in chickens. Therefore, the GALT is largely represented by Peyer's patches, which in turn are an important site for IgA synthesis and also contain a large subpopulation of B lymphocytes that are committed to secrete IgA [168]. The secretory IgA antibody is produced in the GALT by plasma cells and then selectively transported through the epithelial cells into external secretions [156].

In chickens, most of the Peyer's patches are scattered through the jejunum, but one of them can consistently be found in the distal ileum, in a position that is 5 to 10 cm anterior to the ileocecal junction. They can be recognized as rough and swollen oval-shaped white patches with some redness in the texture and about 1 cm in length [170]. In birds with coccidiosis, the Peyer's patches are extremely apparent and easy to locate from both serosal and mucosal intestinal surfaces. In these birds the patches are usually swollen and reddish, indicating vasodilation. Contrarily, the patches in healthy birds are smaller in size [171]. Better microscopical observation of the Peyer's patches were achieved with the swiss roll technique [171].

Along with Peyer's patches, cecal tonsils also elicit immune response against pathogens in the intestinal tract of avian species. Cecal tonsils are discrete lymphoid nodules located at the proximal ends of the ceca, near the ileocolonic junction [166]. They are the largest lymphoid organ in the avian GALT. Although rudimentary at hatch, they develop into immunologically mature lymphoid organs within the first few weeks of age [172,173]. At hatch, $CD4^+$ T cells are the predominant cell type in the cecal tonsils, which are later replaced mainly by B cells and a few $CD8^+$ T cells as it develops into mature secondary lymphoid aggregate [172-174]. It is commonly accepted that cell populations found in the cecal tonsils are thymus- and bursa-dependent [175-177]. The immune relevance of the cecal tonsils has been correlated with age related involution of the bursa and thymus [178]. Similarly to the Peyer`s patches, the cecal tonsils of the chicken are easily

identified at 10 days post hatching, according to a study, and also undergo involution, with a relative depopulation of the subepithelial zone [166,168].

Structurally, cecal tonsils are similar to Peyer's patches, containing central crypts, diffuse lymphoid tissues, and germinal centers [179]. In the cecal tonsil, both B and T lymphocytes are present in the germinal center, as well as plasma cells expressing the surface IgM, IgG and IgA [173]. The exact function of the cecal tonsils is still unknown, but studies suggest that they are responsible for sampling intestinal antigens and development of adaptive immune response [166,180].

The Meckel`s diverticulum is a reminiscent of the yolk on the small intestine and usually persists as a discrete structure for the lifetime of the chicken. Although it`s exact function is still not known, it is known to contain germinal centers with B cells and macrophages [166,181], and it is more important thorough the initial development of the chicken and absorption of maternal antibodies from the yolk [181].

The epithelium and the lamina propria are two anatomic compartments containing immune system cells. These two compartments are anatomically separated by a basement membrane. While the leukocytes located in the epithelium are mainly T cells and, to a lesser extent, non-T and non-B cells, the leukocytes located in the lamina propria are enriched with immunoglobulin-producing B cells [182,183]. The intestinal leukocytes from chickens are composed of 80% lymphocytes, 10 to 15% monocytes, approximately 5% other mononuclear cells and less than 1% polymorphonuclear leukocytes and plasma cells [166]. Some mononuclear cells isolated from the epithelium and lamina propria were reported to be immunoglobulin-positive, with the percentage being higher among lamina propria lymphocytes, 29.5%, as compared to intraepithelial and spleen lymphocytes (7.9% and 19.4%, respectively) [166].

Surface IgA plasma cells are mostly seen in the lamina propria and are actively transported into external secretions. While MHC class I expression is evident on all cells in the intestine, MHC II antigen-expressing cells are located mainly in the lamina propria. $CD8^+$ cells are located scattered in both the epithelium and in the lamina propria [184-186].

Within the GALT, the immune responses are highly coordinated and include lymphocyte stimulation, cytokine secretion and activation of resident immune cells [161]. GALT can be subdivided in inductive and effector sites. Inductive sites of GALT are the primary site for antigen recognition and immune activation [187]. Peyer's patches are known as mucosal inductive sites for humoral and cellular immune responses in the GTI [188].

Following pathogen recognition and immune activation, activated B and T cells migrate to the lamina propria, that serves as the effector site for immune responses. These mucosal effector tissues consist mainly of T cells, predominantly $CD4^+$ T cells, but they also contain a large number of B cells and plasma cells, mainly of the IgA isotype [156].

One of the most interesting features of the mucosal immune system is the intraepithelial transport of polymeric IgA intro the external secretions. External transport of secretory immunoglobulins from the blood or tissues into external mucosae of the digestive, respiratory, genitourinary and nasolacrimal tracts contributes the major source of immunoglobulins to the intestinal tract [156]. Therefore, GALT performs three major functions in response to a coccidial infection: (1) antigen processing and presentation, (2) intestinal antibody production, and (3) co-stimulation of cell mediated immunity [187].

Localization of *Eimeria* in the intestine is dependent on the developmental stage as a result of cellular invasion or phagocytosis. The entering of the protozoa causes tissue damage and induces an immune response. An inflammatory response driven by cytokines is induced after the

presentation of antigens, resulting in the expansion of helper and effector cells, which induce a protective adaptive immune response [189]. At this point, the immune response can be divided in two categories: innate and adaptive immunity. The innate immunity is non-specific and driven by natural killer (NK) cells, macrophages and granulocytes. In the case of *Eimeria*, there is an increased NK activity in the duodenum and jejunum as compared to cecum and ileum following *Eimeria acervulina* infection [190]. This response recognizes molecular structures present on the surface of pathogens, also called Pathogen Associated Molecular Patterns (PAMPs) that are recognized by Toll-like receptors [189]. Macrophages are also part of the innate immunity, however they are involved in coordinating adaptive immune responses by interacting with parasites during their passage through intestinal mucosa and stimulating inflammatory responses [191].

Antigen processing and presentation is mainly achieved in the Peyer's patches of the lamina propria. There is an overabundance of lymphocytes in the Peyer's patches, and they are organized in the form of germinal centers, similarly to those in other lymphoid tissues. within the Peyer's patches, antigen uptake and processing are done by specialized modified epithelial cells, called M cells, that internalize luminal particles within their reach by pinocytosis. The particles are transported in vacuoles and released in the subepithelial space of the M cell pockets to engage with professional APCs, such as memory B cells, dendritic cells and macrophages, and $CD4^+$ T cells [192]. Immature dendritic cells are located in the subepithelial dome region of the Peyer's patches, where they capture antigens from the M cell transcytosis and initiate the adaptive mucosal immune response [192,193], following with migration of APC to the lymphoid follicles and presentation of the antigens in the form of peptides in the context of MHC classes I and II to naïve $CD4^+$ and $CD8^+$ T lymphocytes, respectively [192]. This process generates antigen specific B cells in the local germinal centers under the cytokine control of $CD4^+$ T cells (helper). Primed B cells

then migrate out of the inductive sites of the Peyer's patches to the effector sites of the mucosa via the blood circulation [194]. Therefore, the adaptive immune system is simultaneously acting and plays a fundamental role in the dynamics of coccidial infection, including both, cellular and humoral immune mechanisms [187,195]. The organization in the chicken implies that the primary sites for antigen presentation are in the subepithelial zone, which is characterized by a high MHC class II expression, whereas the low MHC-II expression in the follicles indicates that fewer APC are actively participating in antigen presentation [171].

B and T lymphocytes are actively working on antigen recognition. B lymphocytes express surface immunoglobulin (Ig) molecules with meticulous specificity for antigens, while T lymphocytes recognize processed antigens on antigen presenting cells (APC). Upon recognition and binding of an antigen to a B cell expressing surface Ig, cell division and clonal expansion occur to ensure that immunoglobulins with identical antigen specificity are replicates and secreted from differentiated B cells. T cells, in contrast, are able to recognize small fragments of antigens that have already been processed by APC only when in conjunction with gene products encoded by self-MHC genes. This binding of antigen, T-cell receptor (TCR) and MHC complex induces a series of events that result in T cell activation [196].

After detection of antigens in the lumen of the intestine, antigen-sensitized IgA-committed B and T cell leave the Peyer's patches via efferent lymphatics, passing through the mesenteric lymph nodes and entering the blood circulation through the thoracic duct. Successively, from the bloodstream, this IgA-committed B cells migrate to distant mucosal tissues, including lamina propria regions of the GIT, where they differentiate into plasma cells [156]. In contrast to Peyer's patches, only a small number of surface IgA B cell are found in the cecal tonsils. Rather, the cecal tonsils contain mainly surface IgG and surface IgM B cells [166].

In more details, following oral administration of antigens, such as *Eimeria*, there is an activation of helper T lymphocytes and IgA precursor B cells in the GALT, especially in the Peyer's patches. These activated cells migrate to mucosal effector sites such as the lamina propria of the intestine and to the upper respiratory tract to mediate antigen-specific secretory IgA antibody responses. This activation of B and T cells in the GALT and their migration to the effector sites for the development of the mucosal immune responses is called the common mucosal immune system [197]. This part of the mucosal immune system consists of two separate but interconnected compartments, there are mucosal inductive sites, which include the NALT (nasal associated lymphoid tissue) and the GALT, which are strategically located in places where they encounter environmental antigens, and mucosal effector sites, which include the lamina propria of the intestine and the upper respiratory tract [197].

M cells are responsible for the surveillance and uptake of antigens followed by transport to the underlying lymphoid tissue [195]. It is still not clear, however, if M cells express surface major histocompatibility complex (MHC) molecules and are able to present antigens to T cell or stimulate the secretion of immunoglobulin A (IgA) to generate an antibody response [161]. One of the most interesting features of the mucosal immune system is the intraepithelial transport of polymeric IgA into the external secretions, external transport of secretory immunoglobulins from blood or tissue fluids into the external mucosae of the alimentary, respiratory, genitourinary and nasolacrimal tracts contributes an important source of immunoglobulins to the intestine [156].

The response of the immune system to an infection can be as fast as three hours. This response is primarily characterized by an accumulation of heterophils, and also other polymorphonuclear leukocytes, in the intestinal villi, which are the sites of invasion of *Eimeria* spp. [198]. Additionally, following exposure to antigens, significant architectural changes occur in

the GALT, which include and increased permeability, increased cell infiltration, augmented proliferation of crypt cells and production of mucin enzymes, besides immunoglobulins [199]. Specific immunity to coccidiosis is primarily conferred by populations of T cells, and it is well understood that cell-mediated immunity is protective and centrally important in a coccidia infection, even though some studies have reported that humoral immunity also plays a role in protection against *Eimeria* spp. [191,200-202].

T cells play a critical role in protective immunity against *Eimeria* infection, [203]. A potent T cell-dependent protective immune response against *E. tenella*, *E. maxima* and *E. acervulina* infection of chickens is observed following *in vivo* administration of exosomes, cell-derived phospholipid-enclosed vesicles isolated from dendritic cells of the intestinal cecal tonsils and pulsed in vitro with *Eimeria* antigens [204]. Exosomes secreted from antigen presenting cells (APCs) play a functional role in mediating innate and adaptive immune responses to microbial pathogens [205]. Exosomes from serum have been shown potential as an alternative strategy for controlling chicken coccidiosis. Naïve chickens immunized with $CD8^+$ and $CD8^-$ serum exosomes showed increased numbers of intestinal and spleen IL-2, IL-4, IL-6 and IFN-γ and greater protective immunity and may extend immunological memory to re-infection [153]. Infection with different *Eimeria* species induce different cytokine production [4], however the increased production of IL-8 seems to be a constant factor. In chickens, IL-8 plays a role in initiation of inflammation although generally leading to damage of the epithelium, due to the influx of heterophils, allowing the entry of pathogens [4]. On the other hand, an early and robust IL-8 response may confer the ability to limit *Eimeria maxima* infection in the intestine [206].

Although T cells play the major role in immunity against *Eimeria* infection, B cells are indispensable [1]. The humoral immune response is part of the adaptive immunity that is mediated

by secreted antibodies, produced by B lymphocytes generated in the Bursa of Fabricious, part of the GALT. Secreted antibodies attach to antigens on the surface of invading microorganisms, signaling for the destruction through antibody-dependent cell-mediated cytotoxicity (ADCC) [207]. The humoral immune response is useful on the extracellular stages of the *Eimeria* infection [189].

In vitro, it has been shown that chicken embryo fibroblasts (CEF) and bone marrow macrophages possess a microbiostatic activity because they are able to prevent the intracellular replication of *E. tenella* following activation with chicken culture supernatants containing strong IFN activity, such as Reticuloendoteliosis virus - transformed chicken T-cell line and Concavalin A-stimulated chicken spleen cells [208]. Although the exact mechanism by which the development of *E. tenella* is inhibited by IFN-γ in fibroblasts and macrophages *in vitro* is still not known, it is extrapolated that the mechanisms might be the same as it happens in mammalian species, the L-arginine-dependent production of nitric oxide with the subsequent conversion to nitrite and nitrate [209], the toxic oxygen metabolites, previously reported to be involved in the killing of *Trypanosoma cruzi* [210], and the inhibitory effect is the tryptophan deprivation due to IFN-γ-activated cells reported for *Trypanosoma gondii* [211]. Infection of chickens with *E. tenella* and *E. maxima* is correlated with the higher expression of the cytokines COX-2, iNOS and IFN-γ in the intestinal mucosa at 6-7 days post-infection [212,213]. Comparing the levels of IFN-γ in two different chicken lines, Yun, et al. [214] found that the chicken line SC, which is more resistant to primary infections of *E. acervulina* and *E. tenella*, has higher IFN-γ mRNA expression in cecal tonsils and intraepithelial lymphocytes at days 6 and 8 post-infection, as compared to the chicken line TK (more susceptible to *E. acervulina* and *E. tenella* infection). The presence of high quantities of IFN-γ in the mucosa is capable of stimulating the synthesis of proinflammatory cytokines and chemokines [212], activation of lymphocytes and enhances expression of MHC class II genes [215,216].

IFN-γ also induces iNOS in several cell types, including epithelial cells [217] and macrophages [218]. Although free radical species are produced in response to *Eimeria* infection, their importance in the clearance process is yet to be determined. For instance, the usefulness of the radical species seems to depend on the species of *Eimeria* involved in the infection. Laurent, et al. [212] found that iNOS mRNA expression is more important for *E. tenella* rather than *E. maxima* infection in chickens, what might contribute to the presence of hemorrhages due to vasodilatation induced by NOS that are frequently observed after *E. tenella* infections [219]. Chickens infected with *Eimeria tenella* have also demonstrated increased numbers of IL-4, IL-10 and IL-17A cytokines. IL-17A promotes the migration of parasitized epithelial cells by facilitating their separation from the epithelial layer and movement away from the epithelium into the lamina propria. This step on *Eimeria* pathogenesis is required for the maturation of *E. tenella* schizonts contributing to severe cecal lesions [213].

Lymphocytes in cecal tonsils consist of 45-55% B cells and 35% T cells and are involved both in antibody production and cell-mediated immunity [166]. Their immunological maturation and overall size are dependent on the degree of antigenic stimulation in the intestine [220]. In chickens, a variety of specialized lymphoid organs such as the Bursa and cecal tonsils, and cell types (lymphoid, epithelial, natural-killer) have evolved in the intestinal tissues to defend against harmful intestinal pathogens [221]. Therefore, besides the roles of the cytokines in protection against *Eimeria*, the intensity of infection in chickens is speculated to be attributed to the variation in $CD4^+$ / $CD8^+$ cell ratio [214]. There is a different induction of T cell response according to the species of *Eimeria* infecting the chicken. While infection with *E. acervulina* induces mainly duodenal $CD8^+$ T cells and macrophage response, *E. maxima* and *E. tenella* infections induce mainly $CD4^+$ T cells and macrophage response [4].

It has been previously show that immunization of breeding hens with either *Eimeria* spp. infection or purified gametocyte antigens results in the passive maternal transfer of anti-parasite IgY from hen to hatchlings and that maternal antibodies reduce *Eimeria* infection in the progeny [222,223]. The vaccination of hens with a subunit vaccine, comprised of affinity-purified gametocyte antigens derived from *Eimeria maxima* oocyst wall, conferred high titers of antibodies in the hens and maternal antibodies in the progeny, significantly reducing oocyst shedding upon challenge with *E. tenella* in the progeny and improving bird performance [224,225]. Moreover, IgY egg yolk powder prepared from hyperimmunized hens was able to reduce parasite shedding and improve performance in chickens fed with the powder mixed with feed and challenged with the three major *Eimeria* species: *E. acervulina*, *E. tenella* and *E. maxima* [201].

A number of factors, including previous exposure to the pathogen, has a significant impact in the success of an infection. It is speculated whether *Eimeria* spp. uses host cells also as method of transport within the body. Early reports suggest that macrophages and intra-epithelial lymphocytes are responsible for sporozoite transport [49,226-229]. Further studies identified that $CD8^+$ cells were seen harboring *E. acervulina* sporozoites [230,231], suggesting that these cells are actually transport methods utilized by *Eimeria* spp. there has also been a detection of a significant number of sporozoites inside macrophages, but as these cells are phagocytic, and it is not clear whether sporozoites can continue their infectious process or have a method to exit macrophages. Following a secondary infection, Trout and Lillehoj [232] found an accumulation of sporozoites inside $CD8^+$ cells, and suggested that these sporozoites were unable to exit these cells and continue their cycle. This was confirmed in a consecutive studied in which $CD8^+$ depleted chickens had a significant decrease in oocyst shedding following *E. tenella* and *E. acervulina* infection [232]. Altogether, these

data suggest the role of lymphocytes in the transport of sporozoites from intra-epithelial lymphocytes (IEL) to enterocytes, and that this transport is inhibited in immunized birds.

Entry of sporozoites into $CD8^+$ cells seem to be an active process orchestrated by the sporozoite, because heat killed sporozoites do not enter these cells and $CD8^+$ T lymphocytes are non-phagocytic [156]. This suggests a dual role of $CD8^+$ cells as protective mechanism and also transporter of sporozoites.

The role of GALT and the production of antibodies against *Eimeria* spp. has been previously addressed on this review, however it is still unclear how the interplay between humoral response against coccidiosis works during infection. *In vitro* studies have shown that immune sera increases the phagocytosis of both sporozoites and merozoites by macrophages in cultures [233]. Studies *in vivo*, on the other hand, are contradictory to studies *in vitro*, and indicate that antibodies play only a minor role in immunity to coccidiosis, as shown in bursectomized chickens [234-236].

It is plausible that antibodies are able to reduce the invasion of some species of *Eimeria* spp. or even enhance the destruction of these developmental forms of the parasite in the lumen of the intestine by secretion of antibodies and blocking the contact of sporozoites and merozoites with enterocytes. A draw back on this theory is the fact that *Eimeria* sporozoites enter the cells very rapidly once in the intestine, therefore shifting the theories towards the minor importance of antibodies during *Eimeria* infection.

Other several studies were also conducted in the past in an attempt to elucidate the role of antibodies in the life cycle of *Eimeria*, including the hyperimmunization of hens, which conferred excellent protection of up to 100% against primary challenge infection with the homologous *Eimeria* spp. in the offspring chicks [223].

A mix of cross-reactive, protective antigens has been identified in *Eimeria* spp. and has shown to induce a high-titer protective antibody response against the sexual stages of the parasite and is claimed to help in the control of the disease when used as a subunit vaccine [225].

Furthermore, coccidiosis continues to be an expensive disease in poultry production and understanding *Eimeria* spp. and their modus operandi is essential for new and creatives approach to combat the disease. GALT is probably the most important immune structure during *Eimeria* spp. infection in chickens, actively participating in the recognition and development of the immune response against the parasite. Besides some similarities in the infection process, different species of poultry coccidia operate differently, reshaping the immune response in their own ways in order to complete a successful cycle inside the host. During the past years, advances in molecular biology, genomics, proteomics and metabolomics have enabled researchers to better understand the biology of these parasites as well as their interaction with the host. Further advances will clarify even more the specific points of this interaction and how we can use this on the battle against coccidiosis in poultry.

EPIDEMIOLOGY

Coccidiosis is a widespread disease of growing chickens worldwide. The disease can seriously limit the development of poultry production either in large scale and backyard systems. Seven species of *Eimeria* can infect the chicken. All of them affect a specific intestinal segment [45]. *Eimeria necatrix* has been recognized as the most pathogenic species infecting the chicken, however *E. tenella* is more common and exerts a greater impact on poultry production [237-239], with increased prevalence in poultry production areas in the United States, possibly related to the use of live attenuated vaccines [240].

In regards to variability between strains, it has been shown that *E. tenella* has limited variability, suggesting a more restricted population structure, whereas *E. acervulina* and *E. mitis* present notable variation in population structure [241]. Underlying factors responsible for such variation between species may include a faster generation time and greater fecundity for *E. acervulina* and *E. mitis* as compared to *E. tenella* [242]. Moreover, *E. tenella* strains in USA, UK and Ireland have little genetic variation and are considered to be closely related [243].

Eimeria is usually introduced in the facilities through contaminated equipment, vehicles coming from other poultry operations or by the movement of service personnel between older and new facilities. A surprising aspect is related to the presence of disinfection systems at the farms' entrances. In an epidemiological study conducted in India, there was no reduction in coccidiosis outbreaks due to the presence of disinfectants in the farm entrance, what is attributed to the high resistance of oocysts to many commonly used disinfectants [244]. It is virtually impossible to eliminate the disease once a house has been contaminated [245]. Studies performed in broiler chickens have shown that exposure to sporulated oocysts usually begins shortly after chickens are placed in the litter [246-249], in which the oocysts counts are usually low during the first two to three weeks, rapidly increasing to a peak between four and six weeks, decreasing at low levels again by seven to eight weeks. Therefore the peak of infection in the growing bird is between two and six weeks of age [250].

Coccidiosis is transmitted between hosts by the ingestion of feed, water, and litter contaminated with thick-walled oocysts that are shed in the feces of infected animals and spread by fomites or personnel moving between houses [251]. The flock size is believed to have an important impact on the level of disease. It is possible that larger flocks are associated with increased

prevalence of coccidiosis due to having more animals producing large quantities of oocysts in a confined area, expanding the potential of transmission and infection [59].

The level of infection is also age dependent, with higher index of infection in younger chickens as compared to older chickens [244,252]. There is also a strong impact of breed and flock purpose on infection rates, where non-indigenous commercial breeds and broilers are more likely to be positive as compared to indigenous (rustic) chickens or layers [244]. It has been reported an increase in number of coccidiosis cases in months with high level of humidity and temperature in Pakistan [252], however others have reported higher frequency of coccidial infections in autumn and winter in the Netherlands, when temperatures are lower [59], therefore it seems that it is less probable that temperature is a limiting factor in coccidial infections, in accordance to previously observed [244].

There is a significant difference among the level of infection (crowding effect) among different species of *Eimeria* in poultry. In a comparison among the seven species of *Eimeria*, Williams [51] estimated that the crowding thresholds were 903, ≤16, 39, ≤14, ≤16, ≤16 or 72 sporulated oocysts, respectively, for *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* or *E. tenella*. This is due to the high productive potential of *E. acervulina* [51] and its ability to suppress other species in mixed infections [52]. Other factors affecting the fluctuations in species composition are due to fluctuations in immunity [253] or differences in specific effects of anti-coccidial drugs (ionophores) [59,254,255]. The infection levels vary from hundreds to millions of oocysts per gram of feces and do not correlate directly with the degree of clinical signs [59]. Moreover, there is a possibility of misdiagnosis of the disease and higher incidence of subclinical coccidiosis, what might be related to the prophylactic administration of anticoccidials [256].

The litter is known to have significant participation in the infection cycle of *Eimeria* [257,258], and it is not surprising that there is a lower incidence of coccidiosis in chickens reared in wire cages as compared to the ones reared in the floor [244]. Nonetheless, caged chickens are still found to frequently excrete oocysts, possibly a consequence of the time kept on litter while young and subsequent low level of recycling, hindering the development of robust immune protection [244,258]. Although the litter has an important role in the infection process, the frequent removal of waste during flock cycles may exacerbate oocyst transmission as consequence of litter disturbance [244].

DIAGNOSIS

Many methods used to diagnose and classify the species of *Eimeria* spp. have been extensively reviewed in the literature, such as oocyst morphology, pre-patent period, site of infection or minimum sporulation time, however all of these methods are labor intensive, time consuming and can be very difficult and unreliable with mixed infections due to the overlapping characteristics between species [259,260]. The most reliable identification methods rely on molecular tools and are essential in delimiting species and inferring phylogenetic relationships among species. Over the past 20 years, several polymerase chain reaction (PCR) assays have been developed targeting multiple regions of the genomic *Eimeria* DNA. The most popular PCR assays for detection and classification of *Eimeria* use the 5S ribosomal RNA (rRNA) [261,262], the internal transcribed spacer regions 1 and 2 (ITS-1 and ITS-2) [259,263-268], 18S rRNA [256,267,269], and cytochrome c oxidase subunit I (COI) [270]. COI has shown to be useful in delineating recent speciation events and may be useful in establishing evolutionary events [271]. COI has been widely used as a genetic target for DNA barcoding, a standardized, sequence-based identification procedure that exploits generic differences in specific gene regions [272,273]. In a comparison between

the utility of relatively short mitochondrial COI sequences and the complete nuclear 18S rRNA, Ogedengbe, et al. [32] determines that mitochondrial COI sequences can differentiate common species of coccidia in chickens as reliably as the sequencing of the 18S rRNA. A quantitative real-time PCR (qPCR) assay that utilizes primers targeting the ITS-2 DNA sequence was developed by Vrba, et al. [259]. This assay allows the quantification of the degree of infection of the seven important *Eimeria* species that infect the chicken, working as well in mixed infections. A similar assay was developed for Australian *Eimeria* strains [274]. These primers have been used as diagnostic and research tools, allowing for the genetic differentiation of species in the field, working as well for mixed infections [259]. The primary sample for PCR is fresh excreta, from which DNA is extracted utilizing specific steps to inhibit DNA degradation with enzymes present the feces. A key step towards the use of *Eimeria* species PCR as a sensitive and reproducible discriminatory tool for use in the field is the production of a standardized protocol that includes sample collection and DNA template preparation. Three steps have been shown of considerable importance: First, the sporulation of the oocysts in a 2% potassium dichromate solution, increasing DNA concentration in the sample. Second, concentration of oocysts by salt flotation in saturated saline solution, improving PCR sensitivity. Third, extraction of DNA using specific stool kits including a mechanical homogenization step, adsorbing substances that can degrade DNA and inhibit downstream enzymatic reactions. Following DNA extraction, the procedure shall be carried over with the utilization of the appropriate sets of primers [275]. Field studies utilizing PCR have been conducted in several countries including Brazil, Canada, China, India, Japan, Norway and USA [59,240,256,276-279].

PREVENTION AND CONTROL

Besides biosecurity, conventional coccidiosis control relies heavily on chemoprophylaxis with administration of drugs with anticoccidial activity, which is a tremendous cost to the industry, estimated to be as high as U$127 million annually in United States [280]. Alternative methods of disease mitigation are needed due to increasing government restrictions on the use of coccidiostats, emergence of drug-resistant parasites and the high costs of new drug development [281]. Additional methods of disease control include vaccination and disease-resistant chicken lines [160,242,282-286].

Management strategies that disrupt the eimerian faecal-oral lifecycle, such as housing poultry on raised wired floors, reduce *Eimeria* occurrence and outbreaks of disease [287], However this method is not commonly utilized in the production of broiler chickens.

Different anticoccidials have distinct modes of action and target singular stages of *Eimeria* life cycle. The most common active principles used in coccidiosis control include but are not restricted to ionophores (eg. bambermycin, lasalocid, maduramicin, monensin, narasin, nystatin, salinomycin, semduramicin), toltrazuril, sulfanilamide, amprolium, nicarbazin and diclazuril. Toltrazuril, applied via drinking water, acts upon all intracellular stages in either schizogony or gamogony cycles [288]. Sulfanilamide although effective, was found to be toxic if used for long periods [289]. Nicarbazin was introduced in 1955 [290] and is still in the market, currently used in the starter feed provided to broilers [291]. Although the mode of action of nicarbazin is not yet elucidated, it is believed to involve inhibition of oxidative phosphorylation mechanisms in coccidia [292]. Amprolium acts by inhibiting the uptake of thiamine by second generation schizonts [293], whereas quinolone drugs inhibit parasite respiration by blocking electron transport in the parasite mitochondrion [294]. Ionophores bind with cations forming the cation-ionophore complex which is transported through the cell membrane [295], affecting the movement of ions across the membrane

[296], disturbing the balance of physically important ions and avoiding the emergence of resistance [297]. Shumard and Callender [298] report the discovery monensin, a polyether ionophore drug. This discovery was followed by the synthesis of other formulations, including lasalocid, narasin and salinomycin, which, like monensin, have a broad spectrum of activity against many different species of *Eimeria* [299]. Currently, the use of ionophores and a combination of nicarbazin with narasin are widely used for the control of coccidiosis in poultry [203]. One important point to be considered when feeding anticoccidials to chickens is the concomitant occurrence of other diseases, such as complicated respiratory diseases, infectious bursal disease and mycotoxicosis, as these can instigate a significant decline on feed consumption, therefore reducing the ingestion of anticoccidial medication and diminishing the overall effect of the anticoccidial program [300].

More recently, in consequence of the drug restrictions in animal production, several herbal compounds have been studied for the prevention of coccidiosis. Plants of the genus *Aloe* are known for their medicinal activities. *Aloe vera* inhibits invasion and replication of *E. maxima* in the intestine of chickens, suggesting its beneficial proprieties to control coccidiosis [301]. *Aloe secundiflora* was shown to significantly reduce intestinal lesions and mortality in addition to improvement in body weight to levels similar to the treatment with sulfachloropyrazine sodium, although with only partial reduction in *E. tenella* oocyst shedding [302]. Further investigations are necessary to optimize the efficacy of herbal compounds in the prevention and treatment of coccidiosis.

Different strategies are used to avoid or reduce the emergence of anticoccidial resistance, such as rotating between multiple compounds and the combination of anticoccidial compounds and vaccination [303], the latter being known to reestablish drug sensitivity in *Eimeria* strains in the field [304] due to the reintroduction of sensitivity genes present on the vaccine strains, specially those

strains isolated in the 1950s, previously to the introduction of anticoccidial drugs [305]. Besides these efforts, resistant strains of *Eimeria* have been reported against almost all compounds introduced in the market and appears to be widespread [281,306-308].

Probiotics are classified as live non-pathogenic microorganisms that are capable of maintaining a normal gut microbial population [309,310] through multiple modes of action, including competitive exclusion, pathogen antagonism, and stimulation of the immune system [310,311]. The administration of probiotics is known to reduce oocyst shedding of *Eimeria* [312,313] and lesion scores [314] in chickens. Moreover, the administration of probiotics reduces lesions of necrotic enteritis, a common disease associated with coccidiosis [315]. These reductions seem to be related to the significant decline in numbers of intracellular pathogens due to probiotics supplementation [316,317]. Moreover, the early establishment of beneficial microbiota by probiotics can inhibit pathogen establishment, therefore potentiating a protective effect and enhancing host resistance to infection while reducing the need for prophylactic use of drugs [309,318,319].

Protection to coccidiosis can still be improved utilizing methods already available in the market. The combination of probiotics with live vaccination shows higher weight gain and lower lesion scores post challenge with *E. acervulina* and *E. maxima* as compared to the use of probiotics and vaccination administered solely. Moreover, the addition of probiotics helped birds to compensate for the reduction in growth associated with the utilization of live attenuated *Eimeria* vaccines [320].

Vaccination is a practical alternative to drugs for coccidiosis control [321]. The currently available vaccination strategies consist of controlled infections with either virulent or live attenuated parasites. Protective immunity can be achieved if chickens are infected with a single high dose or multiple low doses (trickle infections) of *Eimeria* [322,323]. The majority of live vaccines

available in the market are non-attenuated, and due to the potential virulence of these strains, it is crucial that all birds are given the required dose in order to reduce the risk of outbreaks following vaccination [324]. A safer alternative is the use of live-attenuated vaccines, composed of strains that have suffered consecutive passages in chicken embryos and selected for precocity, characterized by shortened endogenous life cycle due to the selection of early oocysts, therefore reducing the total amount of oocysts produced during infection [321,325-327]. When applying live vaccination programs, it is imperative that anticoccidial medications are withdrawn from the feed to allow the recycling of oocysts [303]. In spite of the effectivity, the use of live vaccines is restricted by several issues, such as the high production costs, relatively short self-life and the risk of coccidiosis breakdown due to oocyst cycling increasing virulence [153]. Conversely, live vaccines offer a route of protection that circumvents the issue of developing drug-resistant coccidia [324,328]. The presence of oocysts in the litter and excreta after live vaccination is crucial in vaccinated flocks, indicating the vaccine uptake. Vaccine efficacy is directly dependent on the infectivity and fecundity of oocysts, since the protective immunity is induced after two to three consecutive infections [328-330]. Immunity against coccidiosis usually develops within 14 days post live vaccination, whereas, in commercial production, the disease usually affects chickens between 21 to 28 days of age [331].

Subunit vaccines

Subunit vaccines are composed of purified antigenic determinants that are separated from the parasite through various methods. The antigens may consist of recombinant proteins expressed from DNA of various developmental stages of *Eimeria* [303]. Despite some studies reporting the efficacy and safety of subunit vaccines for the control of coccidiosis, the lack of statistical differences in weight gain and feed conversion between vaccinated and non-vaccinated challenged

birds (positive control) evidences the need for improvements on this type of vaccine before the use by the poultry industry [225]. It is unlikely that subunit vaccines will achieve the same level of protection as live vaccines for coccidiosis, especially because different *Eimeria* species induce a protective immune response in singular ways, however it is possible to use a combination of cross-reactive, protective antigens that induce a higher titer of protective antibody response [332]. More recent studies have reported the partial protection granted by multivalent subunit vaccines and the cross protection conferred by the selected proteins used in the formulation [333].

Maternal immunity is due to the large transfer of IgG (or IgY in birds) class antibodies to the progeny. Evidences suggest that IgY antibodies, found in the egg yolks, are the primary effectors of maternal immunity on the chicken [334,335]. As noted by Smith, et al. [336], the levels of maternal antibodies strongly correlate with immunity to *Eimeria* infection, as the progeny of hens previously exposed to *E. maxima* oocysts (20,000 *per inoculum*) showed more than 90% reduction in oocyst production in hatchlings from eggs laid between three and four weeks after vaccination of the hens and 68% reduction in oocyst shedding when the eggs were laid at eight weeks post vaccination of the hens. This drop was circumvented by the use of the adjuvant Arlacel A with the inoculum, therefore reaching 90% reduction in oocyst shedding in the offspring of eggs laid at eight weeks post vaccination of the hens. These results evidence the protection conferred by maternally derived antibodies in controlling coccidiosis in chickens. A vaccine designed for breeder hens was later developed, CoxAbic®, which contains affinity purified gametocyte antigens (APGA) in water-in-oil adjuvant and is injected into the breast muscle of broiler breeder hens. Experiments have shown that the vaccination of hens with APGA provides cross protection to *E. maxima*, *E. acervulina* and *E. tenella* with 50-80% reduction in oocyst shedding [337,338]. These *Eimeria* species are the most pathogenic and it is believed that their control is sufficient to prevent

the deleterious effects of coccidiosis under field conditions [332]. Active immunity due to vaccination of the breeders with APGA lasted up to eight weeks in the progeny. As broiler chickens are bred to live between five to seven weeks, the maternal immunization has the capacity to protect broiler flocks for their entire life time [225]. Moreover, this resistance to infection outlasts the life of maternal antibodies in the progeny and it is believed to be a result of maternal immunity induced by vaccination reducing, but not eliminating, transmission of oocysts, therefore allowing the offspring to develop their own immunity against the sexual stages of *Eimeria* [225]. Immunity against the sexual stages of *Eimeria* has been recognized as strongly effective and the basis for the success of live attenuated vaccines [327].

In ovo administration of live vaccines is also a promising alternative to the control of coccidiosis in poultry, combining uniform distribution of the vaccine with a labor-less method and allowing the chicken to develop very early immunity against the pathogen. Weber and Evans [339] and Weber, et al. [340] report the *in ovo* vaccination of chickens with infective stages of *Eimeria tenella, E. maxima*, *E. acervulina*, *E. praecox*, *E. mitis* and *E. brunetti*, resulting in some level of resistance post homologous challenge at 14 days of age, as shown by improved weight gain and reduced lesion score. Following *in ovo* vaccination, *Eimeria* specific antibody titers generally increase linearly from day 14 onwards, indicating the age-dependent development of the antigen-specific humoral immunity [257]. More recently, the utilization of *Eimeria* proteins for this method of immunization has received more attention. *In ovo* vaccination with the *Eimeria tenella* microneme recombinant gene (EtMIC2) and encoded protein showed increased anti-EtMIC2 antibody titers at days 10 and 17 post-vaccination and associated homologous protective immunity characterized by increased body weight gain and decreased fecal oocyst shedding compared to

non-vaccinated controls. The same vaccination protocol also conferred the birds with immunity to heterologous challenge with *E. acervulina* [341].

New alternatives to coccidiosis control and treatment are still being developed. Passive intravenous administration of anti-IL-17A neutralizing antibodies reduced *E. tenella* multiplication and diminished tissue lesions on the basis of reduction of parasitized epithelial cell motility and the impairment of schizont maturation [213]. Consequently, IL-17A may also be used as a target for *Eimeria* control in the future. Similarly, in induced infections, exosomes from serum have been shown potential as an alternative strategy for controlling chicken coccidiosis in experimental settings, where naïve chickens intramuscularly immunized with $CD80^+$ and $CD80^-$ serum exosomes showed increased numbers of intestinal and spleen IL-2, IL-4, IL-6 and IFN-γ and greater protective immunity as measured by weight-gain, feed efficiency, parasite shedding and intestinal lesions. The administration of $CD80^+$ exosomes may also extend immunological memory to subsequent re-infection [153].

The oocyst wall is impermeable to water based substances, but it is permeable to lipid-based substances and small molecules [251]. Disinfectants can either prevent sporulation [342] or the ability of sporulated oocysts to infect the host. Manny common laboratory chemicals used for disinfection are unable to penetrate the oocyst wall, including potassium dichromate, formalin, ethanol, bleach and sulphuric acid [343]. 10% formalin and 70% ethanol allow for some degradation of oocysts, but this is over the course of four months at 23˚C [344]. Efficacious disinfectants proven to work against *Eimeria* spp. are ammonia, methyl bromide, phenol, and carbon disulphide [345].

Several drugs are currently commercialized for the control of coccidiosis in poultry. For sporozoites of *Eimeria tenella*, invasion of host cells and gliding motility has been shown to be inhibited by cytochalasin D and 2,3-butanedione monoxime. Microneme secretion, however, has

been shown to be unaffected by these drugs, indicating that this secretion is not coupled to the actinomyosin motor [16].

Due to the prokaryotic origins and biological importance of both apicoplast and mitochondria, they have been widely used as drug targets against apicomplexan parasites [23,346,347]. The blockage of the apicoplast protein synthesis using clindamycin and azithromycin in *Plasmodium berghei* was achieved by Friesen, et al. [348]. These defective parasites failed to complete their liver phase. This block in the parasite's life cycle elicited robust and long-lasting protection. The replication of the apicoplast genome in *Toxoplasma gondii* tachyzoites can be specifically inhibited using ciprofloxacin, and this inhibition blocks parasite replication. Furthermore, parasite death occurs with peculiar kinetics that are identical to those observed after exposure to clindamycin and macrolide antibiotics, also proposed as targeting protein synthesis of the apicoplast [349]. Quinolone drugs, such as buquinolate, decoquinate and nequinate inhibit coccidia respiration by blocking electron transport in the parasite's mitochondria [350]. Metilclorpindol also inhibits electron transport in the mitochondria [351]. Another drug acting on the mitochondria is nicarbazin. Although its true mechanism of action is unknown, it has been shown to inhibit succinate-linked NAD reduction in the mitochondria [352]. Besides the unsure mechanism of action of robendine, it is also believed to inhibit oxidative phosphorylation of mitochondria [353]. Toltrazuril, which is applied via drinking water, has been shown to reduce activities of the respiratory chain and to cause an inhibitory effect on the dihydroorotate-cytocrome C reductase [354], and to affect plastid-like organelles [355].

Other drugs act by interfering with essential biochemical pathways of the parasitic cell by affecting an important cofactor. As reviewed by Peek and Landman [303], ethopabate, sulphonamides, pyrimethamine and thiamine analogues (like amprolium), are drugs known to

interfere in essential biochemical pathways, therefore exerting anticoccidial action. Polyether antibiotics are known to alter the transport of cations (Na^+, K^+ and Ca^+) across cell membranes, inducing osmotic damage [356,357].

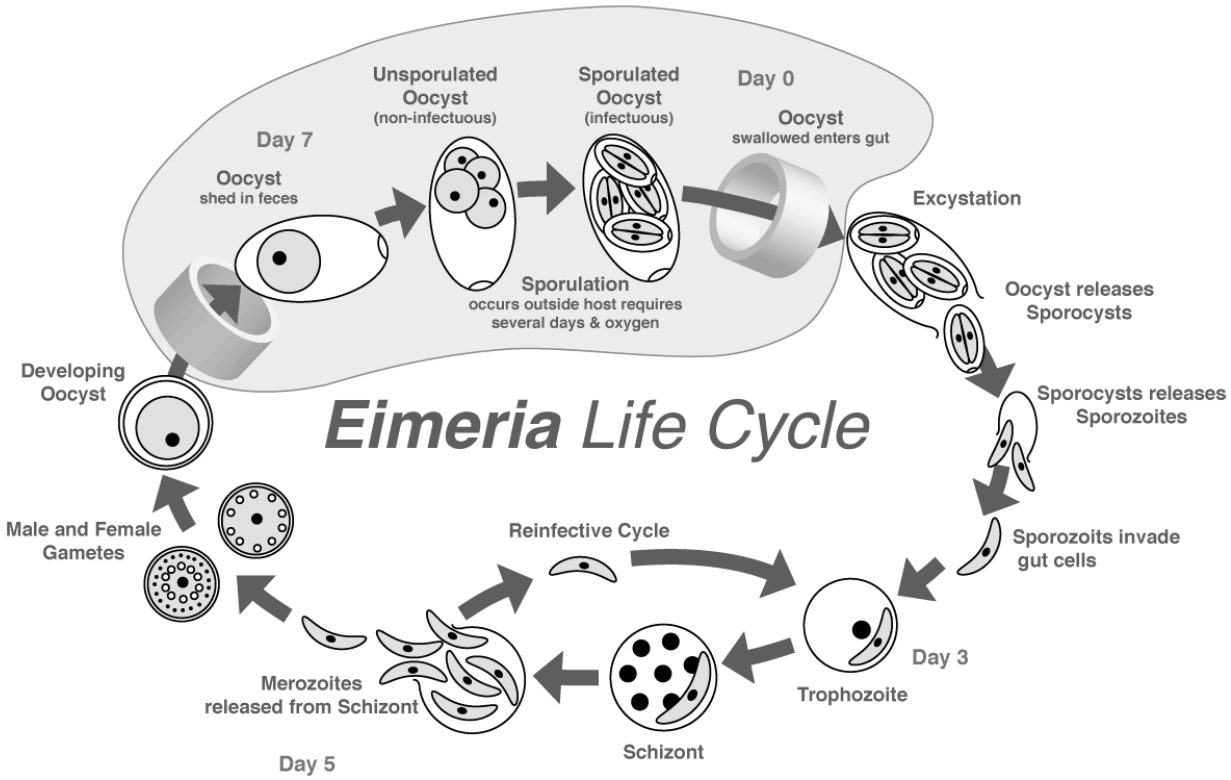

Figure 2.1: Life cycle of *Eimeria* spp. Available at https://www.impextraco.com/sites/default/files/coccidiosis-fig-498632.png

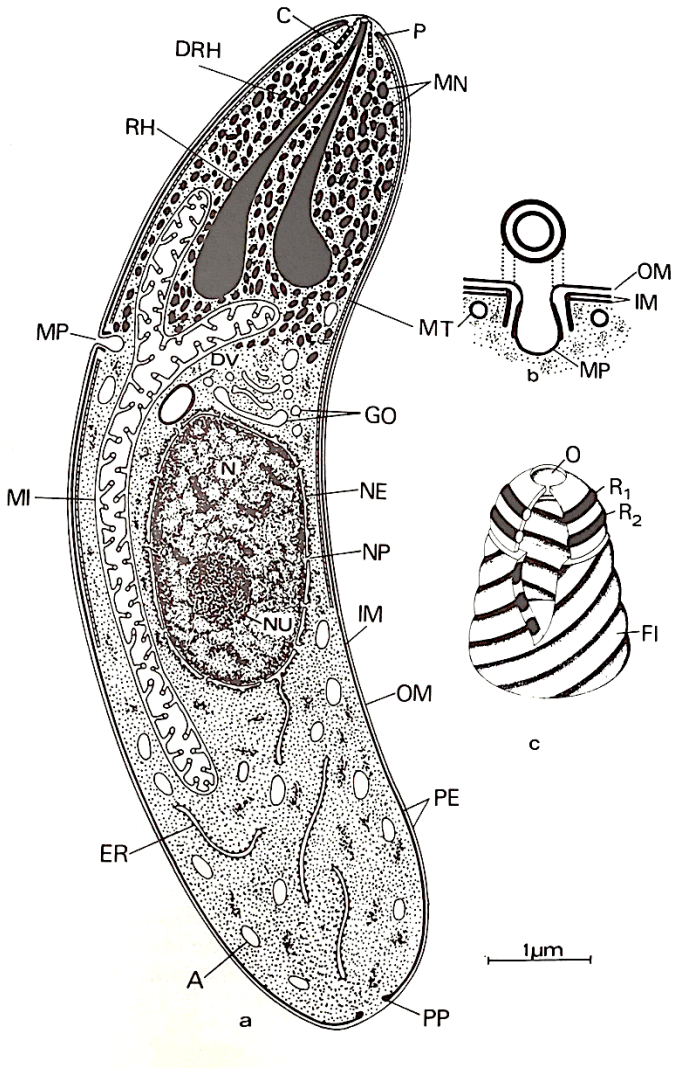

Figure 2.2. Coccidian merozoite showing the principal fine structural features. Available at Chobotar and Scholtyseck [22]. Longitudinal section of the merozoite (**a**); transverse and longitudinal section of a micropore (**b**); and the conoid (**c**). Depicted are: polar ring (P); conoid (C); duct of rhoptries (DRH); rhoptry (RH); micropore (MP); mitochondria (MI); endoplasmic reticulum (ER); amylopectin (A); posterior polar ring (PP); pellicle (PE), outer membrane of pellicle (OM); inner membranes of pellicle (IM); nuclear pore (NP); nuclear envelope (NE); Golgi apparatus (GO); microtubules (MT); microneme (MN); nucleolus (NU); nucleus (N); pre-conoidal rings (R_1R_2); fibril (FI);

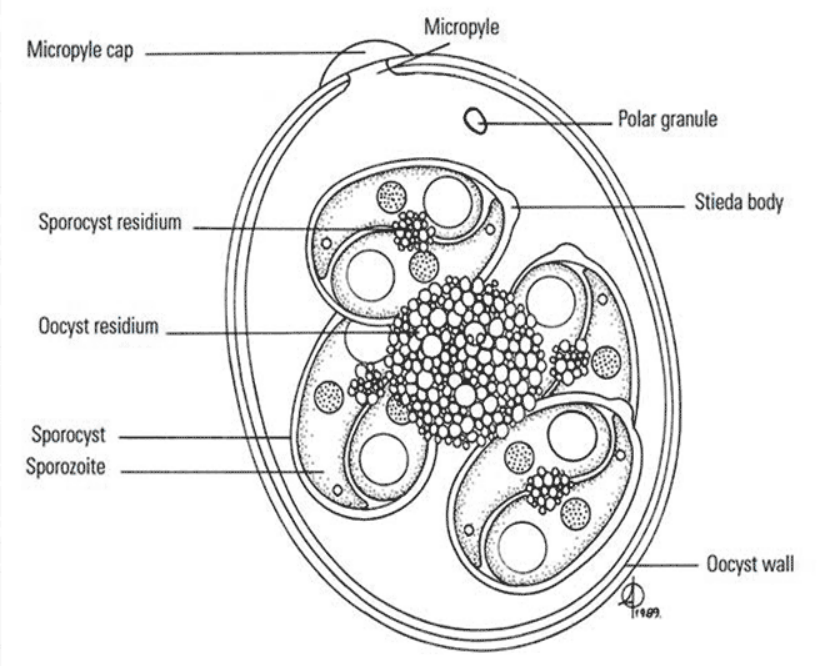

Figure 2.3. Structure of a sporulated *Eimeria* oocyst. Adapted from Levine and Ivens [358]. Available at https://anipedia.org/resources/coccidiosis/1105.

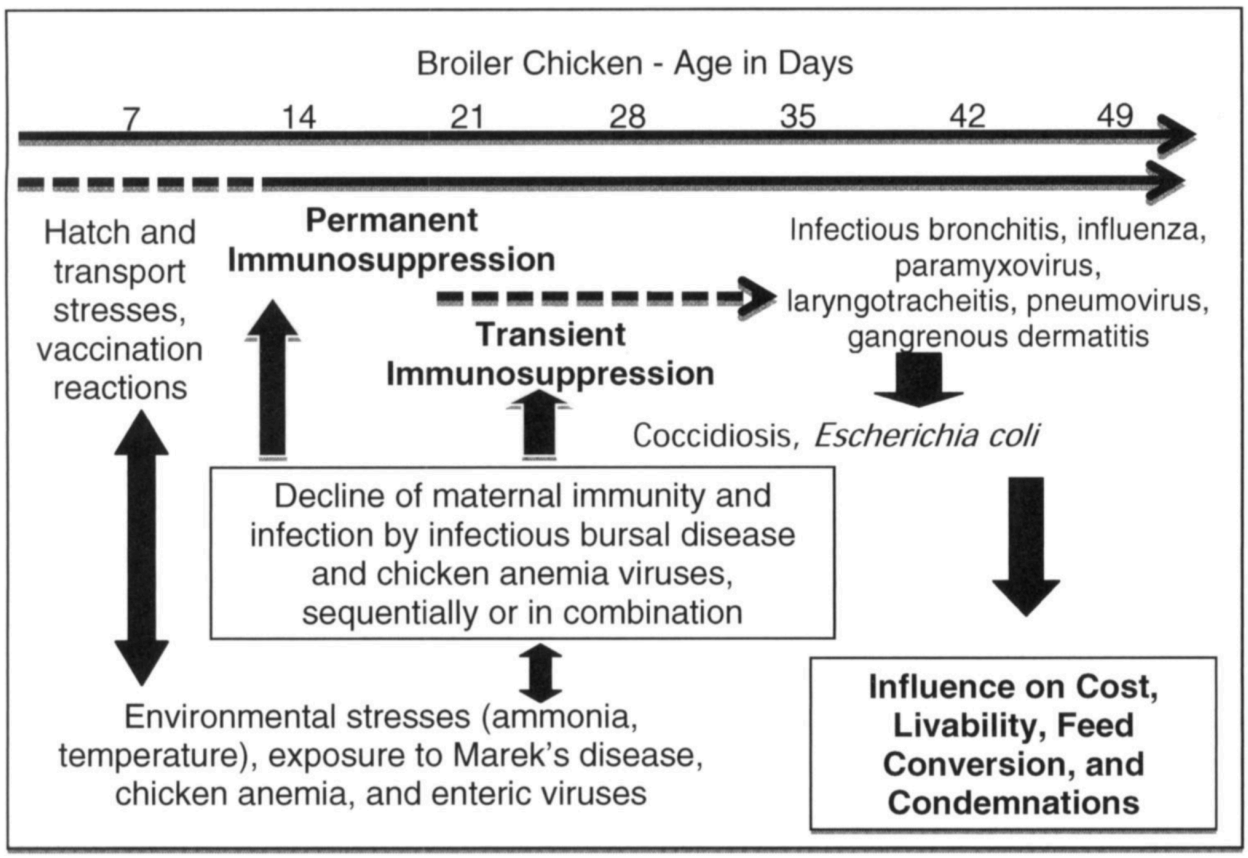

Figure 2.4: Immunosuppressive interactions in broiler chickens. A high-yield broiler chicken might hatch in an energy-depleted state and be subjected to suboptimal handling and transportation, experience delays in acquiring nutrition, and be simultaneously processing the live-attenuated vaccines that were applied in the hatchery and can produce cytolytic effects. Added to that, the brooding environment may not meet thermoneutrality, and placement on reused litter creates the potential for exposure to ammonia and potentially suppressive agents such as Marek disease, chicken anemia and enteric viruses. With the decline in maternal immunity, active infection by infectious bursal disease virus can be permanently immunosuppressive at or before 14 days of age and at least, transient immunosuppressive when it occurs at an older age. Also, with the maternal immunity, chicken infectious anemia can produce a cytolytic infection in the thymus and contribute to the immunosuppression already initiated by IBD. Following this sequence, it is possible for immunocompetency to be compromised in a variable percentage of the flock. This can contribute to increased severity of infection by respiratory viruses and the ability to control secondary respiratory infections by *E. coli*. Coccidiosis and gangrenous dermatitis may likewise emerge as a problem from 25 days of age and older. Collectively, this sequence of events occurs to variable degrees and influences livability, feed conversion, condemnation at processing, and total production cost. Each immunosuppressive influence represents a control point to prevent immunosuppression and improve health and welfare. Available at Hoerr [115].

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CHAPTER 3

THE EFFECT OF INCREASED TEMPERATURES ON VIABILITY, MORPHOLOGY, INFECTIVITY AND DEVELOPMENT OF *EIMERIA TENELLA*

Gustavo H. Schneiders, James C. Foutz, Alberta L. Fuller, Julie Nelson, Romdhane Rekaya, Samuel E. Aggrey. Submitted for publication on *Journal of Parasitology.*

ABSTRACT

Commonly found in backyard and commercial poultry production, Coccidiosis, caused by *Eimeria species* presents a self-limiting intestinal infection based on the number of ingested oocysts. Heat stress (HS) is one of the major environmental stressors in poultry, predisposing broiler chickens to immunosuppression and rendering them susceptible to diseases. There are suggestions that HS reduces *Eimeria* oocyst shedding in chickens, however, the relationship between HS and coccidiosis is not well elucidated. The objective of this study was to investigate the effect of temperature on viability, morphology, infectivity and development of *Eimeria (E.) tenella* in vitro, and merozoite production and oocyst shedding in vivo. In vitro exposure of sporozoites to 55 C for at least 60 min reduced sporozoites viability as shown by morphological changes and rendering them unable to invade MDBK cells. Intracellular development of merozoites was significantly reduced by an increase in 2 C in the optimal temperature of incubation in vitro. Most importantly, the induction of HS in the live chickens caused significantly lower lesion scores, reduced merozoite production and oocyst shedding, resulting in a much less severe disease outcome.

KEY WORDS

Coccidia; Heat Stress; Sporozoite, *Eimeria*, Imaging Flow Cytometry.

INTRODUCTION

Eimeria spp. is an apicomplexan parasite, the causative agent of coccidiosis, a disease of high economic impact in poultry production worldwide. The parasite's life-cycle is comprised of endogenous asexual replication followed by sexual development [1]. The disease is usually

recognized by the presence of bloody or watery diarrhea, moderate to high mortality, loss of weight, poor feed conversion, and other complications. *Eimeria tenella* produces hemorrhage in both caeca, which is accompanied by the presence of white dots (schizonts and oocysts) that can be seen from the serosal surface. *Eimeria tenella* also penetrates deep in the intestinal tissue, producing heavy damage to the mucosa and mucosal layer. The cecal lumen is filled with coagulated blood and necrotic mucosal debris. Microscopically, the epithelium appears denuded with hemorrhages and enlarged cecal pouch that may be also be distended with clotted blood. The cecal wall is often greatly thickened because of edema, infiltration and later scar tissue [2,3].

Heat stress (HS) is one of the major environmental stresses in the poultry industry and results in direct losses to production such as decreased body weight [4,5], protein and fat retention, feed consumption [6], lower meat quantity and quality [7], decreased egg quality and production [8], higher mortality rate and feed conversion [9]. Stress is a predisposing factor of immunosuppression in broilers, offering a good opportunity for normal commensals to induce infection and disease [10-12]. Heat stress has been reported to alter microbial (*Lactobacillus* sp.) composition in chickens [13], and predispose them to enteritis [14], to enhance *Salmonella enterica* serovar Enteritidis attachment and colonization to intestinal tissue and reduce intestinal crypt depth [12]. In coccidiosis, HS has a negative effect on oocysts counts during primary coccidial infections in poultry, as noted by the reduced oocyst output when compared with non-stressed chickens. However, HS did not exert an effect on a second exposure to *Eimeria* infection in chickens older than 27 days [15]. Recently, Ortega et al. (2018) observed that chickens infected with mixed *Eimeria* spp. and raised under HS did not shed any oocysts.

This effect of HS on primary *Eimeria* infections is attributed to the changes in immunity under heat stress conditions. For instance, HS reduces the expression of IL-12 and plasma levels

of IFN-γ, important pro-inflammatory cytokines [16]. In addition, it is common knowledge that coccidiosis tends to be less severe in the summer [17,18], presumably due to effect of the heat on host and parasites. The relationship between HS and coccidiosis is not yet well elucidated. The objective of this study was to investigate the effect of temperature on viability, infectivity and development of *E. tenella* sporozoites in vitro, as well as the development of merozoites and fecal oocyst shedding in chickens infected with *E. tenella* and exposed to HS.

MATERIAL AND METHODS

All experiments conducted in this study were performed under the Animal Use Proposal (AUP) A2015 04-005 approved by the Animal Care and Use Committee (IACUC) of the University of Georgia.

Parasite production and purification

Fourteen-day old commercial Cobb500 broiler chickens (Cobb-Vantress Inc., Cleveland, Georgia) were orally infected with $2.2x10^5$ *E. tenella* oocysts distributed in the feed. Animals were kept at 30 C in batteries with wired floor. Feed (broiler starter with Stafac at 500 mg/kg) and water were offered ad libitum. At 7 days post infection (dpi), chickens were euthanized by cervical dislocation, caeca were collected, minced in 1 L of water, to which of 50 ml of 10% potassium dichromate was added and then sporulated at 48 hr at 26 C with constant oxygenation [19]. Sporulation was confirmed by visual inspection under optical microscopy. After sporulation, oocysts were disinfected with 20% sodium hypochlorite and washed 3 times in PBS to ensure the removal of potassium dichromate and sodium hypochlorite, and then stored in PBS solution with 1% penicillin and streptomycin. Mechanical release of the oocyst wall was performed by manual

physical agitation of oocysts in PBS using sterile 1 mm glass beads for 1 minute, followed by enzymatic excystation of sporozoites by incubation with 0.25% trypsin and 0.75% sodium taurodeoxycholic acid at 41 C for 90 minutes [20], followed by purification by filtration on a 0.2µm tissue culture filter (Nalgene, Rochester, New York) from which the top filter membrane had been removed [21]. The strain of *E. tenella* used on these experiments is known to be sensitive to lasalocid.

Incubation temperature and viability of sporozoites

We used a 2-factorial design to evaluate the effects of temperature and time of incubation on the viability of *E. tenella* sporozoites. Tubes (1.5 ml) containing 300 µl of sporozoites at a concentration of 2.4 x 10^6 sporozoites/ml were incubated at 40, 45, 50 and 55 C for 0, 30, 60, 90, 120 and 150 min. A stained live control, kept at 4 C, and a dead control, incubated with 300 µl lasalocid 0.02% for 2 hr at 37 C [22], were also included. Post-incubation, sporozoites were stained with fluorescein diacetate (FDA) and Propidium iodide (PI) in adapted protocol described by Fuller, et al. [22]. The suspension containing sporozoites was added with 25 µl of FDA 0.00005% and 12.5 µl of PI 0.002%, protected from light and refrigerated at 4C overnight. Sporozoite viability was performed using an ImageStream Amnis imaging cytometer (Millipore Sigma, Carlsbad, California). Viability was determined as emission of FDA, whereas emission of PI determined sporozoite death. Data is depicted as percentage cell population emitting each fluorophore.

Incubation temperature morphology of sporozoites

Sporozoites incubated at different temperatures were morphologically evaluated using aspect ratio, determined by the Ideas software version 6.2 Amnis (Millipore Sigma, Carlsbad,

California). The aspect ratio represents the division of the minor axis of the cell by the major axis, describing how round an object is and classifies cells based on shape change. Aspect ratio is expressed in values ranging from 0 (totally linear) to 1 (totally round).

Flow cytometry analysis

Flow cytometry and imaging were performed on ImageStream Amnis imaging cytometer (Millipore Sigma, Carlsbad, California). For each sample, 10,000 events were acquired. Sporozoites were analyzed in 3 channels: morphology, FDA emission using a 528/65nm filter, and PI emission using a 610/30nm filter. Only samples with a high gradient RMS (measurement for focused cells) were analyzed. A gate was set to encompass the majority of the sporozoites in the sample using the images generated by Bright Field Aspect Ratio vs. Bright Field Area. The FDA and PI gates were drawn based on the intensity of FDA vs. the intensity of PI in live controls stained with FDA only, dead controls stained with PI only, and live non-stained controls.

Infectivity of sporozoites after exposure to heat

Tubes (1.5 ml) containing 1.4 x 10^7 sporozoites suspended in 400 µl of complete cell culture media (RPMI 1640 + 10% FBS and 1% pen-strep - Gibco Life Technologies) were incubated at 40 or 55 C for 60, 120, or 150 min. Post incubation, 200µL of the sporozoite suspension were added to 1 well of a µ-slide 8 wells (Ibidi, Gräfelfing, Germany) containing 90% confluent Mardin-Darbi bovine kidney (MDBK) cells, approximately 10^5 cells/well, followed by incubation at 41 C and 5% CO_2 for 4 hr to allow infection of sporozoites to cells. Post incubation, cells were washed 3 times with PBS to remove free sporozoites and fixed with cold 100% methanol overnight at 4 C. Post incubation, the cell layer was washed 3 times with PBS and incubated for 1

hr at 37 C with mouse anti-sporozoite monoclonal antibodies UGA 1A3 (1:100) [23-25], washed 3 times with PBS and incubated for another hr at 37 C for 1 hr with fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (1:100) and PI 0.00025%. Cell layers were washed 3 times with PBS and mounted using PBS with 1% pen-strep. Intracellular sporozoites were counted using a Zeiss LSM 710 Inverted Confocal Microscope (Zeiss, Thornwood, New York).

Influence of heat on intracellular development in vitro

Thirty-five millimeter plates containing MDBK cells with 100% confluency were infected with $1.3x10^5$ sporozoites of *E. tenella* and incubated at 41 C with 5% CO_2. At 24 hr post infection, the supernatant was removed, and the cell monolayer was washed twice with PBS and new cell media was added. Cells were incubated at either 41 C or 43 C for 48 hr to allow the first round of merozoite development. At 36 hr post infection, the cell layer and supernatant were removed and added to T25 flasks containing 50% confluent MDBK cells, and incubated at their respective temperatures for 48 hr, following with the harvesting of the second generation of merozoites by digestion with 25% trypsin and 75% taurocholic acid for 10 min with 3 subsequent washes in PBS, in modification of protocol described by Xie, et al. [21]. The second generation merozoites were suspended in 200 µl of PBS, counted 5 times on hemocytometer and inoculated in 100% confluent slides containing 10^5 MDBK cells and incubated for 4 hr at 41 C for infection. Post infection unbound merozoites were removed by 3 consecutive washes with PBS, and the cell monolayer was fixed with 100% methanol at 4 C overnight, followed by indirect immunofluorescence as described above. Merozoites were counted in 20 random fields under FITC filter on an Olympus IX71 inverted microscope (Olympus, Center Valley, Pennsylvania) under x20 magnification. Additionally, 10 merozoites from each treatment were longitudinally measured under bright field

microscopy at x20 magnification on a Nikon Labophot microscope (Nikon Instruments Inc., Melville, New York).

Effect of heat stress on *E. tenella* infection in vivo

Fifty-four Cobb500 broiler chickens at 21-days-old, were divided in 2 groups of 27 chickens, infected via gavage with $1.5x10^5$ *E. tenella* sporulated oocysts suspended in PBS. One group was housed at a thermoneutral (TN) environment (25 C), while the other group was housed under HS, with room temperature of 35 C. Both groups were in cages with wired floors. Cloacal temperature was recorded at the time of infection and at 2, 4 and 6 dpi. Nine chickens from each treatment group were euthanized by cervical dislocation at 2, 4, and 6 dpi for the assessment of cecal lesion scores and collection of ceca. At 6 dpi, fecal samples were collected from underneath the cages, pooled and refrigerated at 4 C.

Lesion scores, merozoites counts and oocysts shedding

Eimeria tenella specific cecal lesions were scored on 4 and 6 dpi according to Johnson and Reid [26]. Lesion scores ranged from 0 to 4, where 0 indicates no gross lesion scores, 1 indicates small amounts of orange mucus, 2 represents midgut filled with orange mucus; 3, ballooned thickened intestines with caseous-looking content; and 4 indicates ballooned intestines with blood clots. At 6 dpi, chickens were euthanized by cervical dislocation, caeca were collected and opened longitudinally, the content was gently removed without scraping the epithelia, followed by gentle washing of the ceca in PBS twice to remove fecal material. The lining was scraped thoroughly, and the contents placed in solution containing 0.25% trypsin and 0.75% taurodeoxicholic acid (TDA), incubated for 20 min at 41 C, followed by 3 washes with PBS and 1 filtration with cheese

cloth. After washing the supernatant was discarded and the pellet containing merozoites was resuspended in 1 ml of PBS and counted using a hemocytometer. At 6 dpi, pooled fecal samples were homogenized and 5 g of feces were dissolved in 50 ml of saturated salt solution, with 5 replicates per treatment. The solution was filtered in a coarse strainer to remove big particles. Approximately, 1.5 ml of the filtered homogenized suspension was used to count *E. tenella* oocysts in a Macmaster chamber and expressed in oocysts per gram (opg).

Statistical analysis

Statistical analysis and figures were generated with Prism 7.0 software (Graphpad Software Inc., San Diego, California). D'Agostino and Pearson test was applied to determine normality and determined whether data should be analyzed by parametric or non-parametric tests. Mann-Whitney was used to compare non-parametric data. Parametric data were analyzed by unpaired *t*-test with Welch's correction. All analyses were tested at the 5% significance level.

RESULTS

Effects of temperature on extracellular *E. tenella* sporozoites

Viability: We assessed the viability of *E. tenella* sporozoites incubated at different temperatures across different durations. Intensities of FDA and PI emission in 10,000 events acquired by imaging flow cytometry are shown in Figure 3.1. Similar levels of FDA and PI emission intensity indicate that there was no notable variation in sporozoite viability incubated at 40 and 45 C. However, incubation at 50 C for 60 to 150 min affected the viability of sporozoites slightly as compared to incubating at 50 C for 30 min. The most noticeable reduction in sporozoite viability was observed post incubation at 55 C. At 55 C there was a significant reduction in the

intensity of FDA emission and concomitant increase in the intensity of PI emission from 30 to 90 min. At 120 and 150 min of incubation at 55 C, there was no significant emission of FDA. The proportional emission of FDA versus PI in the sample is shown in Figure 3.2. Sporozoites incubated at 40 and 45 C retained the same level of viability, as noted by FDA emission ranging from 82.1 to 86.3% and PI emission ranging from 1.7 to 2.5%. Incubation at 50 C shows slight reduction in the sporozoite viability as evidenced by the lower emission of FDA at 120 and 150 min (78.8 and 73.9%, respectively) and slightly higher intensity of PI emission (3.1 and 4.2%, respectively). There was a notable shift in the intensity of FDA and PI emission post incubation at 55 C for only 30 min (17.8 and 58.4%, respectively), as compared to other treatments.

Morphology: Sporozoite morphology was determined by comparing aspect ratio vs. area for each sporozoite observation (Figure 3.3). Incubation at 40 and 45 C did not result in significant changes in sporozoite aspect ratio. Similarly, incubation at 50 C for up to 90 min did not alter sporozoite aspect ratio as compared to treatments heated at 40 and 45 C. However, there was a shift with increased aspect ratio post incubation at 50 C for 120 min. A similar shift was observed post incubation at 55 C for only 30 min. Low presence of sporozoites was detected in samples incubated at 55 C for 60 to 150 min. Numerical changes in sporozoite aspect ratio is presented in Figure 3.4. Aspect ratio of sporozoites incubated at 40-45 C was 0.54-0.56, similar to that at 50C which was 0.54-0.58. However, aspect ratio at 55 C was 0.55-0.61. Morphological alterations can also be noted by bright field inspection of the sporozoites (Figure 3.5). While sporozoites incubated at 40-50 C for 60 min present the usual elongated aspect, a more rounded morphology was observed in sporozoites incubated at 55 C for 60 min.

Infectivity: The ability of *E. tenella* sporozoites to infect cells post exposure to HS was accessed in vitro. The presence of sporozoites (green) in the cell monolayer (red) post exposure to

40 C indicates that the infectivity was unaltered by previous parasite's exposure to 40 C (Figure 3.6a-c). In contrast, the lack of binding to cells post exposure of sporozoites to 55 C demonstrates that exposure to this temperature inhibits their ability to infect to MDBK cells in vitro (Figure 3.6d- f).

Intracellular development under high incubation temperature

Incubation MDBK cells infected *E. tenella* and incubation at 43 C significantly reduced second generation merozoite production as compared to exposure to 41 C ($P < 0.0001$), with average production of 202,400 ± 28,680 merozoites/ml at 41 C and 67,200 ± 18,200 merozoites/ml at 43 C (Figure 3.7a). Significant reduction in merozoite counts per microscope field were observed at 43 C as compared to 41 C ($P < 0.0001$). About 9.1 ± 0.84 merozoites were observed per field when *E. tenella* was incubated at 41 C, as compared to an average of 4.2 ± 0.36 merozoites per field observed when sporozoites was incubated at 43 C (Figure 3.7b). There were no notable changes in morphology (not shown) and size of second generation merozoites incubated at 43 C as compared to the control group incubated at 41 C ($P = 0.3498$), with merozoite average size between 48 µm and 45 µm, respectively (Figure 3.7c).

***E. tenella* infected chickens raised in heat stressed environment**

Cloacal temperature was assessed on 2, 4 and 6 dpi. At 2 dpi, cloacal temperature was 41.70 ± 0.32 C and 41.44 ± 0.27 C for HS and TN groups, respectively ($P = 0.0884$). At 4 dpi, cloacal temperatures were significantly higher ($P = 0.0005$) at the HS group (42.68 ± 0.51 C) as compared to the TN group (41.28 ± 0.66 C). The cloacal temperature at 6 dpi was 40.72 ± 0.40 C in the TN group, significantly lower as compared to 43.03 ± 0.45 C of HS group ($P < 0.0001$)

(Table 3.1). There was no difference (P > 0.999) in cecal lesion score at 4 dpi between the HS and TN groups. However, at 6 dpi cecal lesions were significantly higher (P = 0.0092) in TN birds (1.889 ± 1.36) as compared to HS birds (0.333 ± 0.50). At 6 dpi, merozoite counts were significantly lower (P = 0.0002) in HS birds (26.67 ± 72.80) compared to the TN birds (1,457 ± 1,772). Similarly, oocyst counts obtained at 6 dpi were significantly lower (P = 0.0079) in chickens exposed to HS (80.4 ± 73.39) as compared to TN chickens (1,802 ± 797.60) (Table 1).

DISCUSSION

The current study provides evidences of the fate of *E. tenella* under HS. Sporozoites of *E. tenella* were evaluated for viability, morphology, infectivity and development post exposure to elevated temperatures in vitro and in heat stressed broiler chickens. Resistance of *Eimeria* spp. to heat has been historically assessed by exposing unsporulated oocysts to heat and evaluating their sporulation [27] and infection level in chickens [28]. In the current study, viability of *E. tenella* sporozoites was assessed by dual staining with FDA and PI dyes and analyzed under imaging flow cytometry. Both FDA and PI have been shown to be good indicators of *E. tenella* viability [22,29]. We show a slight reduction on the viability of *E. tenella* sporozoites under exposure to 50 C for 60 min, and high reduction on viability after incubation at 55 C for 60 min. This was marked by a reduction in the intensity of FDA emission in sporozoites after exposure to 50 C for 60 min, suggesting that heat causes damage to the sporozoite's membrane allowing the release of fluorescein or the exposure to heat inhibited the sporozoite ability to hydrolyze fluorescein diacetate into fluorescein. Interestingly, the low intensity of FDA emission after exposure to 50 C for 60 min was not accompanied by increased intensity emission of PI. Propidium iodide is known to pigment cells in late stage of apoptosis, therefore the window between low emission of FDA

and high emission of PI suggests that sporozoites are in the early stages of apoptosis after exposure to 50 C for 60 min. Low emission of both FDA and PI post incubation at 55 C for 90 to 150 min was most likely associated with degradation of sporozoites, as the signals for both dyes had faded. By evaluating the sporulation of *E. tenella*, Matsui, et al. [27] reported that the parasite still sporulates post exposure to 50 and 55 C for up to 5 min. The current study collaborates the results of Fish [28], that confirms the inability of sporulated oocysts, exposed to 55 C for 10 min, to infect chickens.

A previous study reported that viability of fresh *E. tenella* sporozoites ranged between 76.1 and 95.6% when analyzed by conventional flow cytometry [22]. The viability reported in the current study has a narrower range, varying between 82.1 and 86.3%, indicating that imaging flow cytometry is a reliable method to determine *E. tenella* viability. Moreover, the use of imaging flow cytometry allows for the observation of single sporozoite morphology and the selection of gates avoiding debris that could skew the results. Normal *E. tenella* sporozoites present a banana-like shape [23]. The sporozoites incubated at 55 C for 120 min presented rod-like shape as compared to controls. The same pattern was observed when sporozoites were incubated at 55 C for 30 min. Changes in the morphology of *E. tenella* sporozoites have been reported post treatment with curcumin (diferuloylmethane), and were correlated with losses in viability [30]. We further evaluated morphological changes using aspect ratio and the results were in concordance with the outcomes under bright field where there were notable increases in the round shapes of sporozoites exposed to 50 C for 120 min. This indicated that the loss of viability was followed by morphological alterations in sporozoites.

Viability of *E. tenella* sporozoites was also studied by their ability to invade MDBK cells in vitro. Sporozoites were able to invade the MDBK cells at 41C, the recommended temperature for cell invasion by *E. tenella* in vitro [31]. However, there was no invasion of sporozoites after

exposure to 55 C suggesting that extracellular exposure of sporozoites to higher temperatures hinders the parasite ability to invade host cells in vitro. The fate of *E. tenella* sporozoites to incubation at temperatures of 50 C and above indicates how resistant the parasite can be in the field to extremely high ambient temperatures.

Cloacal temperature changes by about 1.5-2.0 C between chickens raised under TN and HS condition [32]. When incubation temperature of MDBK cells infected with *E. tenella* was raised from 41 to 43 C there was a significant reduction in the number of second generation merozoites produced. We successfully propagated the MDBK cells that were not infected with *E. tenella* at 43 C to confirm that the cells can tolerate the 2 C increase in the incubation temperature. Thus, the reduction in the merozoites production was the result of the increased in host temperature. It must be pointed out that, the merozoites produced at 43 C had similar sizes compared to their counterparts produced at 41 C.

Anderson, et al. [33] suggests that the increase of 1 to 2 C in body temperature in chickens subjected to HS appears to impair *E. tenella* development. In the current study, HS was confirmed by significant higher cloacal temperatures and clinical signs, such as opened wings and panting. The heat stressed chickens had lower cecal lesion scores, merozoite and oocyst counts as compared to their counterparts housed at TN temperature, confirming the detrimental effects of host HS in the cycle of *E. tenella* in vivo.

Although this study provides evidence of the unfavorable effects of temperature (in vitro) and heat stress (in vivo) on the outcome of *E. tenella* infection, it is important to note that other factors, such as humidity and environmental temperature can have influencing effects under field conditions. High temperature (27- 33C) have a beneficial effect on faster sporulation rate of *E. maxima* oocysts [34], as well as elevated survival rate of sporulated *E. acervulina* oocysts [35].

However, elevated humidity and temperature, such as observed during summers in the United States, also seem to reduce longevity of sporulated oocysts in the litter [36]. Therefore, besides the effect of HS on *Eimeria* spp. infection, other factors such as humidity and litter temperature presumably play a role in the apparent reduced coccidiosis outbreaks on summer months in the United States.

In summary, we report herein the fate of *E. tenella* sporozoites exposure to temperatures up to 55 C. *Emeria tenella* sporozoites incubated at 55 C for 30 min had morphological alterations, lacked the ability to infect MDBK cells. Also, merozoites production was significantly reduced when MDBK cells infected with *E. tenella* were incubated at 2C above regular incubation temperature. The reduction in merozoite production under elevated temperature in *E. tenella* infected MDBK cells was validated in chickens infected with *E. tenella* and raised under HS. In addition to reduced production of merozoites, chickens infected with *E. tenella* and raised in a HS environment also had reduced cecal lesion scores and oocysts production compared to their control counterpart. Host responses to parasitic infections are complex. The molecular, cellular and immune regulations of the life cycle of *E. tenella* in chickens raised in heat stressed environments remain to be investigated

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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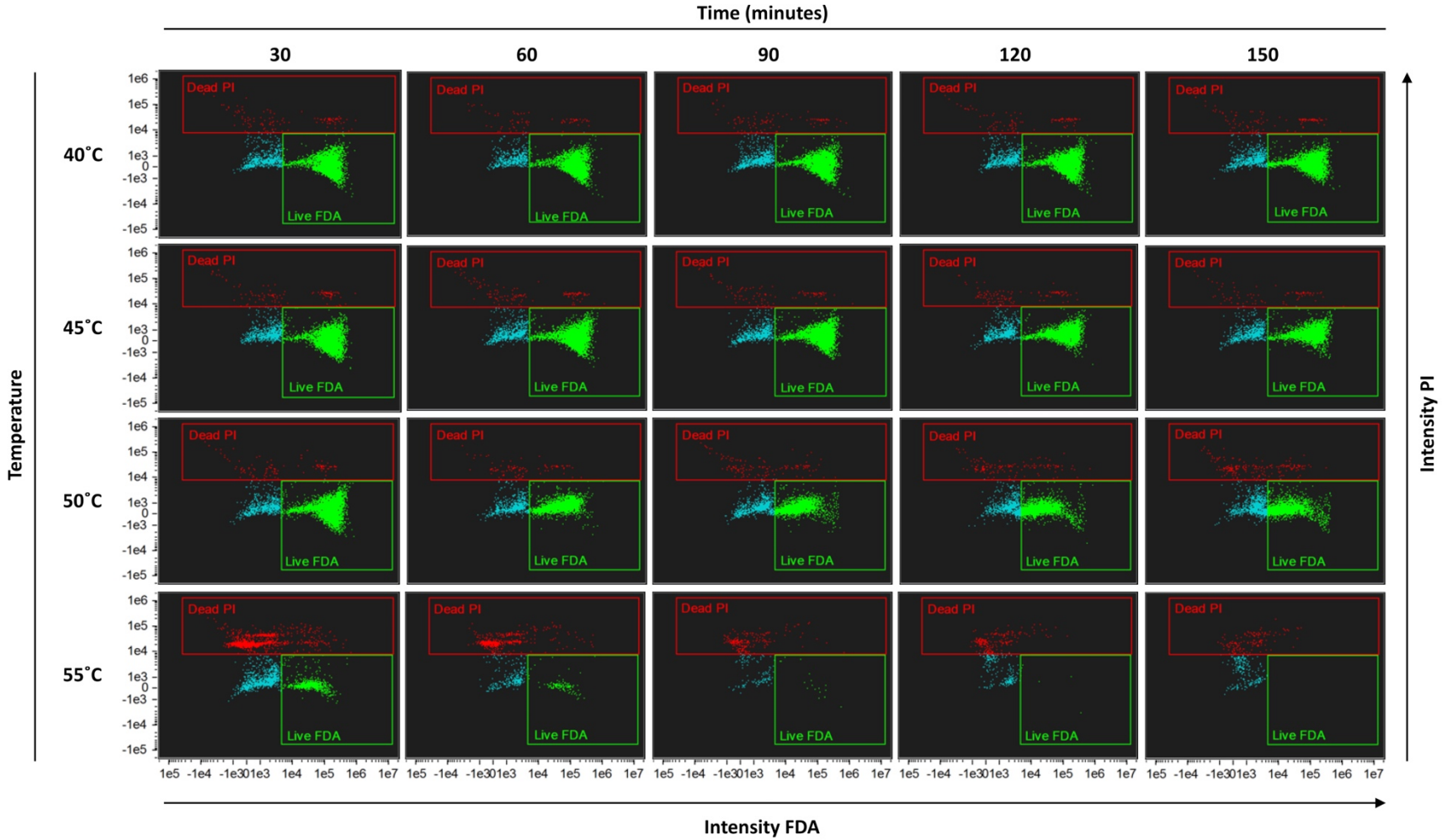

Figure 3.1. Viability of *Eimeria tenella* sporozoites post incubation at different temperatures in vitro. *Eimeria tenella* sporozoites were incubated at 40, 45, 50 or 55C for 30 to 150 min, with 30 min interval between treatments. Graphs depict fluorescein diacetate (FDA-green) and propidium iodide (PI-red) emissions. Samples were analyzed on ImageStream Amnis® imaging cytometer (Millipore Sigma). For each sample, 10,000 events were acquired. FDA emission was evaluated using a 528/65nm filter, and PI emission using a 610-30nm filter. Color version available online.

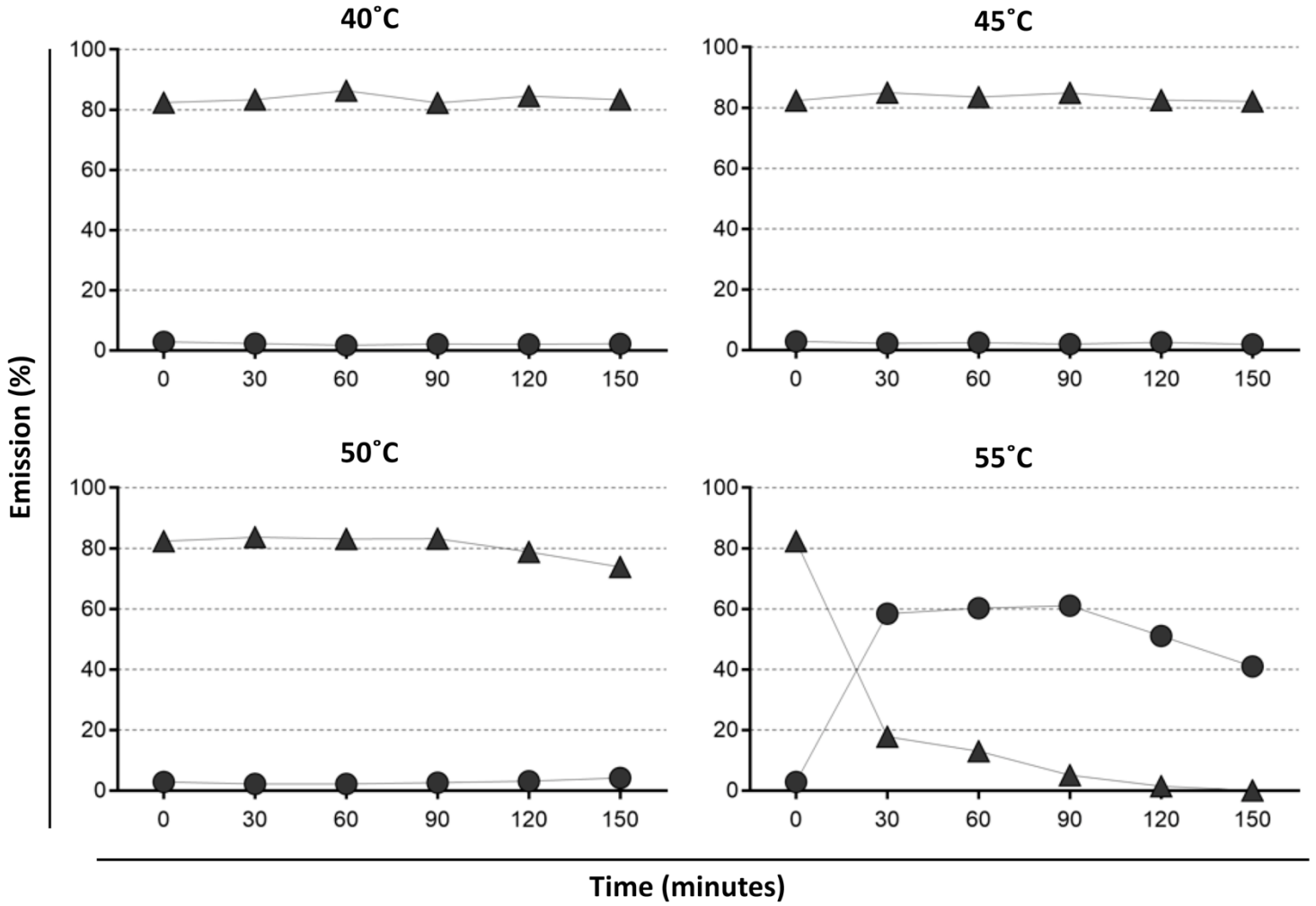

Figure 3.2. Percentage emission of fluorescein diacetate (FDA - triangle) and propidium iodide (PI-circle) by sporozoites submitted to different temperature intensities (40-55 C) for different time periods (0-150 min). Graphic depicts percentage fluorophore emission in the Y axis and time of exposure to respective temperature in the X axis. Ten thousand events where analyzed per sample. Percentage emission was calculated using Ideas software version 6.2 Amnis® (Millipore Sigma).

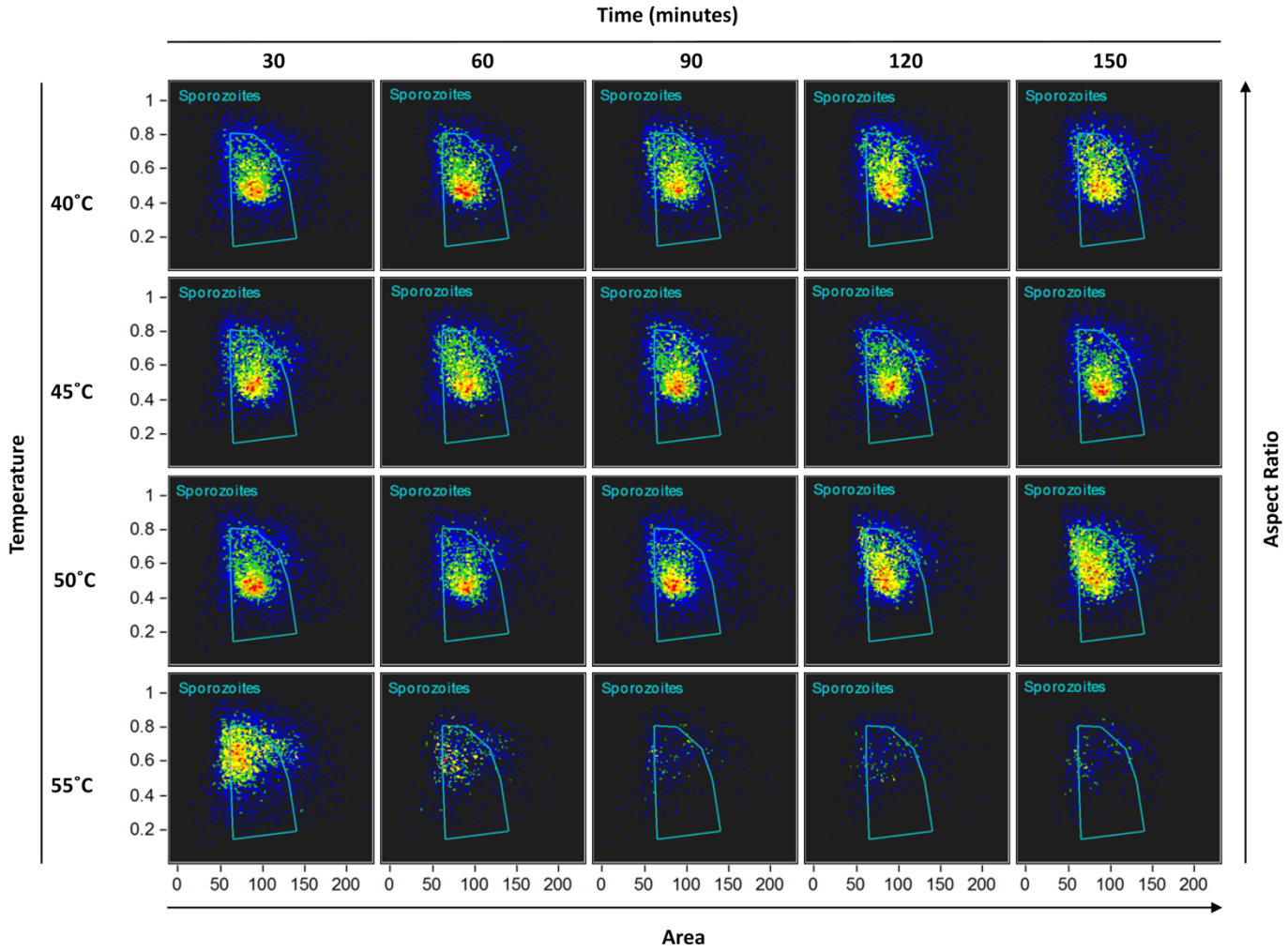

Figure 3.3. Morphology evaluation of *Eimeria tenella* sporozoites post incubation at different temperatures. *Eimeria tenella* sporozoites were incubated at 40, 45, 50 or 55 C for 30 to 150 min, with 30 min interval between treatments. Graphs depicts cellular area in the X axis and aspect ratio in the Y axis. Aspect ratio ranges from 0 (totally linear) to 1 (totally round). Color range depicts the density of cell populations in a given area. Light blue line draws the sporozoite gate at which sporozoites were analyzed. Color version available online.

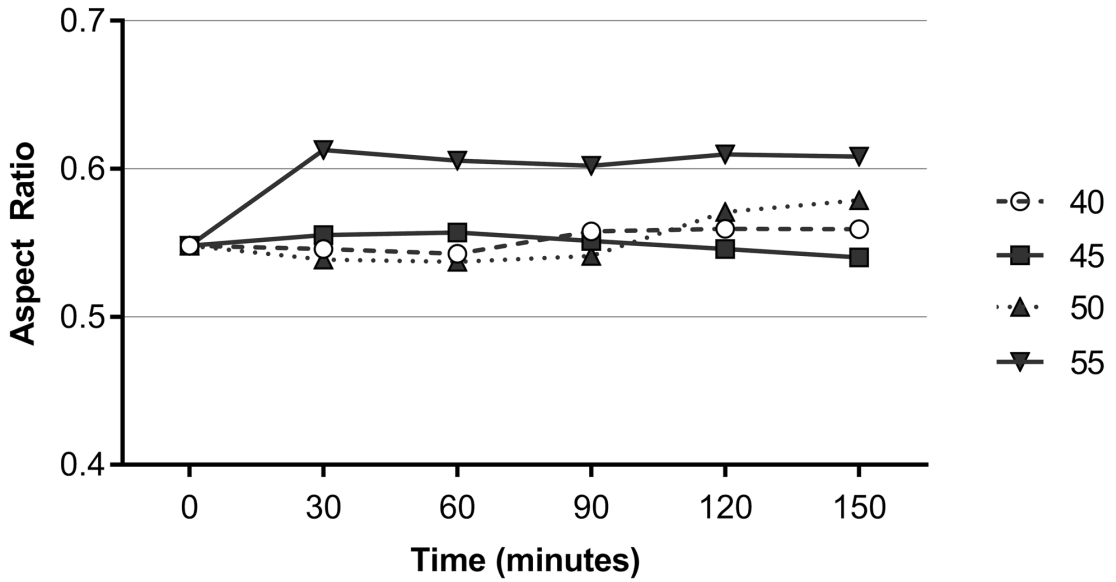

Figure 3.4. Aspect ratio variability in *Eimeria tenella* sporozoites incubated at different temperatures (40 to 55 C). Aspect ratio represents the division of the minor axis of the cell by the major axis, ranging from 0 (totally linear) to 1 (totally round). Graphic depicts the aspect ratio on Y axis and the time of exposure to each temperature on the X axis. Samples were analyzed on ImageStream Amnis® imaging cytometer (Millipore Sigma). For each sample, 10,000 events were acquired.

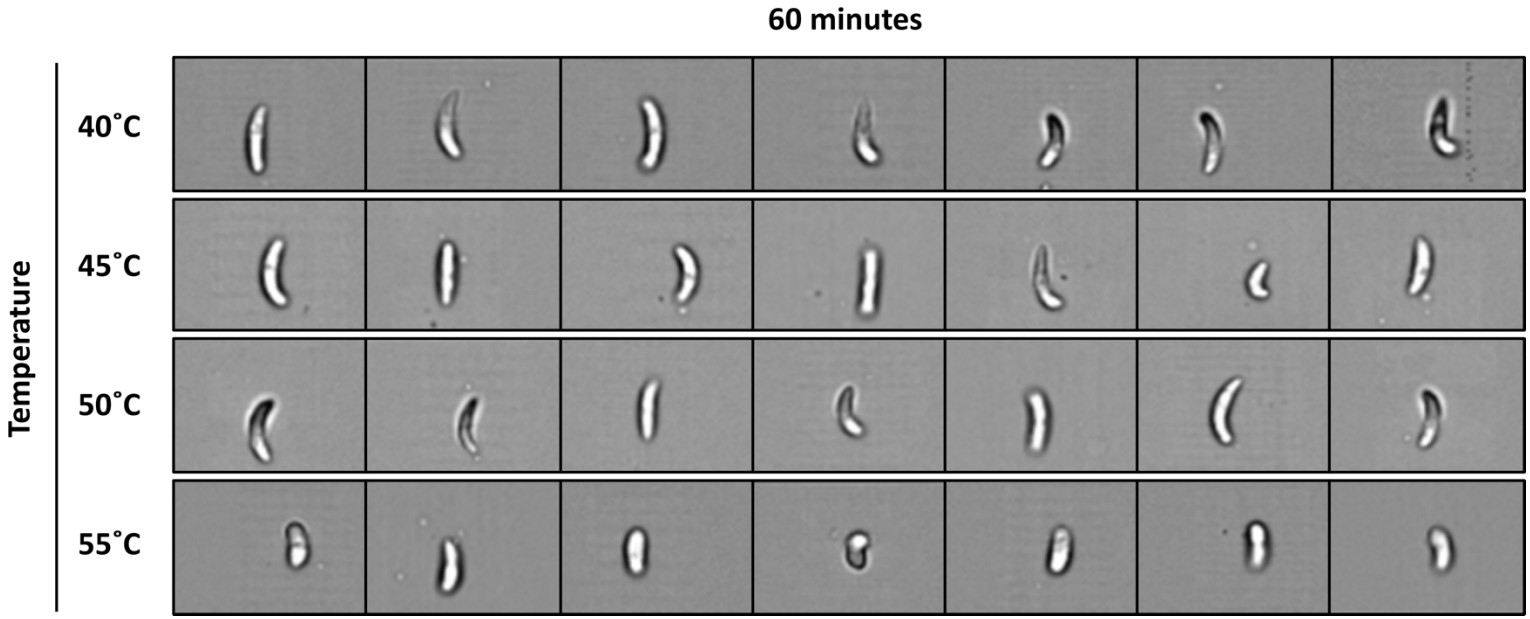

Figure 3.5. Morphology of *Eimeria tenella* sporozoites incubated for 60 min at 40, 45, 50 or 55 C. Morphology was accessed via bright field on ImageStream Amnis® imaging cytometer (Millipore Sigma).

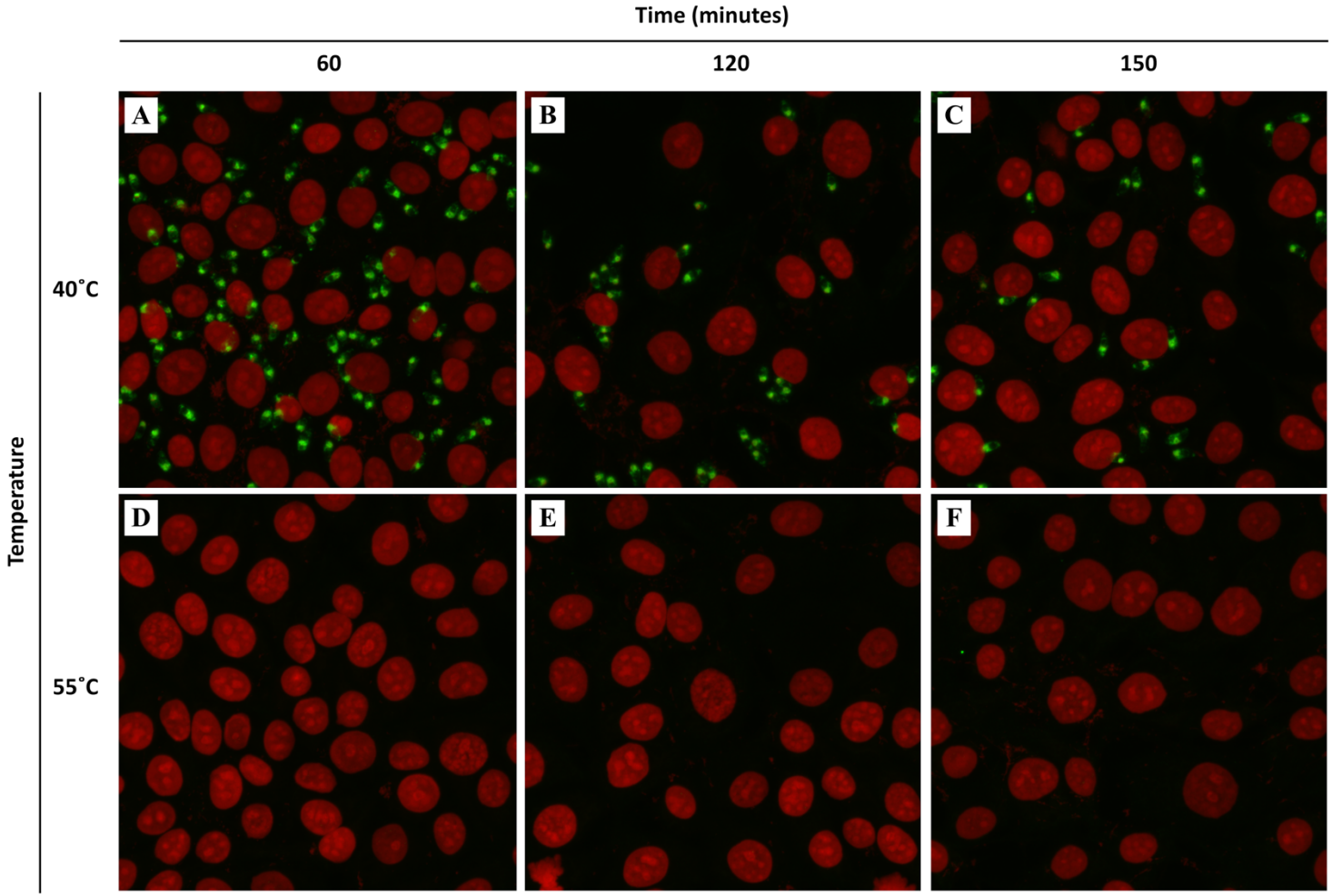

Figure 3.6. Infectivity of *Eimeria tenella* sporozoites post exposure to heat. MDBK cell (red) monolayers 4 hr post infection with *E. tenella* sporozoites (green) previously exposed to heat (40 or 55 C) for 60, 120 or 150 min. Exposure to 40 C does not alter the infectivity of *E. tenella* sporozoites. Contrary, incubation at 55 C inhibits the ability of *E. tenella* sporozoites to infect MDBK cells in vitro. Inverted confocal microscopy, x20 magnification.

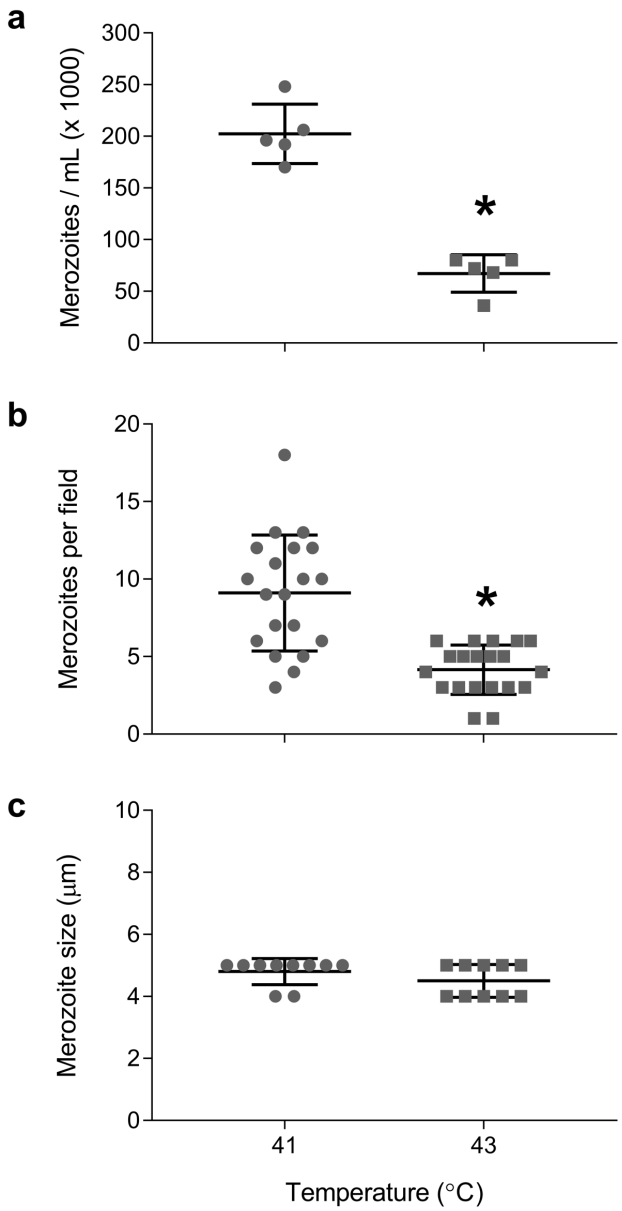

Figure 3.7. Influence of heat on intracellular development of *Eimeria tenella* in vitro. MDBK cell monolayers were inoculated with second first generation merozoites at 41 C. Monolayers were washed with PBS at 24 hr post infection to remove unbound merozoites and incubated with fresh medium at 41 C or 43 C for 48 hr. Cellular contents were digested with Trypsin and Taurocholic acid. (**a**) Mean merozoite counts post digestion of cellular contents is depicted my central horizontal line and standard deviation are depicted by horizontal lines. (**b**) Mean merozoite counts per microscope field at x20 magnification under fluorescent microscopy is depicted my central horizontal line and standard deviation is depicted by horizontal lines. (**c**) Mean merozoite size under bright field microscopy is depicted my central horizontal line and standard deviation is depicted by horizontal lines. The Kruskal-Wallis one-way analysis of variance was performed at 5% level of significance ($P < 0.005$). Significant differences between the groups are indicated by the superscript asterisk.

Table 3.1: Cloacal temperature, cecal lesion scores, merozoite counts and oocyst shedding recorded at 6 dpi in broiler chickens infected with *E. tenella* and housed at heat stress (HS) and thermoneutral (TN) conditions.

	HS	**TN**
Cloacal temperature (˚C)	43.03 ± 0.45	40.72 ± 0.40 *
Cecal lesion score	0.33 ± 0.50	1.89 ± 1.36 *
Merozoite count (/mL)	26.67 ± 72.80	1457 ± 1772 *
Oocyst shedding (opg)	80.4 ± 73.39	1802 ± 797.60 *

Results are expressed as Mean ± standard deviation. * marks statistical significant differences.

CHAPTER 4

HEAT STRESS REDUCES SEXUAL DEVELOPMENT AND AFFECTS PATHOGENESIS OF *EIMERIA MAXIMA* IN MEAT-TYPE CHICKENS

Gustavo H. Schneiders, James C. Foutz, Marie C. Milfort, Ahmed F. A. Ghareeb, Alberta L. Fuller, Susan M. Williams, Romdhane Rekaya, Samuel E. Aggrey. Submitted for publication on *Scientific Reports - Nature.*

ABSTRACT

Coccidiosis, caused by *Eimeria* spp. presents a self-limiting intestinal infection of poultry. Intestinal replication of the parasite causes severe morphological alterations to the host gastrointestinal tract, marked, among others, by the disruption of the intestinal barrier. We have previously reported a significant reduction in merozoite replication and oocyst shedding in *E. tenella in vitro* and *in vivo*. The objective of this study was to investigate the pathogenesis of *E. maxima* infection in broiler chickens under heat stress (HS) and mRNA expression of host cytokines that might affect the curtailed development of the parasite. We herein demonstrate that there is a significant detrimental effect of HS on the pathogenesis of *E. maxima* infection in broilers. There was a restricted replication of the parasite in HS chickens evidenced by significantly reduced oocyst shedding, and disruption of the intestinal blood barrier. Gene expression of parasite genes demonstrated curtailed sexual reproduction of *E. maxima* in HS chickens. There was downregulation of *Eimeria* spp. genes related to gamete fusion, oocyst shedding, mitosis and spermiogenesis. Host gene expression indicates alterations in the cytokine expression that could be related to reduced parasite development *in vivo*.

KEYWORDS

Coccidiosis, poultry, *Eimeria*, heat stress, gene expression.

INTRODUCTION

Eimeria spp. is an apicomplexan parasite, the causative agent of coccidiosis, a disease of high economic impact in poultry production worldwide. The parasite's life-cycle is comprised of several cycles of endogenous asexual replication followed by sexual development that results in

the formation of the oocysts, later excreted in the feces [1]. *Eimeria* (*E.*) *maxima* is one of the seven recognized species of coccidia that infect the chicken. The disease is marked by reduced growth, apathy, diarrhea and in severe cases, mortality. Clinical signs often include emaciation, pallor, roughening of feathers and anorexia. Abundance of yellow-orange mucus and fluid in the distal portion of the jejunum and proximal portion of the ileum, edema, thickening and disruption of the mucosa and sometimes presence of blood in the intestinal lumen are observed at necropsy [2].

Heat stress (HS) is one of the major environmental problems of poultry production in tropical and subtropical regions. Stress is a predisposing factor of immunosuppression in broilers, offering a good opportunity to normal commensals to induce infection and disease [3-5]. Heat stress has been reported to enhance pathogen attachment, colonization, shedding, reduce intestinal crypt depth and impact food safety risks [5-8]. The increase in pathogen colonization in heat stressed chickens is believed to be related to the disturbances in microbiota composition, thereby leading to a loss of protection against pathogenic microorganisms [8].

Contrary to the detrimental effects of HS in the outcome of infection with most poultry pathogens, we have previously demonstrated that the increase in 2˚C in the temperature of incubation of *E. tenella* significantly reduces asexual replication *in vitro* and that HS significantly reduces the outcome of *E. tenella* infection in broilers, as marked by reduction in merozoite production and oocyst shedding [9]. Similarly, HS also significantly reduces *E. acervulina* oocyst shedding in broilers [10]. It remains unclear how HS curtails *Eimeria* spp. replication *in vivo*.

Thus, the objective of this study was to investigate the effect of HS on the pathogenesis of *E. maxima* infection in broilers, as well as differential expression of host cytokines that might affect the curtailed development of the parasite. Together, these data indicate that HS of the host significantly reduces the sexual stages of *E. maxima in vivo*. Moreover, we provide information

on the relevance of HS control in the host response of birds not only for animal welfare and health, but also for their response to pathogens.

MATERIAL AND METHODS

All experiments conducted in this study were performed under the Animal Use Proposal (AUP) A2015 04-005 approved by the Animal Care and Use Committee (IACUC) of the University of Georgia.

Single oocyst cloning

Freshly sporulated *E. maxima* oocysts from a North Carolina field strain were counted suspended in PBS at a concentration of one 500 oocysts/mL. Single oocysts were observed in 2µL droplets under light microscopy and collected using a pipette. Twelve 14 days old-broilers kept in individual disinfected isolators were infected with one oocyst suspended in 200µL of PBS via gavage. Fecal matter was collected from each isolator at 7 days post infection (dpi) and analyzed for the presence of oocysts by salt flotation. Briefly, one volume of feces was solubilized in 9 volumes of saturated salt solution and the supernatant used to verify for the presence of oocysts in a McMaster chamber. The oocysts recovered from one of the chickens were sporulated as previously reported [11], and used to infect a second (p2) and third (p3) passages in chickens. The oocysts from the third passage (p3) were later sporulated and used for the experimental infections of chickens. The purity of the *E. maxima* clone was verified by PCR following protocol reported by Jenkins, et al. [12].

Experimental design

Three hundred 14-days old Ross708 broiler chickens were divided into 30 groups of 10 chickens each and infected via gavage with $2x10^5$ *Eimeria maxima* sporulated oocysts suspended in water and housed at two different temperatures: 15 groups were housed at 20˚C (TNi) and 15 groups at 35˚C (HSi). Similarly, another 300 chickens were mock infected with water and housed at 20˚C (TNc) and 35˚C (HSc). All chickens were raised in batteries with wired floor, with *ad libitum* access to water. Individual body weights and feed consumption were recorded at the day of infection (day 0), at 7 and 14 days post infection (dpi). From 4 to 7 dpi, 10 chickens from each treatment had blood samples collected and were euthanized by cervical dislocation, following with collection ileum samples for histology and gene expression. Intestinal lesions and the presence of *E. maxima* developmental stages (micro-scores) were scored from 4 to 7 dpi. Oocyst shedding was assessed on feces collected from underneath the pens from 4 to 7 dpi and at 14 dpi. Spleen samples collected at zero, 7 and 14 dpi were used to assess T-cell immune response. Quantification of *E. maxima* developmental stages in the ileum was assessed by qPCR from 5 to 7 dpi.

Intestinal permeability

Disruption of the intestinal blood barrier was assessed as previously reported [13]. Briefly, FITC-d was gavaged to chickens at 2.2mg of FITC-d/bird. Two hours post administrations, blood was collected from the jugular vein and stored in tubes protected from the light and kept at room temperature for 3 hours to allow clotting, following with centrifugation (1000g for 15 min) to separate serum. Fluorescence levels of diluted serum (1:1 in PBS) were measured at an excitation wavelength of 485 nm and emission of 528 nm. FITC-d concentration per mL of serum was

calculated based on a standard curve. Serum FITC-d levels were compared across infected and non-infected (control) chickens.

Intestinal lesion scores

Eimeria maxima specific lesions were blindly scored daily according to Johnson and Reid [14]. Lesion scores ranged from 0 to 4, where 0 indicates no gross lesion scores, 1 indicates small amounts of orange mucus, 2 represents mid-intestine filled with orange mucus; 3, ballooned thickened intestines with caseous-looking content; and 4 indicates ballooned intestines with blood clots. For microscopic lesion scores (microscores), a 2.5 cm long portion of the jejunum, proximal to the Meckel's diverticulum was opened and the mucosa scraped with a dissecting knife. The scraping was placed on a slide and covered with a slide. 20 fields were examined at 100x magnification and scored as follows: 0 (no oocyst present), 1 (1-10 oocysts per field), 2 (11-20 oocysts per field), 3 (21-30 oocysts per field), and 4 (more than 30 oocysts per field).

Oocyst counts

Pooled fecal samples were homogenized and 5g of feces were dissolved in 50 mL of saturated salt solution, with 5 replicates per treatment. The solution was filtered in a coarse strainer to remove big particles. 1.5 mL of the filtered homogenized suspension was used to count *E. tenella* oocysts in a Macmaster chamber and expressed in oocysts per gram (OPG).

Nucleic acid extraction

Tissue samples were stored in liquid nitrogen immediately after collection and at -80˚C for long term storage. RNA was extracted using trizol. In brief, 100mg of frozen tissue were

homogenized with 1mL of trizol, followed by phase separation with 0.2mL of chloroform, homogenization, incubation at room temperature (RT) for 3 minutes, centrifugation (12000 rpm, 15 min, 4˚C) and transferring 550µL of the aqueous phase into a tube for RNA precipitation with 0.5mL of isopropanol, homogenization and incubation (RT, 10 min), centrifugation (12000 rpm, 10 min, 4˚C), removal of the supernatant and wash of the pellet with 1mL of 75% ethanol, centrifugation (12,000 rpm, 5 min, 4˚C), removal of the supernatant, incubation (RT, 10 min), and dissolution of the RNA in 100µL of RNase free water followed by incubation (55-60˚C, 10 min) and storage of RNA at -80˚C for downstream applications. RNA was cleaned using RNeasy Mini Kit (Qiagen, Hilden, Germany), following producer`s guidelines, and later reverse-transcribed High Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific, Waltham, MA).

Gene expression, quantification and histology of *Eimeria maxima* in ileum samples

Ileum samples were ground in liquid nitrogen and total RNA was extracted using TRIzon reagent (Invitrogen, Carlsbad, CA), purified with RNeasy Mini Kit (Quiagen, Valencia, CA) and treated with RNase-free DNase (Quiagen, Valencia, CA) according to the manufacturer's instructions. The RNA was suspended in RNase-DNase-free water and concentration measured on a NanoDrop 2000 Spectophotometer (Thermo Fischer Scientific, Wilmington, DE) and stored at -80˚C. Two micrograms of total RNA were reverse transcribed with high capacity cDNA Reverse Transcription Kit according to manufacturer's protocol (Applied Biosystems, Foster City, CA) using a Gradient Mastercycler (Eppendorf, Hauppage, NY) for 10 min at 25˚C, 120 min at 37˚C, five min at 85˚C and final cycle at 4˚C. cDNA samples were stored at -25˚C. Complimentary DNA samples were diluted 1:5 prior to cDNA analysis. Each reaction consisted of 2µL of diluted cDNA, 0.3µL of forward primer (10µM), 0.3µL of reverse primer (10µM), 7.4µL of RNase-DNase-free

water and 10µL of Fast SYBR Green Master Mix (Applied Biosystems, Carlsbald, CA). Primer sequences used for the RT-qPCR assays are listed in Table 4.1. The RT-qPCR conditions were 95˚C for 20s, followed by 40 cycles of 95˚C for 3s and 60˚C for 30s, with Ct values measured at the end of each cycle. In addition, at the end of the reaction, the melting temperature curve of each RT-qPCR was determined. Each biological sample was runt in triplicates using StepOnePlus (Applied Biosystems, Carlsbald, CA). Relative expression of chicken genes was calculated from the amount of the gene-specific cDNA (target) normalized to the amount of chicken β-actin (endogenous control), using the $2^{-\Delta\Delta ct}$ method [15], in which differential mRNA expression was expressed as treatment groups relative to the control group (TNc). Relative expression of *Eimeria*-specific genes was calculated from the amount of the gene-specific cDNA (target) normalized to the amount of 18s (endogenous control), in which differential mRNA expression was expressed as treatment groups relative to the TNc group. Relative quantification of *E. maxima* in ileum samples was calculated from the amount of 18s cDNA normalized to the amount of chicken β-actin, in which differential mRNA expression was expressed as treatment groups relative to the TNc group.

Ileum samples were collected in a daily basis, fixed in 10% buffered formalin for 24 hours or more, processed, embedded in paraffin and 5µm sections were cut and stained with hematoxylin and eosin (H&E) and examined microscopically for the presence of *Eimeria maxima* developmental stages.

T lymphocytes flow cytometry

Spleen were collected and kept in incomplete (no serum added) cell culture media RPMI 1640 with L-glutamine (GE Life sciences, Pittsburgh, PA) on ice until processing. Tissues were homogenized and cells separated in a 70µm Falcon cell strainer (Thermo Fischer Scientific,

Waltham, MA). Two mL of the homogenized tissue suspended in cell culture media were laid on top of 2mL of histopaque 1077 (Sigma Aldrich, St. Louis, MO) and centrifuged at 1200 rpm for 10 min at 10˚C without breaks. Peripheral blood mononuclear cells (PMBC) were collected in 2mL microcentrifuge tubes centrifuged (1200 rpm, 5 min, 10˚C) and washed with cell culture media two times, followed by centrifugation (1200 rpm, 5 min, 10˚C). Cells were counted and diluted to a final concentration of 10^6 cells/mL suspended in flow cytometry buffer (PBS added with 0.5% bovine serum albumin and 0.4% 0.5M EDTA). In a round-bottom 96 well-plate, 10,000 cells were added per well (10µL), and incubated for 20 min at 4˚C with 90µL of CD4-PE (1:300) and CD8-FITC (1:100) conjugated monoclonal antibodies (Southern Biotech, Birmingham, AL) diluted in flow cytometry buffer. Single stained and non-stained controls were included. Post incubation, the plate was centrifuged at 800 rcf for 4 min at 10˚C, followed by removal of the supernatant and fixation of the cells in 200µL of 2% formaldehyde for 15 min. Post fixation, the plate was centrifuged (800 rcf, 4 min, 10˚C), the fixing solution was removed and the cells were resuspended in 200µL of flow cytometry buffer. Samples were analyzed by flow cytometry in up to 24h post fixation. Flow cytometry was performed on a CMM CytoFLEX (Beckman Coulter, Indianapolis, IN) with 5,000 events acquired per sample. Samples were analyzed in 2 channels: FITC emission using a 488-525nm filter, and PE emission using a 561-585nm filter. A gate was set to encompass the majority of single cells using the images generated by bright field. The FITC and PE gates were drawn based on the intensity of FITC vs. the intensity of PE in single-stained and non-stained controls. Cell populations in the samples were determined post compensation with single-stained and non-stained controls. The $CD4^+$:$CD8^+$ ratio was calculated by dividing the number of CD4+ T cells by the number of CD8+ T cells.

Performance parameters

Body weight (BW), feed intake (FI), feed conversion ratio (FCR) and relative growth (RG) were assessed at 7 and 14 dpi. Feed conversion ratio (FCR) was calculated using the following formula: FCR = FI / BW gain. Relative growth was calculated using the following formula: Relative growth = body weight / initial weight x 100, and results expressed as g/g.

Statistical analysis

Statistical analysis of BW, FI, FCR, RG, lesion scores, microscores, FITC-d and OPG was conducted in Prism 7.0 software (Graphpad Software Inc., San Diego, California). D'Agostino and Pearson test was applied to determine normality and determine whether data should be analyzed by parametric or non-parametric tests. Kruskal-Wallis test with multiple comparisons corrected using Dunn's method was used to compare non-parametric data. Parametric data were analyzed by one-way-ANOVA test with multiple comparison's corrected by Bonferroni's method. Gene expression and lymphocyte population analysis was conducted in SAS software (SAS Institute, Cary, North Carolina) by GLM procedure with multiple comparisons corrected with Tukey's method. All tests were performed at the 5% level of significance. All results are expressed as mean ± standard error of the mean.

RESULTS

Coccidiosis and HS cause decreased production parameters

The effects of HS and *E. maxima* infection in BW at 7 dpi (Figure 4.1A) and 14 dpi (Figure 4.1B) are depicted. At 7 dpi, the HSc chickens had lower BW (576 ± 103g) as compared to TNc (631 ± 127g; $p < 0.0011$). The HSi group had lower BW (520 ± 81g) as compared to TNc and HSc

($p < 0.0001$), however not statistically different from TNi ($p > 0.9999$). At 14 dpi, TNi chickens had lower BW (1068 ± 169g) as compared to TNc (1201 ± 125g; $p < 0.0001$), however still higher as compared to HSc (982 ± 91g; $p = 0.0001$) and HSi (981 ± 90g; $p = 0.0001$). While no statistical differences were observed in BW between HSc and HSi at 14 dpi ($p > 0.9999$), both treatments presented significantly lower BW as compared to TNi ($p \leq 0.0001$).

Feed intake at 7 and 14 dpi is shown in Figures 4.1C and 4.1D, respectively. The TNc group of chickens had feed intake at 7 dpi (2,900 ± 220g) higher as compared to TNi (1,671 ± 121g; $p < 0.0001$), HSc (1,931 ± 181g; $p = 0.0007$) and HSi (1,087 ± 120g; $p < 0.0001$). Chickens from the TNi group had feed intake similar to HSc ($p > 0.9999$) and HSi ($p = 0.0953$), however, HSc chickens showed feed intake at 7 dpi higher as compared to HSi ($p = 0.0041$). At 14 dpi, TNc chickens had an average feed intake (4,931 ± 630g) similar to TNi (4,926 ± 544g; $p > 0.9999$) and HSi (3,486 ± 351g; $p = 0.2079$), and higher as compared to HSc (2,169 ± 272g; $p = 0.0007$). Similarly, feed intake was similar between TNi and HSi ($p = 0.2112$), however higher in TNi as compared to HSc ($p = 0.0007$). No differences in FCR were observed between HSc chickens as compared to HSi ($p = 0.3202$).

Feed conversion ratio (FCR) at 7 dpi and 14 dpi are presented in Figures 4.1E and 4.1F, respectively. Chickens from the TNc group had FCR of 0.92 ± 0.04 at 7 dpi, similar to TNi (0.77 ± 0.05; $p = 0.5399$) and HSc (0.84 ± 0.09; $p > 0.9999$), however, statistically when compared to HSi (0.53 ± 0.07; $p = 0.0003$). The TNi chickens had FCR similar to both HSc ($p > 0.9999$) and HSi ($p = 0.0593$), however, HSc had higher FCR as compared to HSi ($p = 0.0067$) at 7 dpi. At 14 dpi, TNc chickens had FCR of 1.72 ± 0.020, similar to TNi (1.49 ± 0.150; $p = 0.7375$) and HSi (1.35 ± 0.119; $p = 0.0865$), however statistically augmented as compared to HSc (1.09 ± 0.078; $p = 0.0004$). Still at 14 dpi, chickens from the TNi group presented FCR similar as compared to HSc

($p = 0.0505$), and HSi ($p > 0.9999$). No statistically differences were observed in FCR between HSc and HSi chickens at 14 dpi ($p = 0.4908$).

Relative growth (RG) at 7 and 14 dpi is presented in Figures 4.1G and 4.1H, respectively. At 7 dpi, the average RG for TNc chickens (195 ± 2.795) was higher when compared to TNi (158 ± 2.668; $p < 0.0001$), HSc (174 ± 2.283; $p < 0.0001$) and HSi (157 ± 1.951; $p < 0.0001$). HSc had RG higher as compared to TNi and HSi ($p < 0.0001$). No differences were observed in RG between TNi and HSi ($p > 0.9999$) at 7 dpi. Relative growth at 14 dpi was significantly higher in TNc (374 ± 3.240) when compared with TNi (322 ± 4.635; $p < 0.0001$), HSc (298 ± 2.402; $p < 0.0001$) and HSi (297 ± 3.119; $p < 0.0001$). Similarly, TNi had RG statistically higher as compared to HSc ($p = 0.0002$) and HSi ($p < 0.0001$). No differences in RG were observed between HSc and HSi at 14 dpi ($p > 0.9999$).

E. maxima-specific intestinal lesions

The peak of intestinal lesion was at 6 dpi (Figure 4.2A), in which lesion scores are significantly higher ($p < 0.0183$) in TNi (3.4 ± 0.40) and HSi (3.6 ± 0.24) chickens as compared to zero recorded in the controls (Figure 4.2B). There were no differences between lesion scores from TNi and HSi ($p > 0.9999$). At 7 dpi (Figure 4.2C), lesion scores in TNi (1.2 ± 0.20) was similar to the control groups (zero; $p = 0.1337$). Although HSi (2.2 ± 0.20) was statistically similar to TNi ($p > 0.9999$), scores recorded for this group were still significantly higher as compared to the controls. Similarly, the highest microscores was recorded at 6 dpi (Figure 4.2D). Microscores at 6 dpi (Figure 4.2E) were similar between TNi (3.8 ± 0.20) and HSi (3.2 ± 0.37; $p > 0.9999$), both significantly higher as compared to zero ($p \leq 0.05$). At 7 dpi (Figure 4.2F) microscores recorded for TNi (1.2 ± 0.37) were not different ($p = 0.1761$) when compared to zero (TNc and

HSc) or to HSi (2.2 ± 0.58; $p > 0.9999$), however, scores recorded for HSi were higher as compared to TNc and HSc ($p = 0.0108$).

Disruption of the intestinal blood barrier precedes oocyst shedding

The peak of intestinal barrier disruption, as measured by FITC-d (ng/mL) translocation from the lumen to the serum, was at 6 dpi (Figure 4.3A). At 5 dpi (Figure 4.3B), the TNc group showed FITC-d concentration (117 ± 6.39) similar to HSc (96 ± 5.08; $p = 0.1929$) and HSi (103 ± 4.48; $p = 0.7990$), however, the TNi (148 ± 9.77) group higher FITC-d concentration compared to TNc ($p = 0.0184$), HSc ($p < 0.0001$) and HSi ($p = 0.0002$). At 6 dpi (Figure 4.3C), FITC-d concentration was higher ($p < 0.0001$) in TNi (313 ± 17.58) and HSi (309 ± 15.75) chickens as compared to TNc (161 ± 1.82) and HSc (150 ± 2.44). No significant differences were detected between TNc and HSc ($p > 0.9999$) or between TNi and HSi ($p > 0.9999$).

Heat stress limits *E. maxima* replication and oocyst shedding

The peak of oocyst shedding was at 7 dpi (Figure 4.3D). While no oocysts were shed from chickens from TNc and HSc groups, oocyst shedding at the HSi group averaged 58,051 ± 13,45 OPG at 7 dpi (Figure 4.3E), demonstrating a tendency to be higher as compared to zero ($p = 0.0795$). Oocyst shedding averaged 172,429 ± 29,13 OPG for TNi chickens, higher as compared to HSi, and the controls TNc and HSc (zero; $p < 0.0001$). Minimal oocyst shedding was detected at 14 dpi (Figure 4.3F), with averages 129 ± 55.02 and 133 ± 63.09 for TNi and HSi, respectively, however these were not statistically different from zero ($p \geq 0.1689$). The presence of *E. maxima* RNA in ileum samples was detected by RT-qPCR at from 5 to 7 dpi with results, expressed as $2^{-\Delta\Delta Ct}$, depicted in Figure 4.4. At 5 dpi, HSi chickens showed significantly lower expression of 18s

(0.08 ± 0.02) as compared to TNc (1 ± 0.20; $p < 0.05$). At 6 dpi the expression of 18s was higher in HSi (2.33 ± 1.36), however, this was not statistically different from TNi (1 ± 0.34; $P \geq 0.05$). At 7 dpi, there was a significantly higher quantification of 18s in HSi (482 ± 458) chickens as compared to TNi (1 ± 0.16; $p < 0.05$). There was no detection of 18s in samples from TNc and HSc.

The development of *E. maxima* in the small intestine was assessed by quantification of the expression of *E. maxima*-specific genes (mRNA) in ileum sections at 5, 6 and 7 dpi, with results depicted on Figure 4.5 as $2^{-\Delta Ct}$. There was no detection of *Eimeria*-specific gene expression in samples from TNc and HSc. At 5 dpi, IMC was significantly upregulated in HSi (12.06 ± 6.42 vs 1 ± 0.06), followed by significant downregulation at 6 (0.02 ± 0.02 vs 1 ± 0.55) and 7 (0.02 ± 0.01 vs 1 ± 0.33) dpi as compared to TNi. The expression of EF-2 was similar between HSi and TNi at 5 (0.87 ± 0.13 vs 1 ± 0.04) and 6 dpi (1.08 ± 0.08 vs 1 ± 0.19), however, HSi showed significant downregulation of EF-2 at 7 dpi (0.14 ± 0.05 vs 1 ± 0.09). The expression of GAM56 was significantly downregulated in HSi at 5 (0.36 ± 0.08 vs 1 ± 0.15), 6 (0.34 ± 0.07 vs 1 ± 0.34) and 7 dpi (0.07 ± 0.02 vs 1 ± 0.10). The expression of GAM82 was significantly downregulated in HSi at 5 (0.57 ± 0.11 vs 1 ± 0.08), 6 (0.39 ± 0.13 vs 1 ± 0.33) and 7 dpi (0.16 ± 0.05 vs 1 ± 0.11). The expression of HAP-2 was significantly downregulated in HSi at 5 (0.24 ± 0.15 vs 1 ± 0.10), 6 (0.17 ± 0.09 vs 1 ± 0.17) and 7 dpi (0.12 ± 0.05 vs 1 ± 0.12). The expression of Prot was significantly downregulated in HSi at 5 (0.49 ± 0.07 vs 1 ± 0.07), 6 (0.14 ± 0.06 vs 1 ± 0.37) and 7 dpi (0.03 ± 0.02 vs 1 ± 0.14).

Eimeria maxima detection by histopathology

There was no histological evidence of coccidia infection in sections from uninfected control chickens (TNc and HSc) throughout the experiment. Mild to severe coccidial enteritis was observed at 7 dpi in chickens from the TNi group. The diagnosis was marked by the presence of *E. maxima* in several stages of development in the lamina propria (Figures 4.6A and 4.6B). On the other hand, chickens from the HSi group were diagnosed as rare to minimal coccidial enteritis, marked by the presence of scattered schizonts in the lamina propria (Figure 4.6C and 4.6D).

Host immunomodulation during HS and *E. maxima* infection

$CD4^+$:$CD8^+$ ratio was calculated from the number of cells labelled with the fluorescent monoclonal antibodies anti-CD4 or anti-CD8 analyzed by flow cytometry. At 7 dpi (Figure 4.7A), $CD4^+$:$CD8^+$ ratio was significantly higher in chickens from the treatments TNi (1.12 ± 0.12), HSc (0.99 ± 0.07) and HSi (1.15 ± 0.14) as compared to TNc (0.55 ± 0.05; $p < 0.05$). Similarly, there was a significantly lower percentage of $CD4^+$ cells in TNc (24.35 ± 1.64) as compared to TNi (33.82 ± 1.74), HSc (38.14 ± 1.62) and HSi (37.38 ± 3.88; Figure 4.7B). There was a significantly higher percentage of $CD8^+$ cells in TNc (44.63 ± 2.53) as compared to TNi (31.28 ± 2.63) and HSi (33.08 ± 2.03), with no statistical difference between TNc and HSc, and between HSc, TNi and HSi (Figure 4.7C). At 14 dpi, $CD4^+$:$CD8^+$ ratio ranged between 0.54 ± 0.05 and 0.99 ± 0.13, with no statistical differences between the treatments (Figure 4.7 D). Similarly, there were no statistical differences in the percentages of $CD4^+$ (Figure 4.7E) and $CD8^+$ (Figure 4.7F) between the treatments.

The mRNA expression of host IL-1, TNF-α, IL-6, IL-10, TGF-B1, TGF-B2, NF-κB-1 and NF-κB-2 was assessed at 5 to 7 dpi, and results depicted on Figure 4.8. There was a significant

downregulation of IL-1 in HSi as compared to TNc, TNi and HSc at 5 dpi, followed by upregulation of IL-1 in TNi at 6 dpi. IL-1 was downregulated in HSc as compared to HSi at 7 dpi, without statistical differences between TNc, TNi and HSC, or between TNc, TNi and HSi. There were no statistical differences in the expression of TNF-α at 5 dpi, however this gene was significantly upregulated in TNi at 6 dpi, and in HSc at 7 dpi. There were no statistical differences in the expression of IL-6 at 5 and 6 dpi between the treatments. At 7 dpi, IL-6 was upregulated in HSc as compared to TNi, however no statistical differences were recorded between TNc, TNi and HSi, or between TNc, HSc and HSi. IL-10 was upregulated in HSi as compared to TNc and HSc at 5 dpi. There was a significant upregulation of IL-10 in TNi at 6 as compared to HSi, TNc and HSc. Similarly, there was a significant upregulation of IL-10 in HSi as compared to the uninfected controls at 6 dpi. The expression of IL-10 remained upregulated in HSi at 7 dpi, however there were no statistical differences in the expression of this gene in TNc, TNi and HSc at 7 dpi. The expression of TGF-β1 was unaltered at 5 dpi, downregulated in HSi at 6 dpi, and upregulated in TNi, and HSi at 7 dpi. The expression of TGF-β2 was downregulated in TNi at 5 and 7 dpi, and in HSi at 6 dpi. The expression of NF-κβ-1 was downregulated in TNi as compared to TNc at 5 dpi, with no statistical differences between TNi, HSc and HSi. There was a significant downregulation of NF-κβ-1 at 6 dpi. NF-κβ-1 was significantly upregulated in HSc at 7 dpi as compared to TNc and TNi. There were no statistical differences in the expression levels of NF-κβ-1 between HSc and HSi, between TNc and HSi, or between TNc and TNi. The expression of NF-κβ-2 was statistically similar between the groups at 5 and 6 dpi. There was a significant upregulation or NF-κβ-2 in HSi at 7 dpi as compared to TNc, but this upregulation was not significant when compared to TNi and HSc.

DISCUSSION

In poultry, *Eimeria* spp. replicates in the intestine causing extensive tissue damage. *E. maxima* causes congestion and edema, cellular infiltration, thickening of the mucosa, with the later generations of schizonts and sexual stages developing deeper in the tissues, causing considerable disruption of the mucosa [2], forming a port of entry for secondary infections such as necrotic enteritis [16]. We have previously reported the shortened life cycle of *E. tenella* under HS *in vitro* and *in vivo*, as evidenced by the reduction in merozoite replication and consequently oocyst shedding [9]. The objective of this study is to understand some of the underlying mechanisms of the detrimental effect of HS on *Eimeria* spp. and on its interactions with the host.

In the current study, HS was confirmed by physiological response of the animals, as marked by panting and opened wings. Our results demonstrate that, at the level of infection tested, *E. maxima* infection in meat-type chickens is as detrimental as HS when infection occurs at 14 days of age, as demonstrated by the similarity in performance parameters evaluated. Between 7 and 14 dpi (14-21 days of age), TNi chickens showed an overall tendency to recover from infection faster, as demonstrated by higher BW, FI and RG as compared to HSi chickens. The recovery from infection coincided with significant reduction in oocyst counts, lesion scores and microscores in TNi as compared to HSi. We also demonstrate that HS by itself exerts severe detrimental effects for poultry production, independent of coccidiosis infection, such as lower development and weight gain. These results are in concordance with literature reports for uninfected heat-stressed broilers, indicating decrease in BW and FI [17]. Interestingly, our data indicates decrease in FCR in HSc and HSi chickens, however, we demonstrate that this decrease is accompanied by reduction in FI and BW, resulting chickens with lower RG.

The peak of intestinal lesions was at 6 dpi, and at that day, both TNi and HSi reached similar levels of lesions, indicating that both groups presented significant parasite replication in the intestine. Moreover, at 7 dpi, TNi showed a tendency towards less severe intestinal lesions, as compared to HSi, indicating a faster recover from infection. Following a similar trend, the peak of microscopic scores was at 6 dpi, with a similarity in scores between both infected groups (TNi and HSi). At 7 dpi, microscopic scores tended (non-significant) to be less severe in TNi chickens as compared to its counterpart, HSi. Altogether these data demonstrate that, at the level of infection tested, heat stressed chickens reach similar levels of intestinal damage caused by *E. maxima* and are rendered unprepared to overcome infection as fast as chickens raised in a thermoneutral environment.

Intestinal damage was also assessed by disruption of the intestinal blood barrier determined by the concentration of FITC-d in the serum. While both groups of infected chickens reached similar levels of intestinal barrier disruption at 6 dpi, only TNi chickens showed significant disruption of the intestinal blood barrier at 5 dpi, indicating a slower replication of *E. maxima* in heat stressed chickens as opposed to the faster replication of the parasite in chickens housed in a thermoneutral environment.

The reduction in oocyst shedding of *E. acervulina* [10] and *E. tenella* [9] under conditions has been previously reported. Similarly, HSi chickens also presented a significant reduction in oocyst shedding at 7 dpi as compared to TNi chickens. Parasite replication was further quantified by RT-qPCR. Our results demonstrate that *E. maxima* replicate faster in TNi chickens as compared to HSi, indicating that of the host also exerts a detrimental effect on *E. maxima*. There is a significant upregulation in 18s at 7 dpi in HSi chickens, as compared to TNi, indicating that the life cycle of *E. maxima* in HS chickens might be in fact extended for a period longer than usual. This delay in

parasite development at 7 dpi is also noticeable on histology sections of the small intestine. While moderate to severe coccidiosis was reported for TNi, marked by the presence of several stages of development of *E. maxima* in intestinal sections, including macrogametocytes and microgametocytes, only rare asexual stages of *E. maxima* were seen in sections from HSi chickens. These are indicatives of the extended asexual development of *E. maxima* in HS chickens. The significant increase in expression of 18s at 7 dpi overlaps with the excretion of *E. maxima* oocyst in the feces. Therefore, this increase is most likely an artifact observed due to the excretion of *E. maxima* from the intestines of TNi chickens.

We evaluated the development of the parasite with a panel of genes related to oocyst production and macrogametocyte development (GAM56 and GAM82), Two putative genes (HAP-2 and Prot) previously related to microgametocyte development in *E. tenella* [18], and two genes (IMC and EF-2) expressed throughout the development of *E. maxima* [19]. Gene expression analysis reveals a significant upregulation of IMC in HSi at 5 dpi and similar expression of EF-2 between HSi and TNi at 5 and 6 dpi, indicating an overall similar level of *E. maxima* asexual reproduction in both treatments. Our analysis also reveals significantly lower expression of *Eimeria* spp. genes related to the sexual reproduction (GAM56, GAM82, HAP-2 and Prot) in HSi as compared to TNi.

IMC is a component of the glideosome, the locomotory system used specifically by apicomplexans to achieve their characteristic gliding motility [19]. The upregulation of this gene in HSi at 5 dpi possibly indicates higher motility of *E. maxima* in the ileum of these chickens as compared to TNi. There was a significantly lower expression of IMC at 6 and 7 dpi, indicating that at those time points, motility of merozoites is compromised in HSi chickens, however further evaluation of motility is necessary for further conclusions. EF-2 is a common immunodominant antigen [20] expressed in *E. maxima* [19]. EF-2 plays a crucial role in the elongation stage of mRNA

translation in eukaryotes, by mediating the translocation of the ribosome relative to the mRNA after addition of each amino acid to the nascent chain [21,22]. The family of proteins is highly conserved and expressed in all eukaryotic cells, playing important roles in mitogenic signal transduction, apoptosis, cell cycle control, and protein utilization [23,24]. The downregulation of this gene in HSi at 7 dpi strongly indicates reduced *E. maxima* replication on this group of chickens. The genes GAM56 and GAM82 code for previously characterized gametocyte antigens and oocyst wall proteins, components of the wall-forming bodies of the macrogametes and incorporated into the oocyst wall [25,26]. The upregulation of these two genes in TNi agrees with the augmented oocyst production as compared to HSi. HAP-2, a microgametocyte-specific gene previously reported in *E. tenella* [18] and *Plasmodium* was also downregulated in parasites from HSi chickens. The expression of this gene is required for gamete fusion and subsequent fertilization of *Plasmodium* [27-29] and is believed to have similar functions in *E. tenella*, mediating membrane fusion between mating gametes [30]. The downregulation of HAP-2 in HSi indicates that there is a significant reduction in parasite fertilization when hosts are exposed to HS. Prot is a histone-like protein that binds sperm DNA, condensing the genome into an inactive state [31]. The gene has been shown to be upregulated in microgametes of *E. tenella* [18]. The downregulation of Prot in the parasite population from HSi chickens is in agreement with the downregulation of the other genes whose expression is specific to the sexual stage of *Eimeria* spp., indicating lower sexual reproduction of *E. maxima* in HSi as compared to TNi.

A previous study shows the effect of *Eimeria* spp on the reduction in white blood cell count, antibody production, lymphocytes numbers, spleen weight, macrophage activity; $CD4^+$ and $CD8^+$ lymphocyte counts [12]. Moreover, Khajavi, et al. [32] demonstrates that the increase in $CD4^+$:$CD8^+$ ratio in HS chickens occurs despite decrease in both $CD4^+$ and $CD8^+$ numbers. Our

data demonstrate that the $CD4^+$:$CD8^+$ ratio increases in chickens upon infection and/or HS, without additive effects. Moreover, HS and *E. maxima* infection cause a significant increase in $CD4^+$ at 7 dpi, while *E. maxima* infection causes a decrease in $CD8^+$ cells at 7 dpi but there were no differences in $CD4^+$ and $CD8^+$ populations between TNi and HSi chickens. Furthermore, our data indicates that the detrimental effects of the host on the outcome of *E. maxima* infection is not due to immunosuppression, as demonstrated by the comparable $CD4^+$:$CD8^+$ ratios in both groups of infected chickens, TNi and HSi, during the first and second weeks of infection. Further studies should determine whether HS during primary infection limits the host's immune response to challenge. Due to the systemic effects of HS in the host, we decided to assess the systemic variation in the $CD4^+$:$CD8^+$ ratio. Future studies should also focus in the intestinal evaluation of T-lymphocytes.

It has been shown that *Eimeria* spp. activates cytokine release and the migration of the inflammatory cells that modulate the host immune system in different ways according to the species [33,34]. Stressors have also been reported to modulate the production and release of cytokines and other inflammatory mediators [35]. To better understand the changes in disease pathogenesis on the current study, we conducted a comprehensive panel analysis of anti-inflammatory and pro-inflammatory cytokines. Heat stress significantly downregulates the expression of pro-inflammatory cytokines (IL-1 and TNF-α) in the ileum of chickens infected with *E. maxima*, with the simultaneous downregulation of NF-κβ-1 and upregulation of IL-10. There is the possibility, however, that this diminished cytokine gene expression is a result of the reduced parasite development in HSi chickens. However, it remains unclear at this point the exact mechanism of cytokine response elicited by HS that limits *E. maxima* development. There was an upregulation of IL-10 at the peak of intestinal lesions (6 dpi) in TNi and HSi. Interestingly, there is an

upregulation of IL-10 only in HSi at 7 dpi, putatively indicating continuation of the replication of this parasite population, corroborating the quantification of *E. maxima* population in the ileum.

Results from our previous studies have reported the significant reduction in merozoite replication and oocyst shedding in *E. tenella in vitro* and *in vivo*. We, herein, also show that there is a significant detrimental effect of HS in the outcome of *E. maxima* infection in broiler chickens. Moreover, the restricted replication of the parasite in HS chickens is shown to be linked to potentially lower gamete fusion, lower parasite fertilization, lower sexual reproduction, resulting in an overall diminished parasite development in chickens reared under HS conditions. We also conclude that, at the level of infection tested, *E. maxima* induces a systemic downregulation of $CD8^+$ lymphocytes, whereas HS and *E. maxima* infection induce upregulation of $CD4^+$ lymphocytes. Also, a very diverse cytokine response shows indicatives of reduced inflammatory response during concomitant HS and infection, suggesting this to be one of the mechanisms resulting in reduced sexual replication of *E. maxima* in heat stressed chickens.

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AUTHOR CONTRIBUTIONS

Conceptualization: S.E.A, A.L.F, G.H.S; Formal analysis: G.H.S and S.E.A; Resources, validation and methodology: G.H.S, J.C.F, M.C.M, A.L.F, A.F.A.G. and S.E.A; Supervision:

S.E.A. and A.L.F; Investigation: S.E.A, A.L.F, R.R. G.H.S; Writing: G.H.S; Review and Editing: S.E.A, R.R. and G.H.S; Funding and administration: S.E.A.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Table 4.1. Primers used for gene expression analysis of *Eimeria maxima*-specific genes and chicken cytokines in intestinal tissue.

Target	Primer sequences	NCBI accession number	Species
18s	F: 5' CGTAACAAGGTTTCCGTAGGT 3' R: 5' CCCGAACGAATCACAACAATATG 3'	AF027724.1	*E. maxima*
EF-2	F: 5' GGCGCTTCAGGAGAGAATTAG 3' R: 5' TAGATCTCCTCGGGTTCCATC 3'	GO305837	*E. maxima*
GAM 56	F: 5' TGAATGGAGTGACCGCTATTG 3' R: 5' CTGCTGCCCTCAGGTTATG 3'	AY129951.2	*E. maxima*
GAM 82	F: 5' CAGAAGGTACTAACAACCCTGTC 3' R: 5' GGGTTGAGGGTTTAGGGTTTAG 3'	AY179510.2	*E. maxima*
HAP-2	F: 5' GAGATGCGTTGGGAGACATAG 3' R: 5' CACAGTCCTTCTGCGAGTTT 3'	XM_013481675.1	*E. maxima*
IMC-1	F: 5' GAGACACAGGAGAACATCATTCA 3' R: 5' CGGATGACCTCTCAACTTCTTC 3'	GO305988	*E. maxima*
Prot	F: 5' TGCTGCTGCACGAGATTATAG 3' R: 5' TTAGGGAGACGAGGTGTATGT 3'	XM_013477344.1	*E. maxima*
β-Actin	F: 5′ AGACATCAGGGTGTGATGGTTGGT 3′ R: 5′ TCCCAGTTGGTGACAATACCGTGT 3′	NM_205518.1	*G. gallus*
IL-1β	F: 5' GTCAACATCGCCACCTACAA 3' R: 5' CGGTACATACGAGATGGAAACC 3'	NM_204524.1	*G. gallus*
IL-6	F: 5' CGTTTATGGAGAAGACCGTGAG 3' R: 5' GAGGATTGTGCCCGAACTAAA 3'	NM_204628.1	*G. gallus*
IL-10	F: 5' AGCTGAGGGTGAAGTTTGAG 3' R: 5' AACTCATCCAGCAGTTCAGAG 3'	NM_001004414.2	*G. gallus*
NF-κβ-1	F: 5' CCTCAACCTCACTTCCTTACTC 3' R: 5' CTTCAGTGTCCAGTCCTTTGT 3'	NM_205134.1	*G. gallus*
NF-κβ-2	F: 5' GACATTGAGGTGCGGTTCTAT 3' R: 5' GATGGCGTACTGCTTGTGTA 3'	NM_204413.1	*G. gallus*
TGF-β1	F: 5' CGCTGTACAACCAACACAAC 3' R: 5' CGGCCCACGTAGTAAATGAT 3'	NM_001318456.1	*G. gallus*
TGF-β2	F: 5' TTCCCTTCCTCCTCTCTCATC 3' R: 5' GATACTCTGTACCAGCCCTTTG 3'	NM_204413.1	*G. gallus*

TNF-α	F: 5’ TTACAGGAAGGGCAACTCATC 3’ R: 5’ GCGTTGATGCTCTGAAAGATG 3’	NM_204267.1	*G. gallus*

18s: 18S ribosomal RNA; EF-2: elongation factor 2; HAP-2: Hapless 2; IMC-1: inner membrane complex 1; Prot: protamine; IL-1β: Interleukin 1β; IL-6: Interleukin 6; IL-10: Interleukin 10; NF-κβ-1: nuclear factor κβ 1; NF-κβ-2: nuclear factor κβ 2; TGF-β1: transforming growth factor β 1; TGF-β2: transforming growth factor β 2; TNF-α: tumor necrosis factor α; F: forward primer; R: reverse primer.

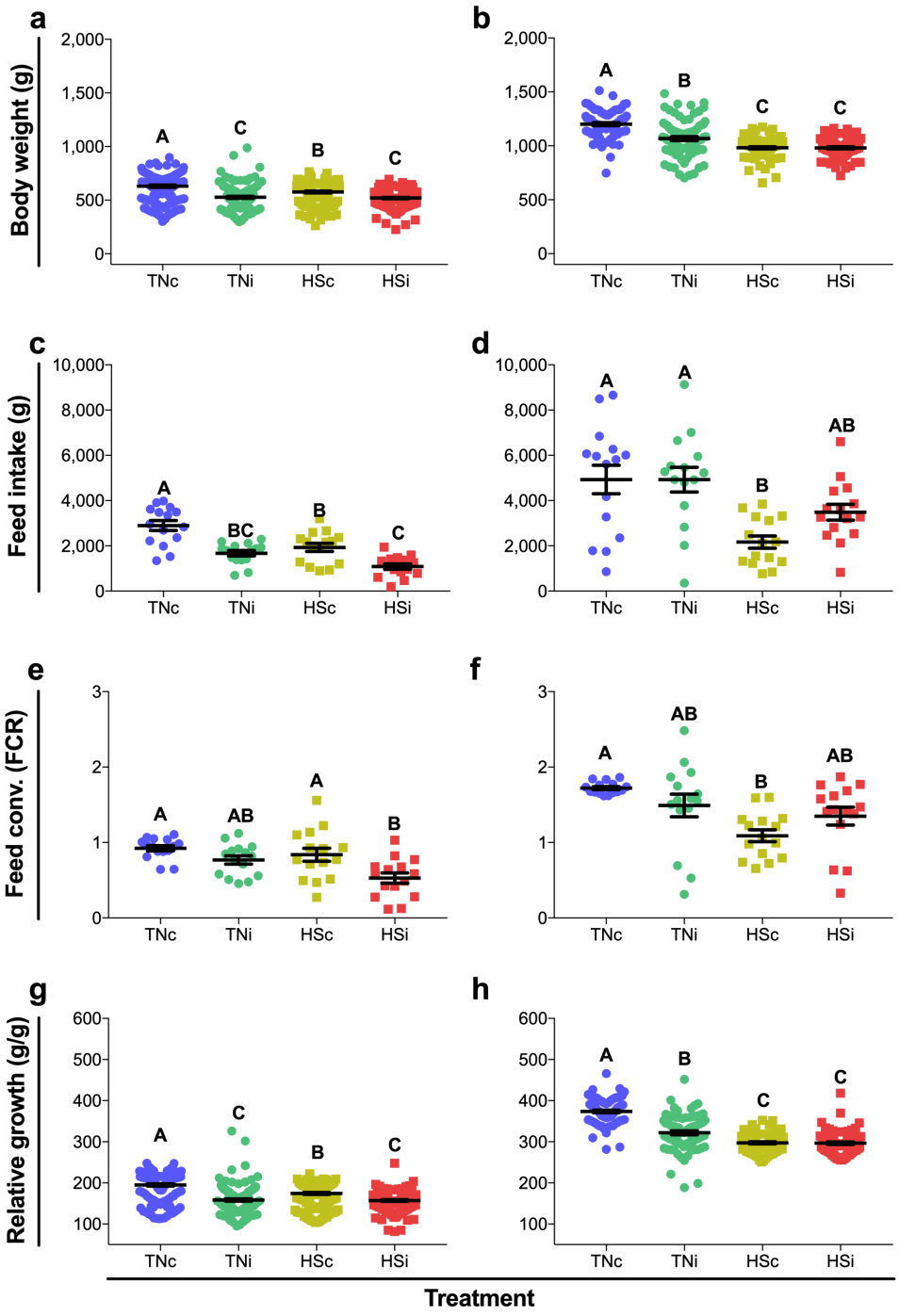

Figure 4.1: Body weight at 7 (a) and 14 dpi (b); feed intake at 7 (c) and 14 dpi (d), feed conversion ratio (FCR) at 7 (e) and 14 dpi (f), and relative growth at 7 (g) and 14 dpi (h). Control chickens were mock infected and housed at thermoneutral (TNc) or heat stress (HSc) environments. 200.000 sporulated oocysts of *E. maxima* were given via gavage to TNi and HSi chickens. Mean and standard error of the mean (SEM) are depicted. Normal data was analyzed by one-way-ANOVA, non-parametric data was analyzed by Kruskal-Wallis. All tests were performed at 5% level of significance ($p < 0.05$). Significant differences between the groups are indicated by different superscript letters.

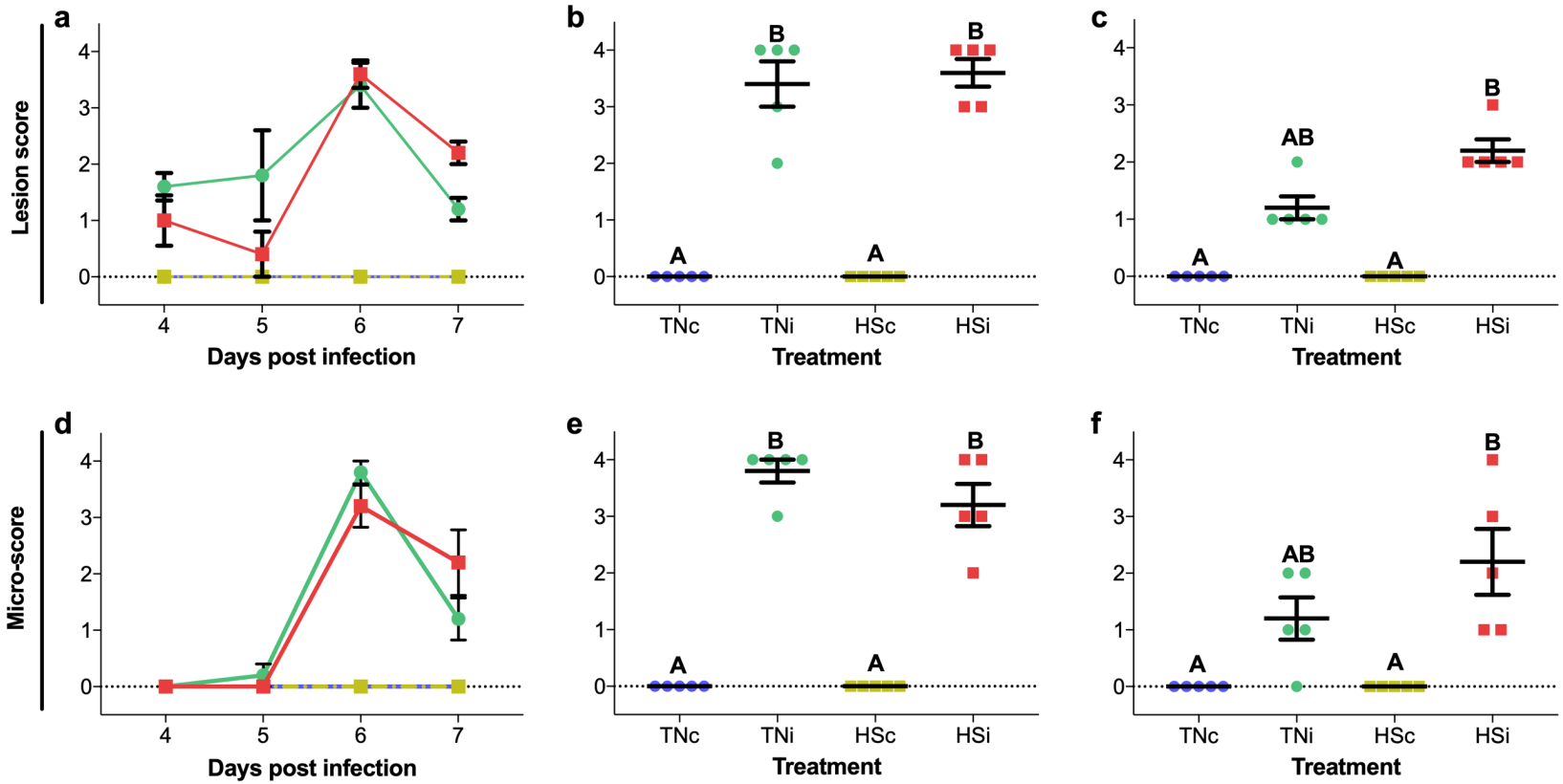

Figure 4.2: Lesion scores from 4 to 7 dpi (a), at 6 dpi (b) and 7 dpi (c). Microscopic scores from 4 to 7 dpi (d), at 6 dpi (e) and 7 dpi (f). of chickens infected with *E. maxima* and housed at thermoneutral (TNi; green) or heat stress (HSi; red) environment, as compared to uninfected chickens housed at thermoneutral (TNc; blue) or heat stress (HSc; yellow) environments, assessed from 4 to 6 dpi. Mean and standard error of the mean (SEM) are depicted. Data were analyzed by Kruskal-Wallis test. All tests were performed at 5% level of significance ($p < 0.05$). Significant differences between the groups are indicated by different superscript letters.

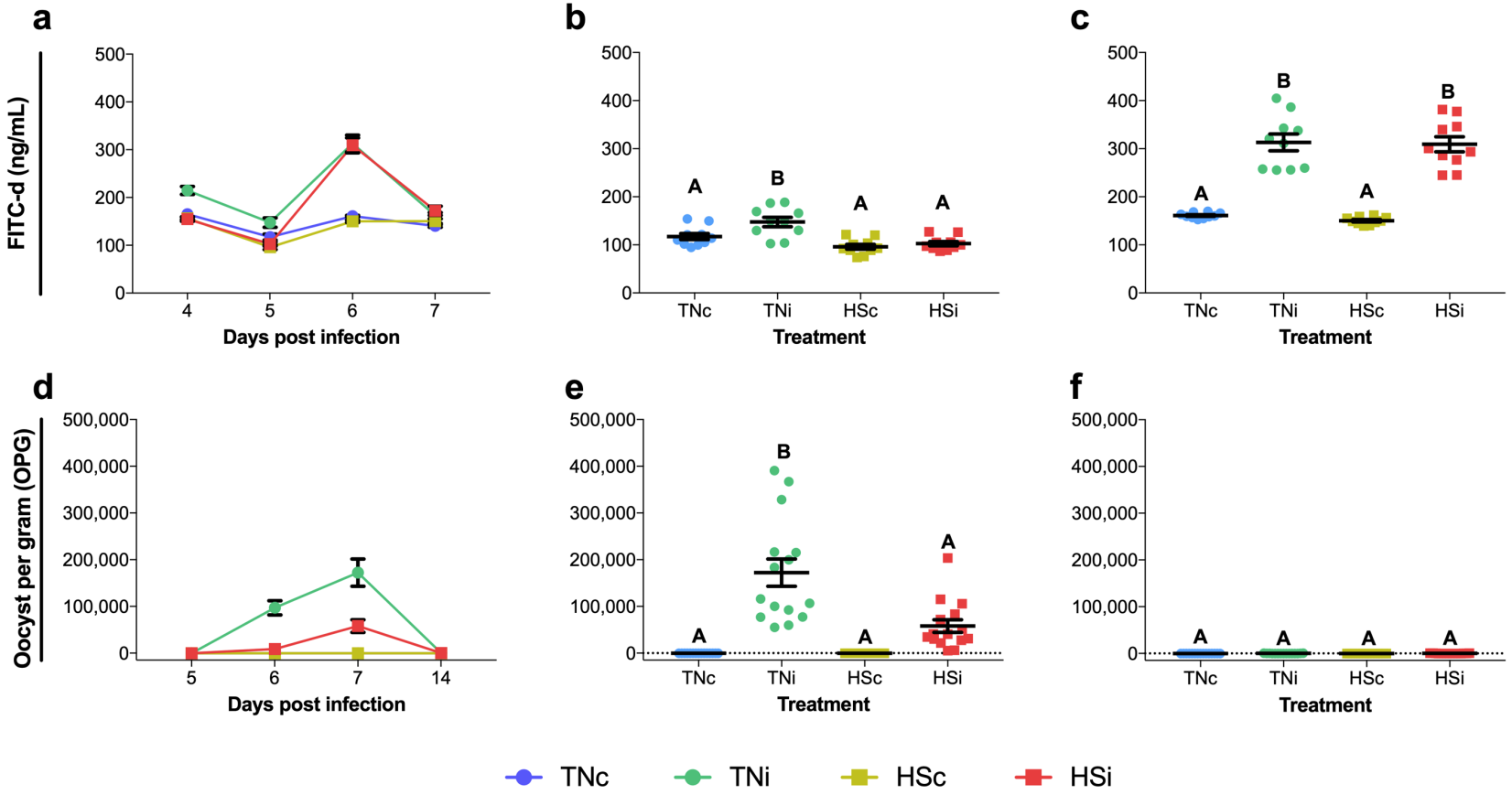

Figure 4.3: Quantification of fluorescein isothiocyanate dextran (FITC-d) in serum samples collected from 4 to 7 dpi (a), at 5 dpi (b) and 6 dpi (c). Oocyst shedding from 5, 6, 7 and 14 dpi (d), at 7 dpi (e) and 14 dpi (f) of chickens infected with *E. maxima* and housed at thermoneutral (TNi; green) or heat stress (HSi; red) environment, as compared to uninfected chickens housed at thermoneutral (TNc; blue) or heat stress (HSc; yellow) environments. Mean and standard error of the mean (SEM) are depicted. Normal data was analyzed by one-way-ANOVA, non-parametric data was analyzed by Kruskal-Wallis. All tests were performed at 5% level of significance ($p < 0.05$). Significant differences between the groups are indicated by different superscript letters.

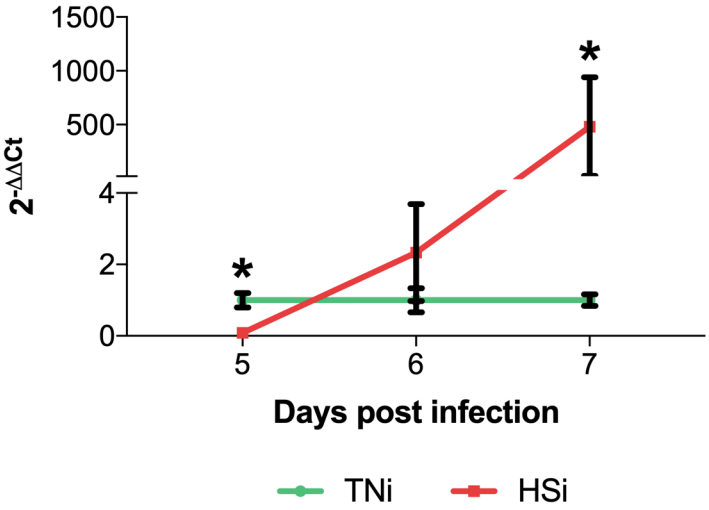

Figure 4.4: Quantification of *Eimeria maxima* mRNA in ileum samples of chickens infected with *E. maxima* and housed at heat stress (HSi; red) environment as compared to infected chickens housed at thermoneutral (TNi; green) environment, assessed by RT-qPCR from 5 to 7 dpi and expressed as $2^{-\Delta\Delta Ct}$. Mean and standard error of the mean (SEM) are depicted. Data were analyzed by GLM procedure with multiple comparisons corrected with Tukey's method at 5% level of significance ($p < 0.05$). Significant differences between the groups within the same day are indicated by the superscript asterisk.

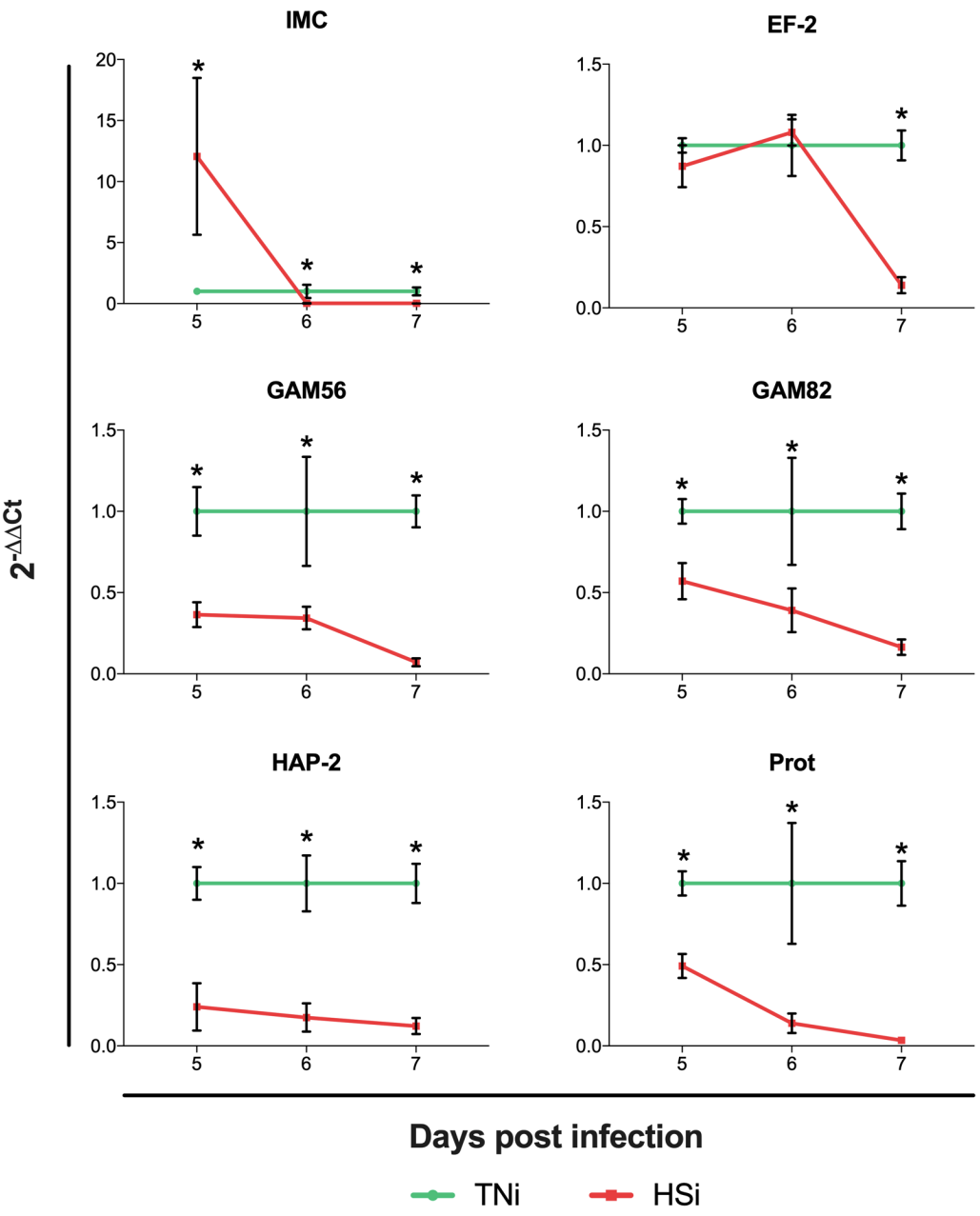

Figure 4.5: *Eimeria* spp. gene expression analysis in ileum samples collected from chickens infected with *E. maxima* and housed at heat stress (HSi; red) environment as compared to infected chickens housed at thermoneutral (TNi; green) environment, assessed by RT-qPCR from 5 to 7 dpi and expressed as $2^{-\Delta\Delta Ct}$. Genes analyzed were inner membrane complex 1 (IMC), elongation factor 2 (EF-2), GAM 56, GAM 82, hapless 2 (HAP-2) and protamine (Prot). Data were analyzed by GLM procedure with multiple comparisons corrected with Tukey's method at 5% level of significance ($p < 0.05$). Mean and standard error of the mean (SEM) are depicted. Significant differences between the groups within the same day are indicated by the superscript asterisk.

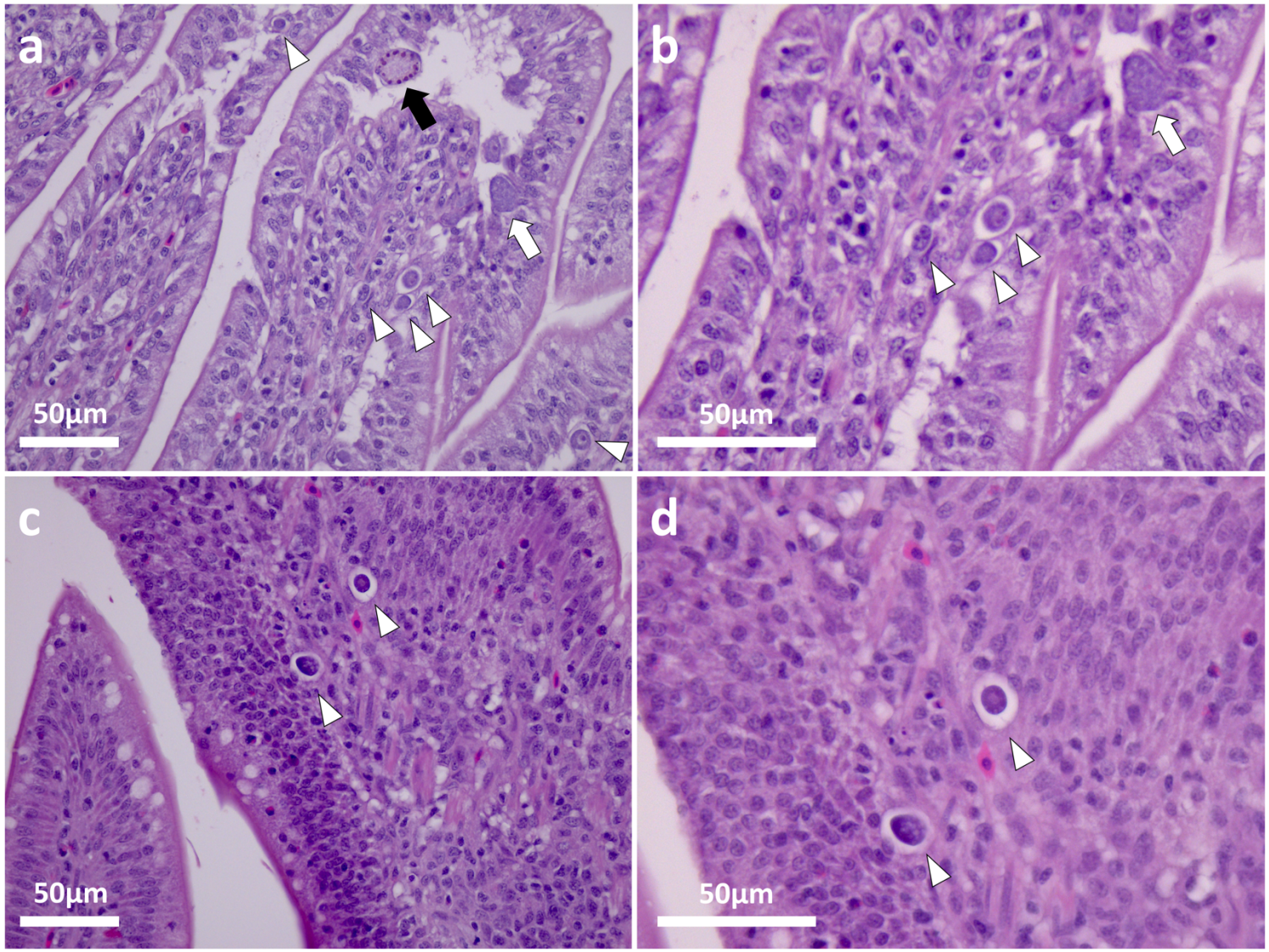

Figure 4.6: Detection of *E. maxima* in histological samples of intestinal sections. Visualization of macrogametocyte (black arrow), microgametocyte (white arrow) and schizonts (white arrowheads) at 7 dpi, at 400x (a) and 630x (b) ileum sections collected from TNi chickens. Visualization of schizonts (white arrowheads) at 7 dpi, at 400x (c) and 630x (d) ileum sections collected from HSi chickens. Tissues were stained with hematoxylin and eosin (H&E), slides analyzed by light microscopy.

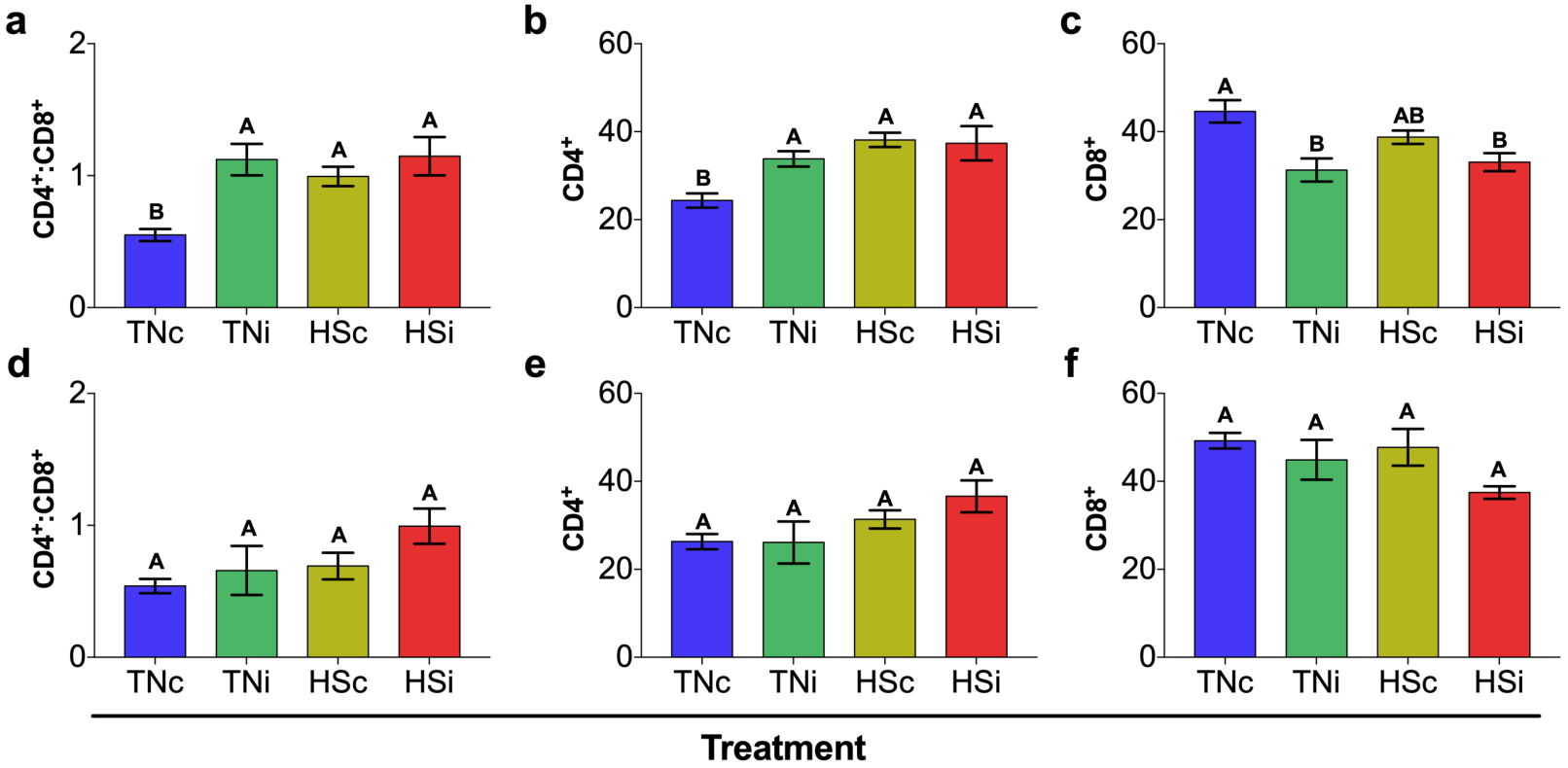

Figure 4.7: T-lymphocyte cell populations and ratio in the spleen of chickens infected with *E. maxima* and housed at thermoneutral (TNi; green) or heat stress (HSi; red) environment, as compared to uninfected chickens housed at thermoneutral (TNc; blue) or heat stress (HSc; yellow) environments. $CD4^+$:$CD8^+$ ratio at 7 dpi (a), $CD4^+$ percentage at 7 dpi (b), $CD8^+$ percentage at 7 dpi (c), $CD4^+$:$CD8^+$ ratio at 14 dpi (d), $CD4^+$ percentage at 14 dpi (e), $CD8^+$ percentage at 14 dpi (f). Data were analyzed by GLM procedure with multiple comparisons corrected with Tukey's method at 5% level of significance ($p < 0.05$). Mean and standard error of the mean (SEM) are depicted. Significant differences between the groups are indicated by different superscript letters.

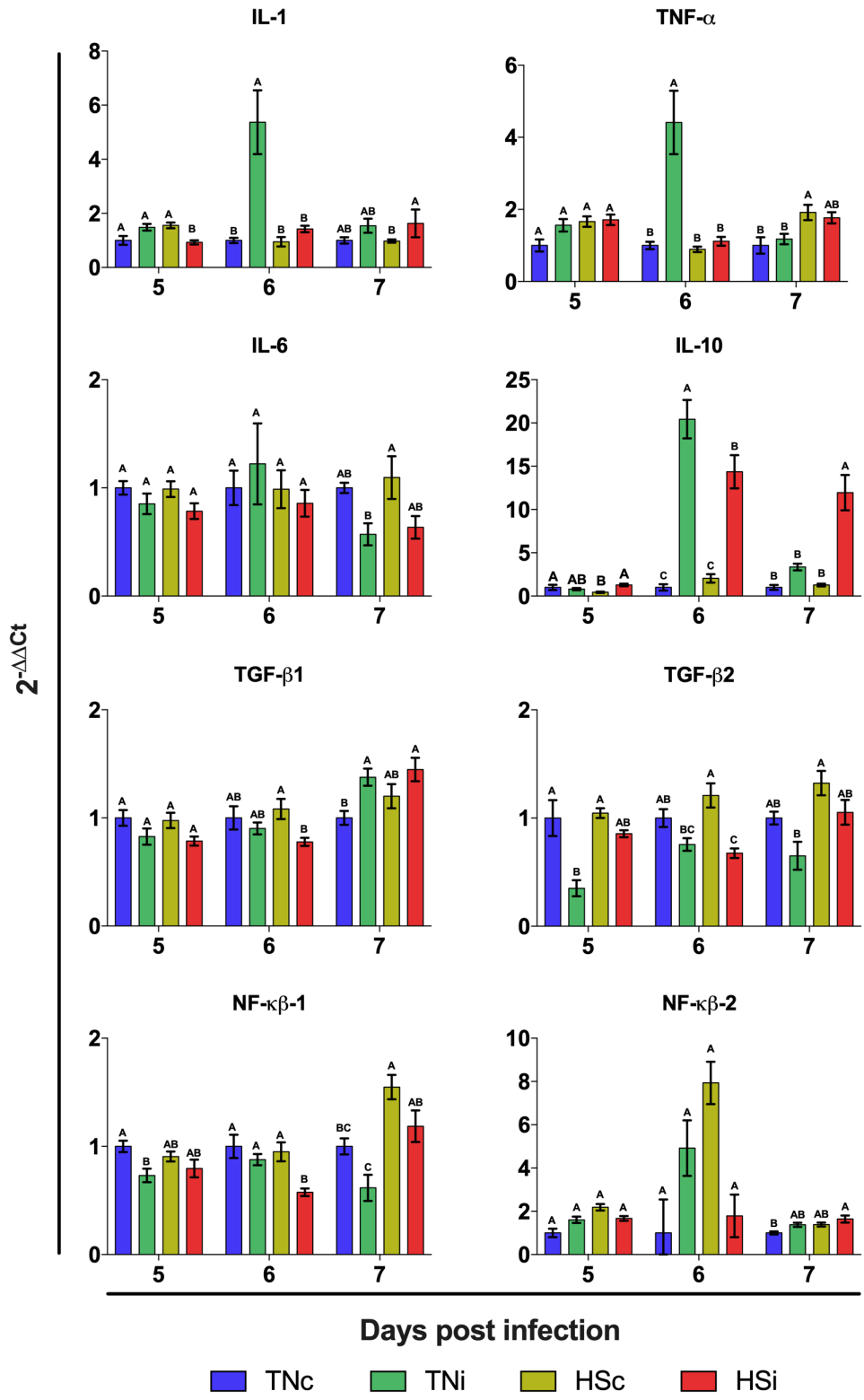

Figure 4.8: Expression analysis of the cytokine genes interleukin 1 (IL-1), tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), interleukin 10 (IL-10), transforming growth factor β 1 (TGF-β1), transforming growth factor β 2 (TGF-β2), nuclear factor κβ 1 (NF-κβ-1), nuclear factor κβ 2 (NF-

κβ-2) in chickens infected with *E. maxima* and housed at thermoneutral (TNi; green) or heat stress (HSi; red) environment, as compared to uninfected chickens housed at thermoneutral (TNc; blue) or heat stress (HSc; yellow) environments, assessed from 5 to 7 dpi. Expression of mRNA was done by RT-qPCR, with results expressed as $2^{-\Delta\Delta Ct}$. Data were analyzed by GLM procedure with multiple comparisons corrected with Tukey's method at 5% level of significance ($p < 0.05$). Mean and standard error of the mean (SEM) are depicted. Significant differences between the groups are indicated by different superscript letters.

CHAPTER 5

ONTOGENY OF INTESTINAL PERMEABILITY IN CHICKENS INFECTED WITH *EIMERIA MAXIMA*: IMPLICATIONS FOR INTESTINAL HEALTH

Gustavo H. Schneiders, James C. Foutz, Marie C. Milfort, Ahmed F. A. Ghareeb, Ufuoma G. Sorhue, Jennifer N. Richter, Alberta L. Fuller, Susan M. Williams, Romdhane Rekaya, Samuel E. Aggrey. Submitted for publication on *Advances in Parasitology.*

ABSTRACT

Commonly found in commercial and backyard poultry productions, Coccidiosis, caused by *Eimeria* spp. presents a self-limiting intestinal infection based on the number of ingested oocysts. Intestinal replication of the parasite causes severe morphological alterations to the host`s gastrointestinal tract (GIT), marked, among others, by the disruption of the intestinal barrier. The objective of this study is to elucidate the dynamics of the intestinal barrier and parasite development, analyzing intestinal permeability elicited by *Eimeria* (*E.*) *maxima* infection and the ontogeny of lesion development throughout the first 10 days of infection. We herein demonstrate that infection with *E. maxima* leads to disruption of the intestinal barrier peaking at 6 days post infection (dpi). *E. maxima* downregulates mRNA expression of occludin and upregulates claudin-1 and JAM-2, indicating vasodilation, altered ion influx and intensive cell replication. This study also presents strong indications of a second peak of *E. maxima* replication following oocyst shedding, without reinfection.

KEYWORDS

Intestinal barrier failure, coccidiosis, poultry, Eimeria, tight junctions.

INTRODUCTION

Coccidiosis is the most important infectious disease in the current poultry production systems, causing significant economic losses for the industry. The disease is caused by Apicomplexan parasites of the genus *Eimeria* spp. The coccidia`s life cycle is comprised of endogenous intracellular asexual replication followed by sexual development [1]. *Eimeria* (*E.*) *maxima* is one of the seven recognized species of coccidia that infect the chicken. The disease is

marked by reduced growth, apathy, diarrhea and sometimes mortality. There is often emaciation, pallor, roughening of feathers and anorexia. Abundance of yellow-orange mucus and fluid in the distal portion of the jejunum and proximal portion of the ileum, edema, thickening and disruption of the mucosa and sometimes presence of blood in the intestinal lumen are observed at necropsy [2].

Due to the complex life cycle of *Eimeria* spp., it is not surprising that several components of the immune system play a role in mitigating the infection. While specific mechanisms such as cellular immunity, especially $CD8^+$, are the most effective against coccidiosis [3-5], non-specific barriers are of fundamental importance, especially in the early days in the course of infection and in the development of cellular immunity [6,7]. Non-specific factors include physical barriers, phagocytes, leukocytes and the complement system. Intestinal forms of the parasite such as meronts, gamonts and oocysts, cause severe morphological alterations to the host`s gastrointestinal tract (GIT), marked by the disruption of the intestinal barrier, distortion, rupture, segregation of adjacent cells, edema, cellular infiltration, sloughing of intestinal epithelia, resulting in the clinical signs observed in the disease [2,5,8].

Tight junctions (TJ) are multi-protein complexes responsible for the regulation of permeability in the intestine via the modulation of its proteins [9], by sealing the paracellular space between adjacent epithelial cells [10,11]. The main clinical sign of *E. bovis* infection in cattle is diarrhea, linked to the downregulation of TJ gene expression [12]. Fluorescein isothiocyanate dextran (FITC-d) is a 3000-5000 Da molecular weight and does not cross intact intestinal blood barrier in high quantities unless the intestinal barrier is compromised [13]. Oral administration of FITC-d has been broadly used to assess the disruption of the intestinal barrier in chickens by measuring intestinal permeability [5,14]. Similarly, the differential expression of TJ proteins such as zona

occludens-1 protein (ZO-1), occludin, claudin 1 and junction adhesion molecule 2 (JAM-2) have been used to assess intestinal permeability in chickens [15,16], however producing conflicting results.

The objective of this study is to elucidate the dynamics of the intestinal barrier and parasite development, analyzing intestinal permeability elicited by *E. maxima* infection and the ontogeny of lesion development throughout the first 10 days of infection, as well as investigating possible biomarkers for intestinal lesion in coccidiosis poultry models.

MATERIAL AND METHODS

All experiments conducted in this study were performed under the Animal Use Proposal (AUP) A2015 04-005 approved by the Animal Care and Use Committee (IACUC) of the University of Georgia.

Single oocyst cloning

Freshly sporulated *E. maxima* oocysts from a North Carolina field strain were counted suspended in PBS at a concentration of one 500 oocysts/mL. Single oocysts were observed in 2µL droplets under light microscopy and collected using a pipette. Twelve 14 days old-broilers kept in individual disinfected isolators were infected with one oocyst suspended in 200µL of PBS via gavage. Fecal matter was collected from each isolator at 7 days post infection (dpi) and analyzed for the presence of oocysts by salt flotation. One volume of feces was solubilized in 9 volumes of saturated salt solution and the supernatant used to verify for the presence of oocysts in a McMaster chamber. Oocysts recovered from one of the chickens were sporulated as previously reported [17], and used to infect a second (p2) and third (p3) passages in chickens. The oocysts from the p3 were

later sporulated and used for the experimental infections of chickens. The purity of the *E. maxima* clone was verified by PCR following published protocol [18].

Experimental design

One hundred and ten 14-days old Ross708 broiler chickens were infected via gavage with $2x10^5$ *E. maxima* sporulated oocysts suspended in water. Another 110 chickens were mock infected with water (control). Five birds each from the infected and control groups were sampled daily for 10 days. On each day the sampled chickens were gavaged with FITC-d for the assessment of intestinal permeability. Chickens were euthanized by cervical dislocation, followed by collection of ileum samples and assessment of lesion scores, microscopic lesion score and body weight. Throughout the course of the experiment, chickens were kept at 25˚C in batteries with wired floor, fed with broiler grower feed, with *ad libitum* access to water. Feces from underneath the cages were collected daily for the assessment of oocyst shedding by salt floatation.

Intestinal permeability

Briefly, FITC-d was gavaged to the sampled chickens at 2.2mg of FITC-d/bird. Two hours post administrations, blood was collected from the jugular vein and stored in tubes protected from the light and kept at room temperature for 3 hours to allow clotting, following with centrifugation (1000g for 15 min) to separate serum. Fluorescence levels of diluted serum (1:1 in PBS) were measured at an excitation wavelength of 485 nm and emission of 528 nm. FITC-d concentration per mL of serum was calculated based on a standard curve. Serum FITC-d levels were compared across infected and non-infected (control) chickens.

Intestinal lesion scores

Eimeria maxima specific lesions were blindly scored daily according to Johnson and Reid [19]. Lesion scores ranged from 0 to 4, where 0 indicates no gross lesion scores, 1 indicates small amounts of orange mucus, 2 represents mid-intestine filled with orange mucus; 3, ballooned thickened intestines with caseous-looking content; and 4 indicates ballooned intestines with blood clots. For microscopic lesion scores, a 2.5 cm long portion of the jejunum, proximal to the Meckel's diverticulum was opened and the mucosa scraped with a dissecting knife. The scraping was placed on a slide and covered with a slide. 20 fields were examined at 100x magnification and scored as follows: 0 (no oocyst present), 1 (1-10 oocysts per field), 2 (11-20 oocysts per field), 3 (21-30 oocysts per field), and 4 (more than 30 oocysts per field).

Oocyst counts

Pooled fecal samples were homogenized and 5g of feces were dissolved in 50 mL of saturated salt solution, with 5 replicates per treatment. The solution was filtered in a coarse strainer to remove big particles. 1.5 mL of the filtered homogenized suspension was used to count *E. maxima* oocysts in a Macmaster chamber and expressed in oocysts per gram (opg).

Nucleic acid extraction

Tissue samples were stored in liquid nitrogen immediately after collection and at -86˚C. DNA was extracted using quick DNA Miniprep Kit (Zymo, Irvine, CA) following manufacturer`s guidelines, and stored at -86˚C. RNA was extracted using trizol. In brief, 100mg of frozen tissue were homogenized with 1mL of trizol, followed by phase separation with 0.2mL of chloroform, homogenization, incubation at room temperature (RT) for 3 minutes, centrifugation (12000 rpm,

15 min, 4˚C) and transferring 550µL of the aqueous phase into a tube for RNA precipitation with 0.5mL of isopropanol, homogenization and incubation (RT, 10 min), centrifugation (12000 rpm, 10 min, 4˚C), removal of the supernatant and wash of the pellet with 1mL of 75% ethanol, centrifugation (12,000 rpm, 5 min, 4˚C), removal of the supernatant, incubation (RT, 10 min), and dissolution of the RNA in 100µL of RNase free water followed by incubation (55-60˚C, 10 min) and storage of RNA at -80˚C for downstream applications. RNA was cleaned using RNeasy Mini Kit (Qiagen, Hilden, Germany), following manufacturer`s guidelines, and later reverse-transcribed high capacity cDNA reverse transcription Kit (Thermo Fischer Scientific, Waltham, MA).

qPCR

Real-time PCR reactions were performed on occludin, claudin-1, JAM2, ZO1 (Table 5.1) in a Step One Plus Real-time PCR machine (Applied Biosystems, Foster City, CA), in a 20µL reaction using 8.4µL of DNase-RNase free water, 0.3µL of each forward and reverse primer, 10µL of PowerUp SYBR Green Master Mix (Thermo Fischer Scientific, Waltham, MA) and 1µL of template DNA. True positive and negative controls for the reaction were included. The samples were tested in triplicates, and the relative amount of *E. maxima* DNA detected per sample was quantified using the $2^{-\Delta\Delta ct}$ method [20]. The parasite load per sample was calculated from the amount of the *E. maxima* DNA (target) normalized to the amount of chicken β-actin (endogenous control). Cycling conditions were 50˚C for 2 min, 95˚C for 2 min, followed by 40 cycles at 95˚C for 15 seconds and 60˚C for 1 min and final melt curve of 95˚C for 15 seconds and 60˚C for 1 min.

Histological analysis

Ileum samples were collected in a daily basis, fixed in 10% buffered formalin for 24 hours or more, processed, embedded in paraffin and 5µm sections were cut and stained with hematoxylin and eosin (H&E) and examined microscopically for the presence of *Eimeria maxima* developmental stages.

Statistical analysis

Statistical analysis and figures were generated with Prism 7.0 software (Graphpad Software Inc., San Diego, California). D'Agostino and Pearson test was applied to determine normality and determined whether data should be analyzed by parametric or non-parametric tests. Kruskal-Wallis test with multiple comparisons corrected using Dunn's method was used to compare non-parametric data. Parametric data were analyzed by one-way-ANOVA test with multiple comparison's corrected by Bonferroni's method. All analyses were tested at the 5% level of significance.

RESULTS

Body weight changes

The presentation of disease was confirmed by the clinical signs shown by the chickens, consisting of apathy, aqueous diarrhea, reduced feed intake and dehydration. Daily body weight is shown in Figure 5.1a. While there are no significant changes in body weight between infected and control birds from 1 to 6 dpi ($p \geq 0.07$), infection with *E. maxima* significantly reduced body weight at 7 (522.1 ± 119.8g vs 663.7 ± 69.5g; $p = 0.0010$) and 8 dpi (509.8 ± 144.2 vs 800.8 ± 135.7g; $p < 0.0001$). Although infected chickens presented lower body weight at 9 dpi (696.4g ±

79.7g) as compared to the control counterparts (783.5 ± 157.2g), this difference was not statistically significant ($p = 0.1583$). At 10 dpi, infected chickens presented significant lower weight gain (730.3 ± 129.8g) as compared to uninfected controls (840.4 ± 167.5g; $p = 0.0022$).

The peak of intestinal lesions precedes oocyst shedding

Uninfected chickens did not shed oocysts throughout the experiment. While there was no shedding of oocysts from infected chickens from 1 to 5 dpi. Comparing to the absence of oocyst shedding from the control groups, shedding was significantly higher at 6 dpi (15753.4 ± 9083.0 opg; $p = 0.0006$) and 7 dpi ($p < 0.0001$), with an average of 64,068.46 ± 38,635.06 opg at the peak of shedding (7 dpi; Figure 5.1b). Although infected chickens shed oocysts 8 dpi (7,434.09 ± 5,529.37; $p = 0.5435$), 9 dpi (424.46 ± 1342.10; $p > 0.9999$) and 10 dpi (967.20 ± 2805.91; $p > 0.9999$), this was non-statistically significant as compared to zero ($p = 0.5435$; Figure 5.1b). *E. maxima*-specific intestinal lesions were scored and results are depicted in Figure 5.1c. While uninfected control chickens did not present lesions characteristic of infection (score 0) throughout the experiment, infected chickens presented significant higher lesion scores from 4 to 7 dpi ($p \leq 0.0116$), with peak at 6 dpi ($p < 0.0001$) and respective average score of 3.6 ± 0.55. Following the same trend, micro-scores, marked by the quantification of *E. maxima* developmental stages in intestinal scrapings (Figure 5.1d), where significantly higher in infected chickens from 2 to 7 dpi ($p \leq 0.0161$), with peak at 6 dpi and respective average lesion score of 4 ± 0 ($p < 0.0001$). Micro-scores where not statistically different at 8 and 9 dpi ($p > 0.9999$), however, infected chickens presented a significantly higher micro-score at 10 dpi (0.8 ± 0.84; $p= 0.0161$). Contrarily, no stages of *E. maxima* were found in intestinal scrapings from uninfected control chickens (micro-score of 0) throughout the experiment.

Eimeria maxima presents two peaks of intestinal replication

The quantification of *E. maxima* DNA in ileum sections was performed through qPCR, and normalized by the presence of chicken β-actin DNA in the sample. Results, expressed as $\log_{10}^{2^{-\Delta\Delta Ct}}$ are expressed in Figure 5.2. While there was no detection of *E. maxima* DNA in samples from uninfected control chickens, parasite load in the samples started to increase at 3 dpi, and was significantly higher from 4 to 7 dpi ($p < 0.0001$) with peak at 7 dpi ($p < 0.0001$) and respective average load of $\text{Log}_{10}3.27 \pm 0.62$. The presence of *E. maxima* DNA was not statistically significant at 8 dpi in infected chickens as compared to uninfected controls ($\text{Log}_{10}0.775 \pm 1.00$ vs $\text{Log}_{10}6.67\text{x}10^{-11} \pm 0.13$; $p = 0.0972$). However, there was another significant increase in *Eimeria* load starting at 9 dpi ($\text{Log}_{10}1.83 \pm 0.94$ vs $\text{Log}_{10}6.67\text{x}10^{-11} \pm 0.11$; $p < 0.0001$) and continuing at 10 dpi ($\text{Log}_{10}2.58 \pm 0.68$ vs $\text{Log}_{10}\text{-}0.123 \pm 0.06$; $p < 0.0001$).

Histological sections reveal continued replication in the small intestine following oocyst shedding

Sections of the small intestine were fixed, stained by H&E and analyzed microscopically (Figures 5.3 and 5.4). There was no histological evidence of coccidia infection in sections from uninfected control chickens throughout the experiment. The first developmental stages of *E. maxima* were detected in intestinal tissue samples from infected chickens at 4 dpi. These were accompanied by the histological signs of coccidial enteritis, characterized by GALT hyperplasia in the lamina propria (Figure 5.3a). At 5 dpi, sexual stages of *E. maxima* were first observed in moderate numbers (Figure 5.3b). At 6 dpi moderate to severe numbers of *E. maxima* developmental stages were observed, including oocysts and macrogametocytes (Figure 5.3c). At 7 dpi, small intestines presented marked multifocal corrosive enteritis with hemorrhage, and the

presence of oocysts and sexual stages of *E. maxima* were observed in rare to moderate numbers (Figure 5.3d). At 8 (Figure 5.4a), 9 (Figure 5.4b) and 10 (Figure 5.4c) dpi, sections evidenced the presence of sexual stages of development of *E. maxima* in mild to moderate numbers.

Disruption of the intestinal barrier

The assessment of intestinal barrier disruption was done through presence of FITC-d in the serum, and results are shown in Figure 5.5. Intestinal permeability was significantly higher at 5 and 6 dpi ($p < 0.0001$). At 6 dpi, the peak of intestinal barrier disruption, the presence of FITC-d in the serum of infected chickens averaged 433.47± 147.43 ng/mL, significantly higher as compared to the average of FITC-d in the serum of uninfected controls (339.38 ± 34.15 ng/mL; $p < 0.0001$). The concentration of FITC-d in the serum of infected chickens was not significantly different as compared to the control group at 7 to 10 dpi ($p \geq 0.7391$).

The expression of TJ genes was assessed from 2 to 7 dpi (Figure 6). The expression of JAM-2 (Figure 5.6a) was not statistically significant from 2 to 5 dpi ($p > 0.9999$) and at 7 dpi ($p = 0.8369$), however, at 6 dpi, infected chickens presented significantly higher mean mRNA expression of JAM-2 ($Log_{10}0.27 \pm 0.06$; $p < 0.0001$) as compared to the mean expression of uninfected chickens ($Log_{10}1.33x10^{-10} \pm 0.08$). While the expression of claudin-1 was non-statistically significant altered from 2 to 3 dpi ($p \geq 0.0598$), it was significantly higher from 4 to 7 dpi ($p \leq 0.0018$), with peak at 6 dpi ($Log_{10}1.03 \pm 0.17$; $p < 0.0001$), as compared to control chickens (zero). Changes in the mRNA expression of occludin were not significant at 2 to 3 ($p \geq 0.1151$) and at 6 dpi ($p > 0.9999$), however, the expression of occludin was significantly reduced in infected chickens at 4 (Log_{10}-0.18 ± 0.13 vs $Log_{10}6.67x10^{-11} \pm 0.07$; $p = 0.0003$), 5 (Log_{10}-0.13 ± 0.11 vs $Log_{10}6.67x10^{-11} \pm 0.09$; $p = 0.0261$) and 7 dpi (Log_{10}-0.13 ± 0.11; $p = 0.0229$), as compared to the

control counterparts. While the expression of ZO-1 gene was slightly elevated at 2 dpi in infected chickens ($Log_{10}0.13 \pm 0.23$ vs. $Log_{10}-1.89x10^{-18} \pm 0.10$; $p = 0.0031$), there were no statistically significant differences between the groups from 3 to 6 dpi ($p \geq 0.1301$). However, there was a significantly lower expression of ZO-1 mRNA in infected chickens at 7 dpi ($Log_{10}-0.197 \pm 0.07$; $p < 0.0001$) as compared to the control group ($Log_{10}6.67x10^{-11} \pm 0.09$).

DISCUSSION

The first line of defense against *Eimeria* spp. is provided by the infected epithelial cells and the cells in close contact with them, such as intra-epithelial lymphocytes and fibroblasts [21]. The maintenance of intestinal integrity is a fundamental aspect for animal health and productivity, since it functions as a nutrient absorption system and as a barrier, preventing systemic infections [22], therefore preventing the incidence of enteric diseases, improving performance and reducing mortality [23]. In poultry, *Eimeria* spp. replicates in the intestine causing extensive tissue damage. *E. maxima* causes congestion and edema, cellular infiltration, thickening of the mucosa, with the later generations of schizonts and sexual stages developing deeper in the tissues, causing considerable disruption of the mucosa [2]. The current study was undertaken to elucidate the dynamics of intestinal barrier disruption as well as parasite development during the course of infection of *E. maxima* in broiler chickens.

The minimum prepatent period for *E. maxima* reported is between 5 [24] and 6 dpi [25], with typical peak of oocyst shedding at 7 dpi [26]. The strain of *E. maxima* used on this study has a prepatent period of 6 dpi and peak of oocyst shedding in the feces at 7 dpi, corroborating with previous reports in the literature. The development of *E. maxima* within the host was assessed at the histological level in sections of the small intestine. The presence of oocysts and sexual stages

of the parasite peaked at 6 dpi in the intestinal wall, and at 7 dpi in the feces. Only mild to moderate numbers of sexual stages of the parasite were observed at 7 to 10 dpi.

Previous studies have reported the sequential decay in oocyst shedding following 7 dpi with *E. maxima* [26-28]. Although non-statistically significant, we observed that oocyst shedding showed a small trend to resume sequential increase at 9 and 10 dpi, indicating a possible restart in oocyst production. This phenomena has not been reported in previous studies, but the continuous presence of oocysts in the intestinal wall following the patent period has been reported and suggested to play some part in the crowding effect of *E. maxima* [29]. In the current study, there was a significant increase of developmental stages in intestinal scrapings at 10 dpi and in the detection of *E. maxima* DNA concentration in ileum sections at 9 and 10 dpi, corroborating the oocyst shedding. Altogether, these data indicate the possible recommence of the infection cycle of *E. maxima* following the first round of oocyst shedding in chickens without reinfection. A study conducted by Dubey and Jenkins [30] describes the formation of fifth generation schizonts at 5 dpi, after the formation of gamonts, supporting the hypothesis that *E. maxima* can continue its replication past the first round of oocyst shedding without reinfection. The possibility of reinfection in this study was minimized be the housing of chickens in wired floor cages and daily removal of waste.

Eimeria spp. is entirely dependent on the survival and continued existence of the parasitized epithelial cell for a definite length of time, therefore, changes in the cell population kinetics of the intestinal epithelium, associated with other morphological changes, are important factors in the outcome of infection [31]. Coccidia infection causes villous atrophy, which leads to reduction of functional epithelial cells available for parasite development [32,33]. Similar changes are believed to occur during *E. maxima* infection, raising the possibility that developmental stages

reminiscent in the intestine could take advantage of the compensatory growth of new epithelial cells to resume their development, giving rise to the second peak of replication. The reduction in cell availability has been considered as a factor equally important, if not more, than the crowding effect in the self-limiting nature of coccidiosis in poultry [29]. Further studies should address the importance of the infectious dose in the continued replication of *E. maxima*.

Integrity of the intestinal barrier has been traditionally assessed *in vivo* by the presence of FITC-d in the serum of chickens following gavage administration of the substance suspended in water. Studies utilizing gene expression as biomarkers to determine intestinal integrity demonstrate a high level of variability in which genes are reliable indicators of intestinal barrier dysfunction, therefore FITC-d administration continues to be the primary marker for intestinal barrier integrity in chickens [16,34]. The peak of damage to the intestinal epithelia reported here was at 6 dpi, as demonstrated by the circulatory levels of FITC-d. This correlates with the highest presence of *E. maxima* sexual stages and oocyst maturation in the epithelia demonstrated by histology and qPCR. Sexual stages of *E. maxima* develop in deeper tissues between 5-8 dpi, an in severe infections cause considerable disruption of the mucosa [2]. Based on the quantification of FITC-d in the serum, the intestinal wall shows the first signs of repair at 7 dpi, prior to the possible relapse of infection.

Reduced intestinal barrier function can result in increased bacterial translocation and inflammatory responses, bacterial chondonecrosis with osteomyelitis, and necrotic enteritis [35-39]. Tight junctions (TJ) are multi-protein complexes which are crucial for the integrity and function of the epithelial barrier, as they exert a dual function linking cells and forming channels that allow permeation between cells, resulting in epithelial surfaces with different tightness [40].

JAM-2 is primarily expressed in endothelial cells, where it plays a dual role in the adhesion and transmigration of lymphocytes and regulation of vascular permeability [41]. It has also been reported that permeability of Mardin-Derby Canine Kidney (MDCK) cells increases upon JAM-2 expression [42], evidencing its role in favoring cellular permeability. Our results show the significant upregulation of JAM-2 mRNA in infected chickens at 6 dpi, demonstrating the augmented vascular permeability caused by *E. maxima* infection. These disagree with previous report in which there was no differential expression of JAM-2 in chickens with intestinal barrier failure as compared to chickens with intact intestinal barrier [16]. However, the concomitant infection by more than one *Eimeria* spp. and utilization of non-starch polysaccharides in the aforementioned study should be considered as possible factors involved on this difference.

Claudin-1 is a member of multiple-span transmembrane proteins called claudins and classified as a "tight" or "sealing claudin", as opposed to the pore forming "leaky" claudins [43]. Upregulation of claudin-1 expression has been observed as a response to inflammation associated with a leaky epithelial barrier [44], where claudin proteins have been proposed to actively contribute to disease pathogenesis. Increase in claudin-1 expression has also been associated with enhanced cellular proliferation [45]. In response to inflammation, altered claudin protein profiles in the TJ are associated with perturbed paracellular movement of fluid and solutes, which is in turn reflected in overall change in epithelial barrier function [43]. This study demonstrates the upregulation of claudin-1 at the peak of intestinal lesions caused by *E. maxima*, indicating a compensatory gene expression in response to the accentuated intestinal permeability.

Occludin is involved in the regulation of inter-membrane diffusion and paracellular diffusion of small molecules [46], and is down-regulated in humans with inflammatory bowel diseases [46,47]. The downregulation of occludin has been reported in the intestinal barrier function

model developed by Chen, et al. [16], by combining infection with *E. acervulina*, *E. maxima* and *E. tenella* with non-starch polysaccharides in broiler chickens. Our results demonstrate no significant variation in occludin mRNA expression at 6 dpi. On the other hand, there is significant downregulation of this gene at 4 and 5 dpi, prior to the peak of *E. maxima* intestinal replication, and again at 7 dpi. This possibly indicates the downregulation of this gene at the lowest levels of parasite replication only. The significant downregulation of occludin at 7 dpi also supports our data demonstrating the recommence of a second cycle of infection. The expression levels of occludin have been shown to be inversely correlated with the translocation of FITC-d from the GIT to the blood [48], and movement occludin from the TJ into the cytoplasmic vesicles occurs frequently during barrier function loss [49].

ZO-1 is a plaque protein that acts as adaptor to connect transmembrane proteins to the perijunctional actomyosin ring [11], and plays a major role in the formation of TJ in epithelial cells [50]. Our results demonstrate minimal differences in mRNA expression of ZO-1 between infected and non-infected chickens, indicating that *E. maxima* infection does not severely affect cytoskeleton rearrangement.

Intestinal health is a crucial factor in animal production, and is closely related to well-being, feed intake and the efficient absorption of nutrients. In poultry, intensive selection for higher weight gain and lower feed conversion ratio has produced breeds that have extremely high feed intake as one of their main characteristics. Among other factors, intestinal infection caused by *Eimeria* spp. severely damages the intestinal integrity, leading to intestinal lesions, malabsorption, secondary infections, and dysbiosis. We herein show that infection of chickens with *E. maxima* results in the differential expression of TJ genes resulting in increased vascular permeability and cellular influx. Changes in the molecular structure of the junctional complexes results in decreased

absorption of nutrients, increased secretory passage of ions and water, causing leak flux diarrhea and augmented passage of macromolecules from the lumen, inducing intestinal inflammation [51]. The differential expression of TJ genes is a valuable tool that can be further developed with the objective of monitoring intestinal health in poultry. It is, however, unlikely that a single biomarker will be sufficient to track all the aspects of intestinal health and deficiencies thereof, hence, combination of multiple biomarkers is the most promising way of tracking intestinal health in a holistic way in the future.

In summary our results indicate that infection with *E. maxima* leads to disruption of the intestinal barrier by downregulation of the expression of occludin and upregulation of claudin-1 and JAM-2. Further studies should address other TJ genes in order to provide a more holistic view of the intestinal barrier dysfunction during coccidiosis. Additional data in the expression of TJ genes should provide deeper understanding of the assessment of efficacy of new methods of therapy and control of coccidiosis in poultry. It is important to consider that other *Eimeria* spp. will likely provide somehow different patterns of dysfunction, based on the cycle and possibly on the infectious dose. We also demonstrate herein a strong indication of a second peak of *E. maxima* replication in the intestines. To our knowledge, this fact has not been previously reported, although previous literature provides strong indicatives of this phenomena. Further research is necessary to prove if intestinal cell availability could be the cause of such observation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Table 5.1. Sequences of real-time PCR primers

Target	Primer sequences	NCBI accession number	Source
β-Actin	F: 5′ AGACATCAGGGTGTGATGGTTGGT 3′ R: 5′ TCCCAGTTGGTGACAATACCGTGT 3′	NM_205518.1	Habashy et al., 2017
Claudin-1	F: 5’ GGATGACCAGGTGAAGAAGATG 3’ R: 5’ GGGTGTGAAAGGGTCATAGAAG 3’	NM_001013611.2	
E. maxima	F: 5’ TCGTTGCATTCGACAGATTC 3’ R: 5’ TAGCGACTGCTCAAGGGTTT 3’	M99058	Vrba et al., 2010
JAM-2	F: 5’ GCAACGCTGACTTCATTCTTAC 3’ R: 5’ CAATGCTTCGGCCTCAATTAC 3’	NM_001006257.1	
Occludin	F: 5’ GAGCCCAGACTACCAAAGCAA 3’ R: 5’ GCTTGATGTGGAAGAGCTTGTTG 3’	NM 205,128	Wang et al., 2018
ZO-1	F: 5’ CACTAGAGGATGAGGAGGAAGA 3’ R: 5’ GGAATGGCTCCTTGTGGTATAA 3’	XM_015278981.2	

JAM-2: Junction adhesion molecule 2; ZO-1: Zona occludens 1; F: forward primer; R: reverse primer.

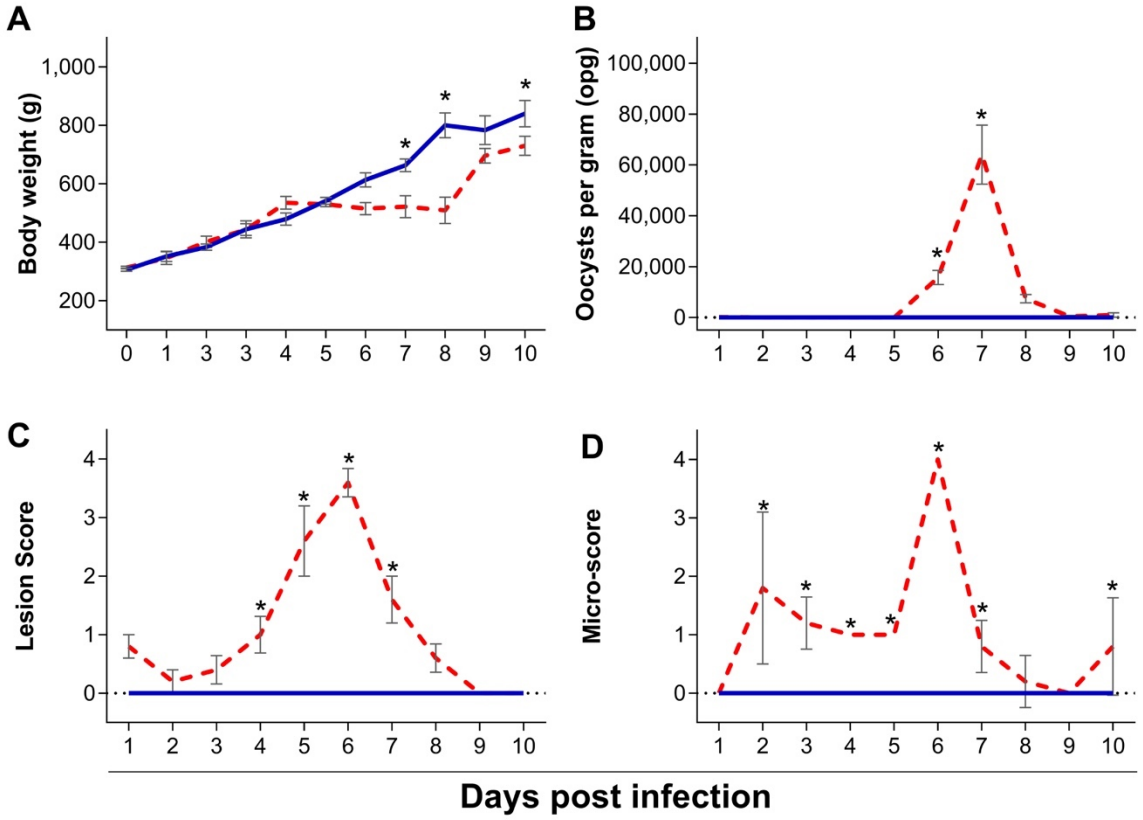

Figure 5.1: Body weight (A), oocyst shedding (B), lesion scores (C) and microscopic scores (D) of chickens infected with *Eimeria maxima* (red dashed line) as compared to uninfected chickens (blue solid line), assessed from 1 to 10 days post infection (dpi). Standard error means (SEM) are depicted by vertical lines. Two-way-ANOVA test was performed at 5% level of significance ($p < 0.05$), with multiple comparisons corrected by Bonferroni's method. Significant differences between the groups are indicated by the superscript asterisk.

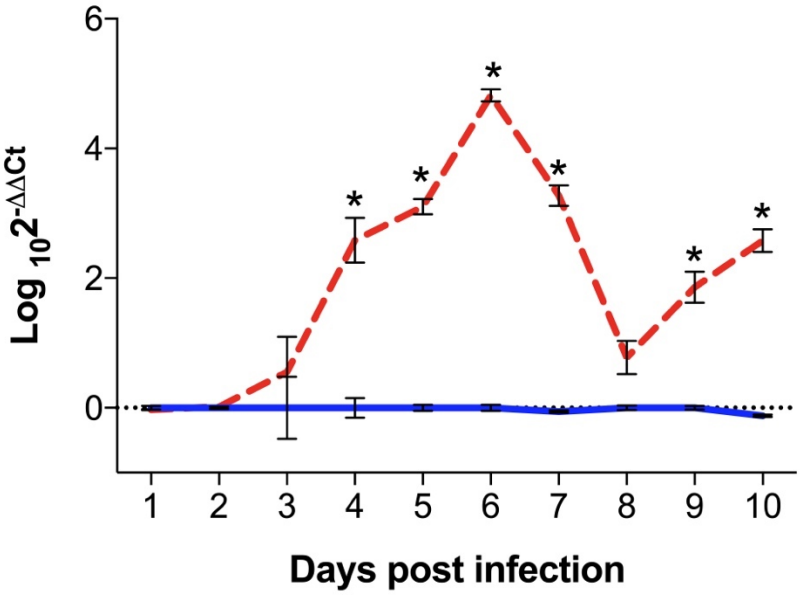

Figure 5.2: Quantification of *Eimeria maxima* DNA in ileum samples of infected (red dashed line) and uninfected (blue solid line) chickens, assessed by qPCR from 1 to 10 days post infection (dpi) and expressed as $Log_{10}2^{-\Delta\Delta Ct}$. Standard error means (SEM) are depicted by vertical lines. Two-way-ANOVA test was performed at 5% level of significance ($p < 0.05$), with multiple comparisons corrected by Bonferroni's method. Significant differences between the groups are indicated by the superscript asterisk.

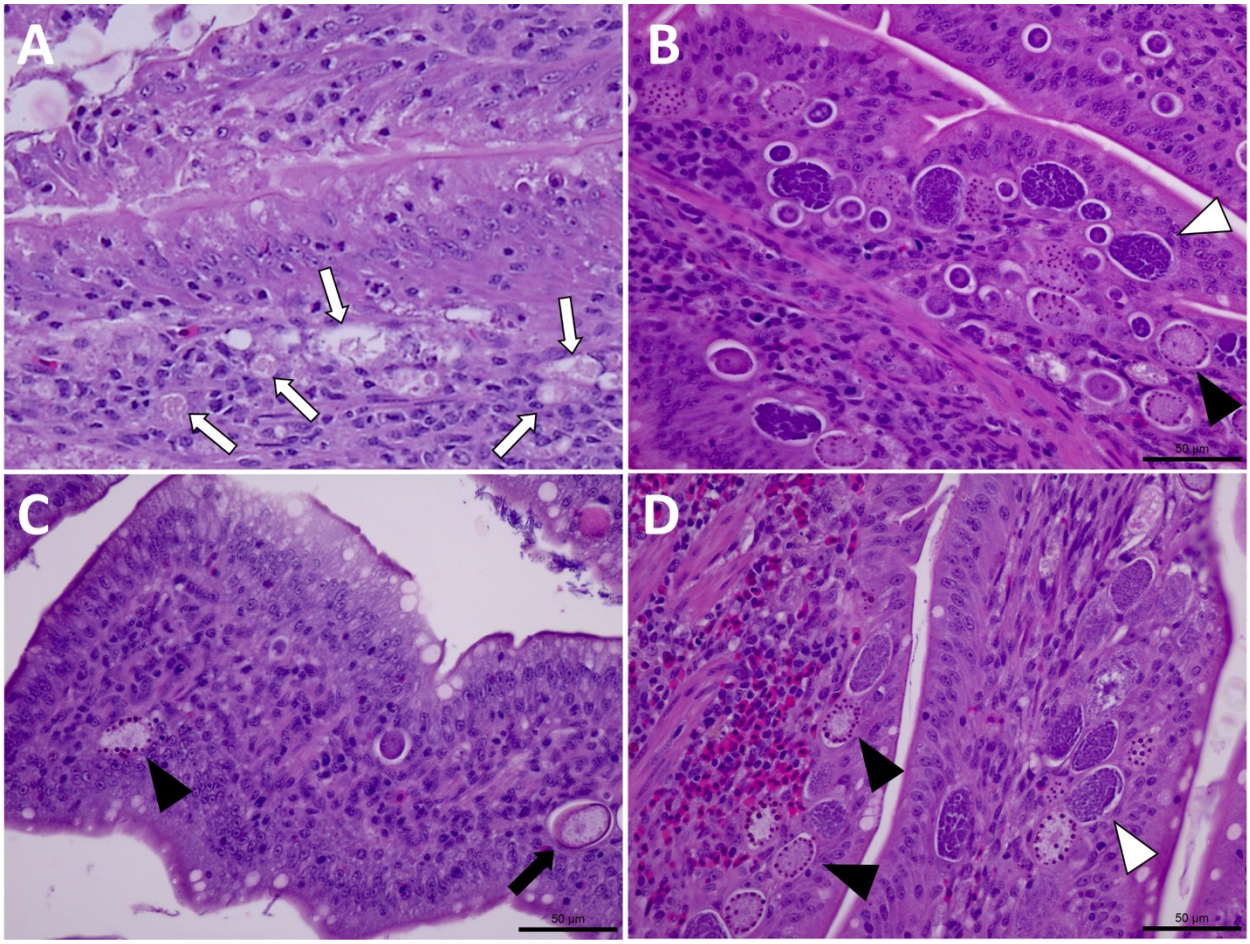

Figure 5.3: Detection of *E. maxima* in histological samples of intestinal sections. (A) visualization of schizonts (white arrows) at 4 dpi, 200x. (B) Visualization of macrogametocytes (black arrowheads) and microgametocytes (white arrowheads) at 5 dpi, 400x. (C) Visualization of macrogametocyte (black arrowhead) and immature oocyst (black arrow) at 6 dpi, 400x. (D) Visualization of macrogametocytes (black arrowheads) and microgametocytes (white arrowheads) at 7 dpi, 400x). Tissues were stained with hematoxylin and eosin (H&E), slides analyzed by light microscopy.

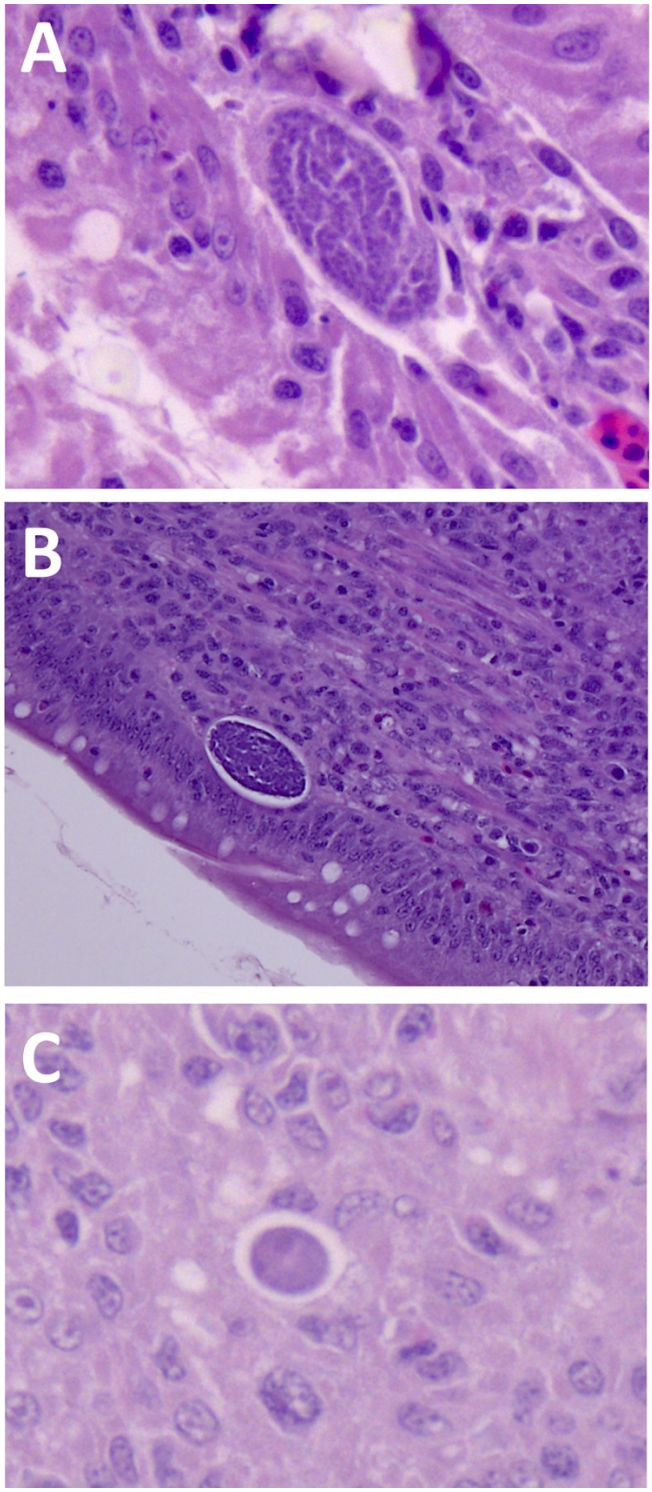

Figure 5.4: Detection of *E. maxima* in histological samples of intestinal sections. (A) visualization of microgametocyte at 8 dpi, 630x. (B) Visualization of microgametocyte at 9 dpi, 400x. (C) Visualization of immature macrogametocyte at 10 dpi, 630x. (D) Tissues were stained with hematoxylin and eosin (H&E), slides analyzed by light microscopy.

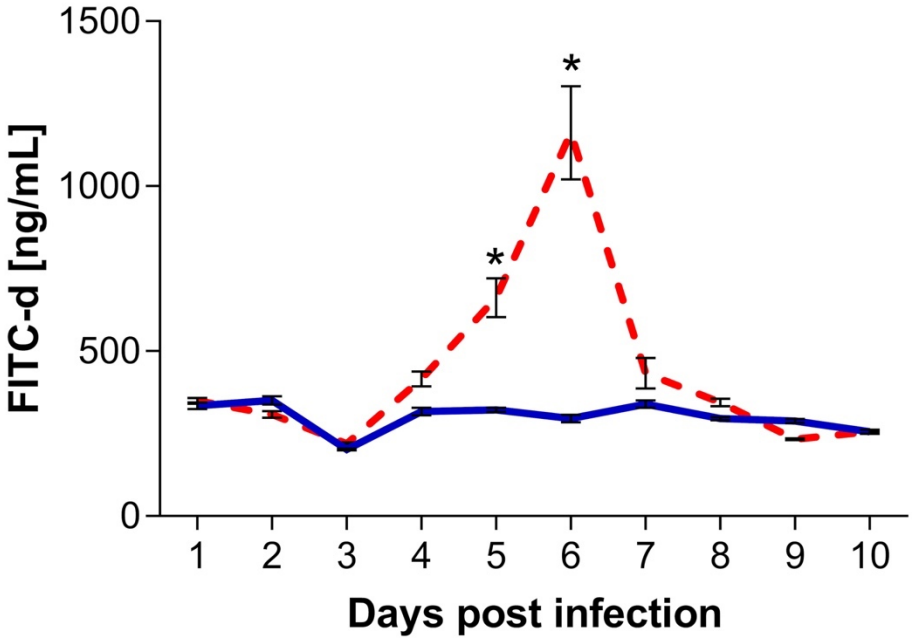

Figure 5.5: Quantification of fluorescein isothiocyanate dextran (FITC-d) in serum samples collected from chickens infected with *Eimeria maxima* (red dashed line) as compared to uninfected chickens (blue solid line). Presence of FITC-d was assessed by ELISA from 1 to 10 days post infection (dpi). Standard error means (SEM) are depicted by vertical lines. Two-way-ANOVA test was performed at 5% level of significance ($p < 0.05$), with multiple comparisons corrected by Bonferroni's method. Significant differences between the groups are indicated by the superscript asterisk.

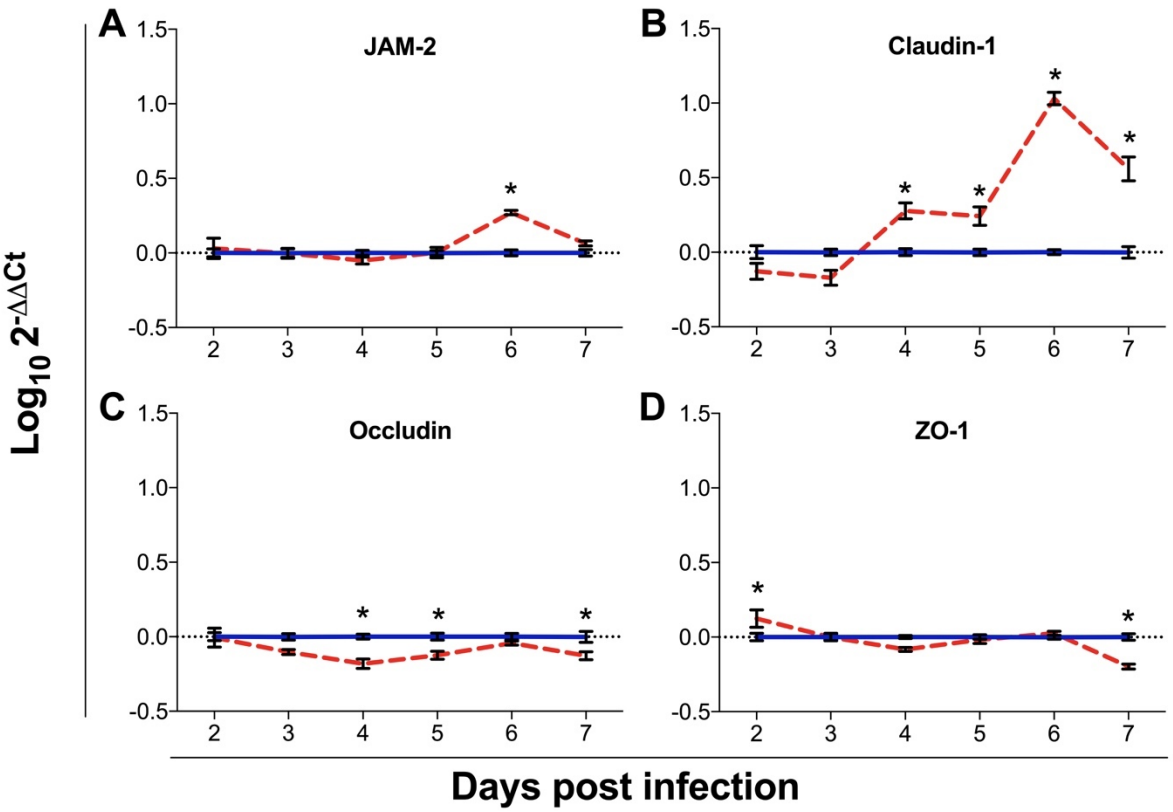

Figure 5.6: Gene expression analysis of the tight junction genes: junction adhesion molecule 2 (JAM-2; A), claudin 1 (B), occludin (C) and Zona occludens 1 (ZO-1; D) in chickens infected with *Eimeria maxima* (red dashed line) as compared to uninfected chickens (blue solid line). Data were assessed from 2 to 7 days post infection (dpi). Expression of mRNA was done by qPCR, with results expressed as $Log_{10}2^{-\Delta\Delta Ct}$. Standard error means (SEM) are depicted by vertical lines. Two-way-ANOVA test was performed at 5% level of significance ($p < 0.05$), with multiple comparisons corrected by Bonferroni's method. Significant differences between the groups are indicated by the superscript asterisk.

CHAPTER 6

DISCUSSION

Eimeria spp. continue to cause outbreaks of the disease worldwide. While several products for prevention and control of the disease are available in the market, new consumer tendencies, for instance the production of organic and drug-free poultry, dictate the need for new methods to control the disease such as alternative adjuvants for immunization of birds, such as the use of host cytokines as adjuvant. In this context, it is important to develop a more comprehensive idea of the pathogenicity of *Eimeria* spp. and its interactions with the host. Heat stress (HS) is a major environmental problem in the poultry industry and has been documented as an immunosuppression factor that offers good opportunity for commensal microorganisms to induce infection and disease, predisposing chickens to enteritis, enhancing *Salmonella enterica* attachment and colonization and to altering intestinal morphology. Previous observations from our study group observed the reduction of oocyst shedding in chickens infected with *Eimeria* spp. in elevated rearing temperature. In addition, it is common knowledge that coccidiosis tends to be less severe in the summer [1,2], presumably due to effect of the heat on host and parasites. The relationship between HS and coccidiosis is not yet well elucidated. This dissertation assesses the interplay between heat stress and *Eimeria* spp. pathogenesis in meat-type chickens.

The objective of the first study (Chapter 3) was to investigate the effect of temperature on viability, infectivity, morphology and development of *E. tenella* sporozoites in vitro, as well as the development of merozoites and fecal oocyst shedding in chickens infected with *E. tenella* and

exposed to HS. Viability was assessed by dual staining with fluorescein diacetate (FDA) and propidium iodide (PI) dyes and analyzed under imaging flow cytometry. We show a reduction on viability of sporozoites after incubation at 55˚C for 60 min, suggesting that heat causes damage to the sporozoite membrane. Losses in viability were correlated with changes in morphology, assessed by aspect ratio, similar to previous reports in *Eimeria tenella* [3]. Sporozoites exposed to high temperatures were also unable to infect MDBK cells. When incubation temperature of MDBK cells infected with *E. tenella* was raised from 41 to 43˚C there was a significant reduction in the number of second generation merozoites produced. We successfully propagated the MDBK cells that were not infected with *E. tenella* at 43 C to confirm that the cells can tolerate the 2 C increase in the incubation temperature. Thus, the reduction in the merozoites production was the result of the increased in host temperature. Furthermore, we confirmed the detrimental effects of HS on *E.* tenella infection *in vivo*, as heat stressed chickens had lower cecal lesion scores, merozoite and oocyst counts as compared to their counterparts housed at TN temperature.

Although this study provides evidence of the unfavorable effects of temperature (in vitro) and heat stress (*in vivo*) on the outcome of *E. tenella* infection, it is important to note that other factors, such as humidity and environmental temperature can have influencing effects under field conditions. High temperature (27- 33˚C) have a beneficial effect on faster sporulation rate of *E. maxima* oocysts [4], as well as elevated survival rate of sporulated *E. acervulina* oocysts [5]. However, elevated humidity and temperature, such as observed during summers in the United States, seem to reduce longevity of sporulated oocysts in the litter [6]. Therefore, besides the effect of HS on *Eimeria* spp. infection, other factors such as humidity and litter temperature presumably play a role in the apparent reduced coccidiosis outbreaks on summer months in the United States.

The objective of the second study (Chapter 4) was to investigate the effect of HS on the pathogenesis of *E. maxima* infection in broilers, as well as investigate differential expression of host cytokines that might affect the curtailed development of the parasite. Our results indicate prolonged replication of *E. maxima* in intestinal tissues, demonstrated by continuing detection of 18s in ileum samples in infected chickens exposed to heat stress (HSi), as opposed to reduction of 18s expression in infected chickens reared in thermoneutral environment (TNi). This is also demonstrated by higher intestinal lesion scores, micro-scores and intestinal barrier disruption in HSi past the peak of oocyst shedding from the control group. Moreover, we assessed the development of *E. maxima* in the ileum by expression of genes specific to *Eimeria* spp. Our results indicate that exposure of the host to HS inhibits the parasite's ability to complete the sexual phase of the development, as there is significant reduction in genes related to gametocyte development and oocyst formation. Altogether these results demonstrate that the reduced outcome of *E. maxima* infection in HS chickens is a result of the inhibition of the sexual stage of development.

In attempt to determine the immune status of the heat stressed host on the reduced outcome of *Eimeria* spp. in poultry, we conducted a cytokine gene expression analysis and comparison of T-lymphocyte populations in the spleen of HSi, TNi, and uninfected controls exposed to HS (HSc) and thermoneutral (TNc) temperatures. Our data indicates that the detrimental effects of the host on the outcome of *E. maxima* infection is not due to immunosuppression, as demonstrated by comparable $CD4^+$:$CD8^+$ ratios in both groups of infected chickens, TNi and HSi, during the first and second weeks of infection. We demonstrate that HS significantly downregulates the expression of pro-inflammatory cytokines (IL-1 and TNF-α) in the ileum of chickens infected with *E. maxima*, with the simultaneous downregulation of NF-κβ-1 and upregulation of IL-10. There is the possibility, however, that this diminished cytokine gene expression is a result of the reduced

parasite development in HSi chickens. However, it remains unclear at this point the exact mechanism of cytokine response elicited by HS that limits *E. maxima* development. Overall, we conclude that, at the level of infection tested, *E. maxima* induces a systemic downregulation of $CD8^+$ lymphocytes, whereas HS and *E. maxima* infection induce upregulation of $CD4^+$ lymphocytes. Also, a very diverse cytokine response shows indicatives of reduced inflammatory response during concomitant HS and coccidiosis infection, suggesting this to be one of the mechanisms resulting in reduced sexual replication of *E. maxima* in heat stressed chickens.

The objective of the third study (Chapter 5) was to elucidate the dynamics of the intestinal barrier and parasite development, analyzing intestinal permeability elicited by *E. maxima* infection and the ontogeny of lesion development throughout the first 10 days of infection, as well as investigating possible biomarkers for intestinal lesions in coccidiosis poultry models. The peak of oocyst shedding was at 7 dpi, similar to the several reports in the literature. Interestingly, our results indicate an increase of developmental stages in intestinal scrapings at 10 dpi, in the detection of *E. maxima* DNA concentration in ileum sections and oocyst shedding at 9 and 10 dpi. Altogether, these data indicate the possible recommence of the infection cycle of *E. maxima* following the first round of oocyst shedding in chickens without reinfection.

Eimeria spp. infection is entirely dependent on the survival and continued existence of the parasitized epithelial cell for a definite length of time, therefore, changes in the cell population kinetics of the intestinal epithelium, associated with other morphological changes, are important factors in the outcome of infection [7]. Coccidia infection causes villous atrophy, which leads to reduction of functional epithelial cells available for parasite development [8,9]. Similar changes are believed to occur during *E. maxima* infection, raising the possibility that developmental stages reminiscent in the intestine could take advantage of the compensatory growth of new epithelial

cells to resume their development, giving rise to the second peak of replication. The reduction in cell availability has been considered as a factor equally important, if not more, than the crowding effect in the self-limiting nature of coccidiosis in poultry [10]. Further studies should address the importance of the infectious dose in the continued replication of *E. maxima*.

Tight junctions (TJ) are multi-protein complexes which are crucial for the integrity and function of the epithelial barrier, as they exert a dual function linking cells and forming channels that allow permeation between cells, resulting in epithelial surfaces with different tightness [11]. We conducted expression analysis in four TJ genes in order to identify possible biomarkers of intestinal integrity in poultry. We herein show that augmented intestinal permeability in *E. maxima* infection is a result of disruption of the intestinal barrier liked to the upregulation of JAM-2, resulting in augmented vascular permeability, and upregulation of Claudin-1 in response to inflammation and enhanced cellular proliferation.

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CHAPTER 7

CONCLUSION

Intestinal health is a crucial factor in animal production, and is closely related to well-being, feed intake and the efficient absorption of nutrients. In poultry, intensive selection for higher weight gain and lower feed conversion ratio has produced breeds that have extremely high feed intake as one of their main characteristics. Among other factors, intestinal infection caused by *Eimeria* spp. severely damages the intestinal integrity, leading to intestinal lesions, malabsorption, secondary infections, and dysbiosis. We herein show that infection of chickens with *E. maxima* results in the differential expression of TJ genes resulting in increased vascular permeability and cellular influx. Changes in the molecular structure of the junctional complexes results in decreased absorption of nutrients, increased secretory passage of ions and water, causing leak flux diarrhea and augmented passage of macromolecules from the lumen, inducing intestinal inflammation. The differential expression of TJ genes is a valuable tool that can be further developed with the objective of monitoring intestinal health in poultry. It is, however, unlikely that a single biomarker will be sufficient to track all the aspects of intestinal health and deficiencies thereof, hence, combination of multiple biomarkers is the most promising way of tracking intestinal health in a holistic way in the future. Meanwhile, measuring the concentration of FITC-d in the serum seems to be the gold standard for assessing intestinal blood barrier disruption.

Our results indicate that infection with *E. maxima* leads to disruption of the intestinal barrier by downregulation of the expression of occludin and upregulation of claudin-1 and JAM-

2. Further studies should address other TJ genes in order to provide a more holistic view of the intestinal barrier dysfunction during coccidiosis. Additional data in the expression of TJ genes should provide deeper understanding of the assessment of efficacy of new methods of therapy and control of coccidiosis in poultry. It is important to consider that other *Eimeria* spp. will likely provide somehow different patterns of dysfunction, based on the cycle and possibly on the infectious dose. We also demonstrate herein a strong indication of a second peak of *E. maxima* replication in the intestines. To our knowledge, this fact has not been previously reported, although previous literature provides strong indicatives of this phenomena. Further research for a period longer than 10 days is necessary to prove if intestinal cell availability could be the cause of such observation.

We herein report the fate of *E. tenella* sporozoites exposure to temperatures up to 55˚C. *E. tenella* sporozoites incubated at 55˚C for 30 min had morphological alterations, lacked the ability to infect MDBK cells. Also, merozoites production was significantly reduced when MDBK cells infected with *E. tenella* were incubated at 2˚C above regular incubation temperature. The reduction in merozoite production under elevated temperature in *E. tenella* infected MDBK cells was validated in chickens infected with *E. tenella* and raised under HS. In addition to reduced production of merozoites, chickens infected with *E. tenella* and raised in a HS environment also had reduced cecal lesion scores and oocysts production compared to their control counterpart. Host responses to parasitic infections are complex.

Additionally, this work shows that there is a significant detrimental effect of HS in the outcome of *E. maxima* infection in broiler chickens. Moreover, the restricted replication of the parasite in HS chickens is shown to be linked to potentially lower gamete fusion, lower parasite fertilization, lower sexual reproduction, resulting in an overall diminished parasite development in

chickens reared under HS conditions. We also conclude that, at the level of infection tested, *E. maxima* induces a systemic downregulation of $CD8^+$ lymphocytes, whereas HS and *E. maxima* infection induce upregulation of $CD4^+$ lymphocytes. Also, a very diverse cytokine response shows indicatives of reduced inflammatory response during concomitant HS and infection, suggesting this to be one of the mechanisms resulting in reduced sexual replication of *E. maxima* in heat stressed chickens.

This work provides new perspectives for studies with coccidiosis in poultry, combining stablished knowledge with molecular biology to provide a bigger overview of the pathogenicity of the infection with focus on heat stress of the host. Understanding the nature of the diminished outcome of infection in heat stressed chickens provides basis for future research seeking new methods of preventing and controlling the disease. It remains to be assessed which other factors of the host might have a relationship with the diminished outcome of *Eimeria* spp. infection in poultry. Moreover, crowding effect, intestinal cell availability and viability as well as infection with other species of the genus *Eimeria* remain to be tested. This work assessed a small panel of host cytokines linked to host response to HS and *Eimeria* infection. Further studies should comprise a more holistic panel of host cytokines and chemokines to provide a more comprehensive overview of the effect of HS in the host that curtail *Eimeria*'s life cycle. It remains to be tested whether HS during primary infection limits the host's immune response to challenge. Due to the systemic effects of HS in the host, we decided to assess the systemic variation in the $CD4^+$:$CD8^+$ ratio. Future studies should also focus in the intestinal evaluation of T-lymphocytes.