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List of abbreviations

°C	Degree Celcius
AMOVA	Analysis of Molecular Varience
ARC	Agricultural Research Council
BAM	Binary version of the Sequence Alignment/Map format
bp	Base Pairs
BTP	Biotechnology Platform
CAGE	Cap Analysis of Gene Expression
cDNA	Complementary Dideoxynuclein Acid
COX	Cytochrome C Oxidase
DEG	Differentially Expressed Genes
EVM	Ethno-Veterinary Medicine
FAO	Food and Agriculture Organisation
FDR	False Discovery Rate
FPKM	Fragments Per Kilobase Of Exon Per Million Fragments Mapped
F_{ST}	Fixation Index
GIN	Gastrointestinal Nematode
GIT	Gastro Intestinal Tract
GO	Gene Ontology
GTF	Gene Transfer Format
GWAS	Genome-Wide Association Studies
H	Number of Haplotypes
HD	Haplotype Diversity
HE	Haematoxylin and Eosin
IEC	Intestinal Epithelial Cells
ITS	Internal Transcribed Spacers
KEGG	Kyoto Encyclopedia of Genes and Genomes
KZN	KwaZulu-Natal
LSM	Least Squares Means
MAPK	Mitogen-Activated Protein Kinases
MDS	Multidimensional Scaling Plots

MHC	Major Histocompatibility Complex
MPSS	Massively Parallel Signature Sequencing
n	Number
NCD	Newcastle Disease
NGS	Next-Generation Sequencing
Nm	Gene Flow
NRF	National Research Foundation
PAMP	Pathogen-Associated Molecular Patterns
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PRR	Pattern Recognition Receptors
PUFA	Polyunsaturated Fatty Acids
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
S	Segregating Sites
SAGE	Serial Analysis of Gene Expression
SAM	Sequence Alignment Map
SE	Standard Error
Seq	Sequence
SNP	Single Nucleotide Polymorphisms
SP	Species
TLR	Toll-Like Receptors
VCP	Transitional Endoplasmic Reticulum Partial
WAAVP	World Association for the Advancement of Veterinary Parasitology

Chapter 1:

General introduction

1.1. Background

Village chickens *(Gallus gallus domesticus)* play an important role (Acamovic *et al.*, 2005) in food security and are owned by village communities in rural areas of South Africa (Thekisoe *et al.*, 2004), other African countries and the developing world. They play a vital role through their contribution to social-economic and cultural lives of smallholder farmers (Dolberg & Petersen, 1999; Pedersen, 2002) as sources of food and income, as token of appreciation for services rendered and as gifts to visitors (Kusina & Kusina, 1999). Their role in national economies is vital to improve the nutritional status and income of smallholder farmers and landless communities (Permin *et al.*, 2002; Muchadeyi *et al.*, 2004).

An estimated 80% of the poultry population in Africa is kept under traditional scavenging systems (GuÈye, 1998; McAinsh *et al.*, 2004), characterized by low productivity and high mortality (Phiri *et al.*, 2007; Mwale & Masika, 2009) that is caused by lack of adequate nutrition and proper management and exposure of chickens to diseases, parasites and predators (dogs, cats, snakes, eagles, hawks and thieves) (Minga *et al.*, 1989; Kusina *et al.*, 2001; Pedersen, 2002). For this study, Limpopo and KZN province were chosen because they are major chicken producing provinces in South Africa and also they were selected based on the willingness of the farmers for voluntary participation.

Gastrointestinal parasites have a negative influence on chicken productivity and flock size (Magwisha *et al.*, 2002). There are about one hundred helminth species that have been recognized in wild and domestic birds. These helminths can damage the intestinal mucosa and reduce growth rate and/or egg production even to the best fed, housed and genetically improved chickens (Irungu *et al.*, 2004a). The common internal parasites that infect chickens include cestodes, nematodes and coccidia (Mwale & Masika, 2011). Diseases like coccidiosis can

cause vast economic losses to poultry farmers if not controlled (Soulsby, 1982; Permin *et al.*, 2002).

Ascaridia galli has been reported as the most prevalent parasitic species affecting village chickens. Mwale and Masika (2011) reported the prevalence of *A. galli* to be 14.25% in Qolora village and 31.43% in Nontshinga village of Centane District in the Eastern Cape province of South Africa. High prevalence of *A. galli* was also reported in guinea fowls in South Africa (Junker & Boomker, 2007). *Ascaridia galli* is of economic importance due to the high treatment cost of affected birds as well as the reduction in feed efficiency and weight gain, that lower overall flock productivity (Permin *et al.*, 1999). Okulewicz and Złotorzycka (1985) showed that *A. galli* inhibited the natural bacterial micro flora of the gastro-intestine of chickens and acts as a vector that can lead to secondary infections such as *E. coli* (Okulewicz & Złotorzycka, 1985; Chadfield *et al.*, 2001; Dahl *et al.*, 2002; McDougald, 2005; Permin *et al.*, 2006). The reported prevalence of *A. galli* parasites vary in different production systems (Permin *et al.*, 1999) with majority of chickens raised in extensive production system showing high prevalce of *A. galli* parasites as compared to intensive production system.

Genetic resistance of a host to disease and parasites has the potential to be used control and manage animal health and in to improve productivity (Delany & Pisenti, 1998). Use of genetic resistance in disease control in poultry was previously limited due to the extensive routine application of chemotherapeutics (Zekarias *et al.*, 2002). The advantages of genetic resistance is further emphasised by the emergence of virulent and drug-resistant pathogens and restrictions on the use of antimicrobials (Zekarias *et al.*, 2002). Breeding of genetically resistant animals, in combination with good hygiene practices are now considered a relatively low risk strategy to control diseases (Wigley, 2004). Due to genetic improvements being permanent and cumulative, they are a long-term, cost-effective, and environmentally friendly solution to maintaining poultry health (Lamont, 1998; Lamont *et al.*, 2003). An example of breeding for disease resistance was shown by Salter & Crittenden (1989) who developed a flock resistant to the Avian Leukosis Virus (ALV) subgroup A, by inserting a dominant gene for resistance to ALV into the germ line of the chickens. Lyall *et al.* (2011) also detected and established a flock of chickens that suppressed the transmission of Avian Influenza using transgenic methods.

1.2. Problem statement and justification

Parasitism is a problem in indigenous chickens raised under low input scavenging production systems (Mwale & Masika, 2011). Severe cases of parasitism can cause mortality (Soulsby, 1982). In South Africa, a number of village chickens in the different agro-ecologies are infested by gastro-intestinal parasites resulting in reduced nutritional status and potential income of rural households (Mukaratirwa & Khumalo, 2010; Mwale & Masika, 2011). It is imperative that characterization of parasites from different production systems is undertaken to better understand the dynamics of infections. Previous studies utilized mitochondrial DNA sequences as a marker for studying genetic variability and population structure in livestock and parasites (Muchadeyi *et al.*, 2008; Nejsum *et al.*, 2008; Katakam *et al.*, 2010).

Traditional control of animal gastrointestinal nematode infections depends on treatment with anthelmintics (Woolaston & Baker, 1996), which are often expensive and even unavailable in low input production systems. Other disadvantages of using this control method is the residual effects of drugs remaining in meat which lead to serious animal welfare and food safety problems (Woolaston & Baker, 1996; Sangster, 1999; Brown, 2007). Therefore alternative control strategies need to be adopted (Heckendorn *et al.*, 2009).

Genetic control strategies are a viable option for controling parasitism particularly for chickens raised under low-input prodution systems. Understanding the host-parasite inter phase is a prerequisite to develop such control strategies. Resistance to parasites is a complex trait influenced by the genes of the host animal and the environment they find themselves in (Warner *et al.*, 1987). Furthurmore, there is a dearth of information available on the genetic resistance to gastrointestinal parasites in village chickens in South Africa and most developing countries, which makes it difficult to select for resistance to parasites in chicken flocks or implement effective control strategies. Parasites resistance has not been prioritised in poultry breeding at present. Only a few studies, such as those on *Eimeria* (Lillehoj *et al.*, 2007) and *A. galli* (Dalgaard *et al.*, 2015) were performed in order to understand the host protective immune responses and subsequent application to vaccine development. Natural acquired immunity against *A. galli* is not well described, but reports exist on variability in disease susceptibility (Permin & Ranvig, 2001; Kaufmann, 2011).

The chicken was the first livestock species to be sequenced (ICGSC, 2004), and led the way in both avian biology and agriculture animal species disease control and, in particular, selection for genetic resistance. Knowing the specific points of genetic variation in the chicken genome will accelerate identification of the genes that control health in poultry, by studying associations of the genomic variation with traits of host resistance to disease (Burt, 2005).

The availability of tools to understand host-pathogen interactions will allow significant progress in identifying and manipulating genetics for the improvement of resistance to gastro-intestinal nematodes (GIN) infections (Dalgaard *et al.*, 2015). Access to molecular and genomic approaches opens up opportunities to understand genetics, environmental and the host-pathogen interactions and can assist in the development of new control strategies. Whole genome and transcriptome sequencing technologies provide opportunities to investigate the genes that play a role in potential resistace to parasite in extensively raised chicken populations distributed across the environmental gradient. In recent years the rapid advances in RNA-seq technologies, have resulted in more genomes sequenced faster and cheaper than ever before (Morozova *et al.*, 2009; Wang *et al.*, 2009; Metzker, 2010). A number of studies have reported differential gene expression in response to gastrointestinal tract infections in livestock (Li *et al.*, 2011a; Bai *et al.*, 2012) and chickens (Kim *et al.*, 2008). Genes such as fatty acid binding protein and several apolipoproteins (APOA1, A4, B100, and C2) were also over-expressed in resistant animals. It would therefore be interesting to investigate the regulation of these gene families in this study.

Village chicken populations are raised under extensive systems of production where nutritional, climatic and health challenges vary amongst production systems. A study by (Permin *et al.*, 1999) have reported differences in the prevalence of *A. galli* between different farming systems where 64% was observed in free range/organic systems, 42% in deep-litter systems and 5% in conventional cages. Characterisation of the production systems practiced in the different village chicken keeping provinces is therefore presequisite to building a better understanding of genes with possible involvement in the resistance of village chickens to *A. galli* parasite. Such analysis would facilitate informed comparisons of resistance profiles of populations raised in different farming systems and give insights on how chickens from different farming systems respond to gastrointestinal parasite infection. It is also assumed

based on differences in climatic and other environmental factors, that different provinces in different agro-ecological zones will host different *A. galli* parasite. A study by (Höglund *et al.*, 2012) for example indicated that *A. galli* paraistes were sub-structured across farming systems in Denmark and Sweden. An undestanding of the diversity of parasites in affected populations is therefore important in establishing the relationship between *A.galli* populations from two different provinces. A holistic approach is therefore required when undertaking genomics studies in village chicken and other livestock populations.

1.3. Aim and objectives

The main aim of this study was to build an understanding of the genetics of resistance to *A galli* infection in village chickens of Limpopo and KZN provinces in South Africa. The objectives included:

- To investigate the village chicken production systems and identify the most prevalent gastrointestinal parasites in Limpopo and KwaZulu-Natal provinces of South Africa
- To investigate the genetic diversity and population structure of *A. galli* parasites from the two different agro-ecological zones of the country
- To apply transcriptomics to investigate gene expression profiles in the small intestine of *A. galli* infected and uninfected chickens from Limpopo and KZN provinces of South Africa
- To investigate the differences in gene expression profiles between the duodenum, jejunum and ileum of *A. galli* infected chickens in Limpopo and KZN provinces of South Africa

1.4. Thesis organization

This thesis is presented in seven stand alone chapters containing an introduction to the thesis (Chapter 1), followed by a review of literature on the thesis topic (Chapter 2) and three experimental Chapters prepared as publications (Chapters 3-5). The thesis concluded with a

critical review, discussion, future studies, conclusions (Chapter 6) and addendum (Chapter 7). References for manuscripts that were prepared or submitted to different journals were reformated to give a single format for this thesis.

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Chapter 2:

Literature review

2.1. Introduction

It is estimated that up to 70% of poultry products in the developing world are produced by resource-limited farmers and in family-managed poultry systems, of which 80% are found in rural areas under scavenging system (GuÈye, 1998; Branckaert *et al.*, 2000). The scavenging production system predisposes the chickens to diseases and parasites especially helminthes (Swatson *et al.*, 2003; Acamovic *et al.*, 2005; Mungube *et al.*, 2008). Various drugs for controlling parasites and treating diseases of chickens have been effectively developed and applied globally (Maphosa *et al.*, 2004). However, rural farmers often does not use commercial drugs due to poor access, driven by geographic distance between suppliers and rural communities, limited veterinary extension services and the costly prices of these veterinary drugs (Mwale & Masika, 2009; Nyoni & Masika, 2012). Continued and improper use of veterinary drugs is also discouraged because of the residual effects of drugs in meat products and parasite developing resistance to drugs. Genetic control strategies are a more sustainable disease management strategy particularly for smallholder farmers that have limited resources (Lamont, 1998). Characterisation of parasitic pathogens as well as the host response to infection are crucial to the development of alternative control strategies, such as breeding of resistant host of development of protective vaccines. This study aims at characterising village chicken production systems and the role that village chickens play in communities. This chapter also includes a review on literature with regards to opportunities and challenges of applying genomics and transcriptomics in studying genetic diversity in village chicken populations.

2.2. Village chicken production systems

2.2.1. The distribution and characteristics of village chickens

There are approximately 21 billion poultry existing worldwide (FAOSTAT, 2014). Some 1.8 billion chickens are found on the African continent with a total of 200 million found in South Africa (FAOSTAT, 2014), kept by commercial and smallholder farmers. Although other poultry species which include ducks, turkeys, guinea fowl, quail and pigeons are important, village chickens are the most important and major poultry species bred for food security (Acamovic *et al.*, 2005). Village chickens are commonly reared in the rural communities of South Africa under extensive systems of production (Swatson *et al.*, 2001; van Marle-Köster *et al.*, 2008; Mtileni *et al.*, 2009; Mukaratirwa & Khumalo, 2010; Nyoni & Masika, 2012).

Previous genetic diversity studies on South African village chickens suggested that village chicken populations hold valuable genetic diversity (van Marle-Köster *et al.*, 2008; Mtileni *et al.*, 2011b; Khanyile *et al.*, 2015) that have been developed over many years, and survived successfully under extreme and unusual environmental conditions. These studies revealed a high level of genetic variation within and among the village chicken populations and identified maternal origins of South African chicken populations. Furthermore, the studies indicated that village chickens contribute genetic variation that is distinct from diversity exhibited by commercial and specialized chicken populations. Mtileni *et al.* (2011a) reported that the South African conserved and field chicken populations share some ancestral maternal lineages, which suggests that these populations could be from the same maternal lineages.

2.2.2. Characteristics of village chicken production systems

Village chickens are also known as rural, backyard, indigenous, scavenging, traditional, local, native or family chickens (Sonaiya, 2000; Permin *et al.*, 2002; McAinsh *et al.*, 2004; Oka *et al.*, 2007; Moreki, 2010). These chickens are often left to scavenge for their feed and water around the homestead and in the fields after crop harvests for an average of 5 to 11.0 h per day between 5am-6pm (Maphosa *et al.*, 2004). Some village chickens are then confined at night or left to sleep on trees or bushes (Maphosa *et al.*, 2004). In some production systems, the chickens are occasionally supplied with feed supplements (Maphosa *et al.*, 2004; Moreki, 2010) that ranges from yellow maize, kitchen wastes, cracked grains, maize bran, sunflower cake, grower's mash for chicks and/or wheat (Muchadeyi *et al.*, 2004; Nyoni & Masika, 2012). This production system is characterized by substandard management, lack of adequate nutrition, low production performance, slow growth rate, late sexual maturity (Gondwe &

Wolly, 2007; Phiri *et al.*, 2007; Mapiye *et al.*, 2008; Nyoni & Masika, 2012) and to high mortality (Mungube *et al.*, 2008). Their mean annual egg production is estimated at around 60 small eggs (Sørensen, 2010). The low productivity of indigenous chickens expressed in terms of egg production, egg size, growth and survivability of chicks kept under traditional production system could be attributed to incidence of diseases and predation, lack of genetic improvement and management factors (Sonaiya, 2000; Mengesha *et al.*, 2008).

In the village chicken production system chickens are exposed to the extreme environmental conditions that negatively influence production. The environmental conditions play a selective role (natural selection) in the village chicken population by eliminating animals that cannot utilize poor quality feeds and those that are susceptible to diseases (Ekue *et al.*, 2006; Mtileni *et al.*, 2009). Genotypes that are adapted to the environment and are able to make use of the available feed and those that can resist disease will be able to survive in such extreme environments.

It is proposed reported that there is constant disease pressure on scavenging chickens, due to the different ages mixed in a flock and possible disease transfer from wild birds as well as other poultry species that use the same land (Acamovic *et al.*, 2005). Parasitism ranks high among factors that threaten village chicken production (Muchadeyi *et al.*, 2007). As chickens get exposed to parasites during scavenging, parasitic infections are ubiquitous and high infection loads have been reported resulting in clinical disease (Oniye *et al.*, 2000). Due to minimum intervention provided by resource-limited farmers, relatively high mortality are experienced.

Village chickens are mainly used for household consumption (Sonaiya *et al.*, 1999; Nyoni & Masika, 2012) and plays a role in traditional ceremonies and festivals (Mtileni *et al.*, 2009). Chicken meat and eggs provide animal protein to man and can give extra cash income when sold at the market (Kalita *et al.*, 2004; McAinsh *et al.*, 2004; Njenga, 2005; Nyoni & Masika, 2012). Cocks are also used as alarm clocks in the villages (Kusina & Kusina, 1999). Another important role of village chickens is the provision of manure (Nyoni & Masika, 2012). Fifteen adult chickens produce about 1.0-1.2kg of manure per day (Aini, 1990). Manure from chickens is applied in vegetable gardens, and is regarded to be of high value for vegetables in comparison to other manure obtained from goats or cattle (Maphosa *et al.*, 2004; Muchadeyi *et al.*, 2004). Chickens are also useful in the control of weeds when they graze young grass and other

vegetation. Chickens are therefore an important component in integrated farming systems (Barua & Yoshimura, 1997; Birech, 2002).

2.2.3. Challenges to village chicken production systems

There are a number of factors affecting village chicken production. Poor nutrition due to lack of supplementation is a major challenge to scavenging chickens. Chickens are left to scavenge to meet their nutritional needs (Muchadeyi *et al.*, 2004; Mwale & Masika, 2011). In event of supplementation, it is often thrown onto the ground further exposing chickens to internal parasites (Nyoni & Masika, 2012). Supplementary feed are mainly based on the farmers' judgment (Mapiye *et al.*, 2008; Nyoni & Masika, 2012) and not the nutritional requirements of chickens and tent to vary from household to household. In a 2012 study, supplementary feed was reported to range from as little as one handful (approximately 100 g) of yellow maize grain to about five handfuls (approximately 500 g) per day (Nyoni & Masika, 2012).

Parasitism ranks high amongst the factors affecting the health of village chickens, due to existence of conditions such as inadequate hygiene and rainfall, humidity, ambient temperature and compromised biosecurity (Smyth, 1976; Swatson *et al.*, 2003). The prevalence of gastro-intestinal parasites in village fowls have been studied in different countries (Njunga, 2003; Bowdridge, 2009; Kaufmann, 2011) and to a lesser extent in South Africa (Mwale & Masika, 2011). Different species of endo-parasites have been identified such as *A. galli* and *H. gallinarum* (Ssenyonga, 1982; Poulsen *et al.*, 2000; Permin *et al.*, 2002; Muhairwa *et al.*, 2007). Poultry endo-parasites of economic importance include *Eimeria* species and helminthes (Norton & Ruff, 2003). *Ascaridia galli, Capillaria spp.* and *H. gallinarum* are the most commonly encountered helminthic species (Permin *et al.*, 1999; Irungu *et al.*, 2004b; Kaufmann & Gauly, 2009). Heavy *A. galli* infections may obstruct the small intestine and cause mortality (Ramadan & Znada, 1991) and have been associated with reductions in egg production in laying hens and in overall growth in chickens (Soulsby, 1982; Ramadan & Znada, 1991). Parasites can lead to secondary infections (Okulewicz & Złotorzycka, 1985; Chadfield *et al.*, 2001; Dahl *et al.*, 2002; Permin *et al.*, 2006) and transmit diseases like histomoniasis, cestodosis and ascariodiosis (Soulsby, 1982; Fatihu *et al.*, 1990). *Ascaridia galli* may also play a role in transmission of Salmonella infections (Chadfield *et al.*, 2001; Eigaard *et al.*, 2006).

2.3. Parasite infection in village chickens

2.3.1. Classification of A. galli parasite and A. galli infections in village chickens

Ascaridia galli (Schrank, 1788, Freeborn, 1923 (synonyms *A. lineata* Schneider, 1866; *A. perspicillus* Rudol- phi, 1803) is a common parasitic nematode found in domestic and a number of wild fowl and shows a worldwide distribution (Permin *et al.*, 1999; Magwisha *et al.*, 2002; Marín-Gómez & Benavides-Montaño, 2007; Luna-Olivares *et al.*, 2012). This parasite is classified under the group of ascarid worms (phylum Nematoda; Class Secernentia; order Ascaridida; family Ascaridiidae). The *A. galli* populations in Denmark and Sweden are reported to be sub-structured according to farms and geographical areas, with the genetic differentiation of populations contained within individual hens (Gasbarre *et al.*, 2001). A previous study that sequenced *A. galli*, *A. columbae* and *Ascaridia spp*. (GHL-2012) reported this species share an identical arrangement of mitochondria (mt) genes that differ significantly from other nematodes (Alvarez *et al.*, 2008). A separate study that looked at the phylogenetic relationships of these two species, observed *A. galli* and *A. columbae* grouping separately from other nematodes (Mair *et al.*, 2004). A study by Li *et al.* (2011b) investigated phylogenetic relationships of nematodes classified in the Ascaridoidea using a total evidence parsimony analysis of a combination of morphology and one mitochondrial and two nuclear genes. Findings from this study indicated that *Ascaris lumbricoides* and *Ascaris suum* are sister taxa that share a most recent common ancestor with *Parascaris equorum*, which is a large ascarid affecting horses (Li *et al.* (2011b).

Ascaridia galli is prevalent in chickens, turkey, geese, guinea fowl and a number of wild birds but chickens are the primary host. Of all the nematodes of poultry, *A. galli* is known to be the largest (51-116 mm) and most pathogenic (Kates & Colglazier, 1970; Kawai & Akira, 2001; Lacy & Stow, 2011) and its predilection site being the small intestine (Soulsby, 1982). Reports from Denmark and other European countries showed that the majority of chickens kept in free-range systems are infected with *A. galli* (Permin *et al.*, 1999; Jansson *et al.*, 2010; Kaufmann, 2011). Infection with *A. galli* may directly contribute to economic losses due to weight loss, reduced growth rates and decreased egg production (Permin & Ranvig, 2001; Permin *et al.*, 2006). Mortality has also been observed in severe cases (Permin *et al.*, 2006). In addition, *A galli* damages the intestinal mucosa, which results in blood loss and compromised immunity leading to secondary infections (Permin *et al.*, 1999). High infection loads of this parasite result

in the blockage of the small intestines, which at to mortality (Ramadan & Znada, 1991; Permin *et al.*, 2006). Symptoms of heavily infected chickens include droopiness of wings, bleaching of the head, ruffled feathers, emaciation and diarrhea that might be accompanied by anemia and intestinal obstruction in very heavy infections (Ackert & Herrick, 1928)

2.3.2. The life cycle of A. galli

The life cycle of *A. galli* is illustrated in Figure 1.1. Eggs are passed with the faeces of the host, where they develop outside the host animal. These reach the infective stage (L_3) after 10 to 20 days (depending on temperature and relative humidity), (Ackert, 1931; Riedel, 1947; Reid & Carmon, 1958; Reid, 1960). For the eggs to proceed into the infective stage, a minimum of five days at 32-34°C is required when the eggs are incubated in water (Reid, 1960). The eggs may not survive after 22 hours at temperatures between -12°C to -8°C, (Ackert, 1931), but can however, survive a winter with moderate frost (Cruthers et al., 1974). Temperatures above 43°C are lethal for eggs at all stages (Ackert, 1931; Reid, 1960). In deep litter systems, eggs probably can remain infective for years depending on the temperature, humidity, pH and ammonium concentration (Hansen *et al.*, 1953; Koutz, 1953; Reid, 1960; Cruthers *et al.*, 1974; Matter & Oester, 1989).

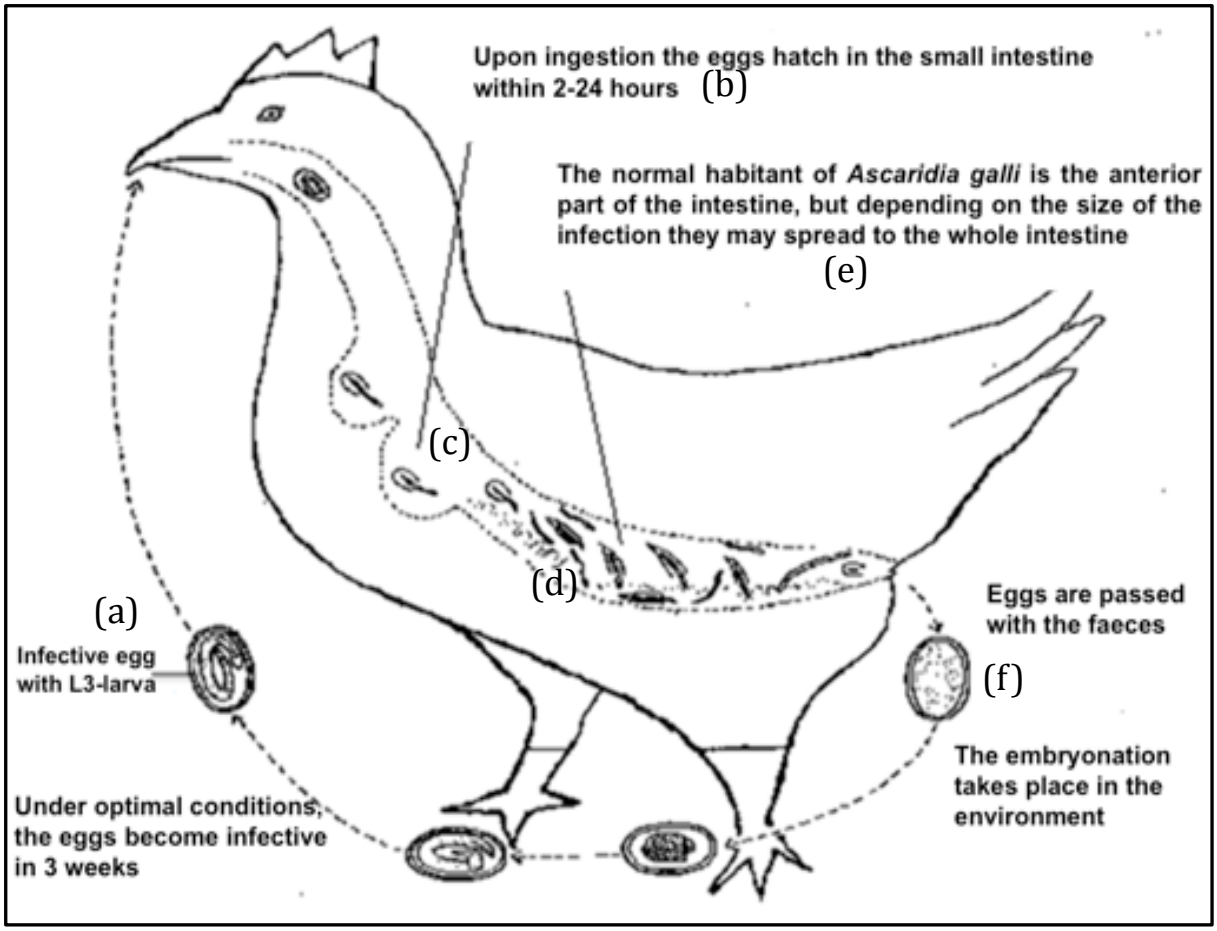

Figure 1.1: General life cycle diagram of *A. galli* (Adapted from Permin, 1997)

The life cycle of *A. galli* is completed when the infective eggs (a) are ingested by new hosts through contaminated feed or water. The eggs containing the infective L_3-larvae stage are mechanically transported to the duodenum (b). The larvae are protected by the three layers covering the eggs until they reach the duodenum or jejunum, where they hatch within 24 hours (Tugwell & Ackert, 1952; Soulsby, 1982; Kaufmann, 1996; Idi, 2004). The larvae will embed themselves into the mucosal layer of the intestine (c) (Herd & McNaught, 1975; Luna-Olivares *et al.*, 2012). During hatching the mature coiled larvae protrude the anterior end of the egg through an opening in the shell moving out to the lumen of the intestine (d) (Ackert, 1931; (Ferdushy *et al.*, 2013). The larvae then enter the histotropic phase where they embed themselves into the mucosal layer of the intestine (e) (Permin & Hansen, 1998). Thereafter, the larvae will mature in the lumen and be passed with the faeces of the host (f) (Anderson, 1992; Chadfield *et al.*, 2001; Idi, 2004).

2.4. Disease and parasite control strategies in chickens

2.4.1. Methods for disease and parasite control in chickens

Traditional control of animal parasitic nematodes relies upon treatment with drugs called anthelmitics (Woolaston & Baker, 1996). This type of control is threatened by the widespread occurance of drug resistances in nematode populations (Jackson & Miller, 2006) their relatively high costs and residual products contaminating animal products. The repeated use of antiparasitic drugs inevitably leads to the development of resistance in the target organisms, and this systematically occurs within approximately ten years following the introduction of the chemicals to the market (Brown, 2007). There are only three anthelmintic drug classes available to treat GINs, the imidazothiazoles, benzimidazoles and macrolides (Bowman, 2003). Resistance has developed in parasite populations to each of these three drug classes (Howell *et al.*, 2008). Consequently, most farmers have resorted to the use of alternative traditional means to control parasites and treat diseases in chickens (Anthony *et al.*, 2005; Nyoni & Masika, 2012). However, majority of these traditional medicines have not been validated hence their safety and efficacy need to be assessed to ensure the health of consumers..

2.4.2. Role of the immune system in disease/ parasite resistance in chickens

The immune system is the host defense mechanism against infectious diseases (Bacon *et al.*, 2000). The immune system of the intestine has a complex task of reacting to pathogens and harmful compounds as well as tolerating the constant flow of food antigens and the abundant non-pathogenic microflora. The immune system includes the rapid responding innate immunity and the slow responding adaptive immunity (Abbas & Lichtman, 2005). The rapid innate immune response is active in the first few days of infection (Abbas & Lichtman, 2005). Innate immunity is capable of removing the infectious agents shortly after the infection. Innate immunity can lead to a series of signaling events resulting in the induction of dendritic cell maturation, which is responsible and necessary for activation of adaptive immune responses (Lamont *et al.*, 2002; Werling & Jungi, 2003; Pasare & Medzhitov, 2005).

Adaptive immunity is the second line of defense after innate immunity. It is also called specific immunity due to its extraordinary capacity to distinguish among different, even closely related microbes and molecules. For immune response occurring in the intestine, intestinal epithelial

cells (IECs), lymphocytes and macrophages are the barriers encountering invasion. The two types of lymphocytes: T-cells and B-cells are components of adaptive immunity. B-lymphocytes produce antibodies and are responsible for the humoral immune system whereas T-lymphocytes take care of the cellular immune system (Lee, 2006). They are both triggered by antigen into action. T-cell respond to antigen by producing clones of T-cells: cytotoxic T-cells, helper T-cells and suppressor T-cells. IECs have traditionally been regarded as passive cells primarily responsible for maintaining integrity of the intestinal barrier. However, it is now widely appreciated that they are also important regulators of innate and acquired immunity (Goto & Ivanov, 2013).

The genes responding to pathogens frequently belong to the innate immune system, or are involved in host cellular processes (Jenner & Young, 2005; Aldridge *et al.*, 2006). Gene expression level changes due to pathogen in particular cells of the host. These changes can be different between susceptible and resistant hosts (Liu *et al.*, 2001).

The chicken immune system has several mechanisms to protect against and combat pathogenic infection (Erf, 2004; Sharma, 2008). Heterophils are the avian equivalent of neutrophils and are the first cell type to rapidly localize to sites of infection (Kogut *et al.*, 1995). The proper signaling by cytokines increases the effectiveness of immune response and T-helper cells. *Ascaridia galli* infection in chickens results in polarization towards a type 2 immune reaction, including augmented expression of interleukin-4 and interleukin-13 and diminished interferon-gamma expression (Bao *et al.*, 2000). The intestine, as a first barrier, is in contact with commensal and pathogenic microorganisms (Abreu, 2010). These responses allow the intestine to contribute to the defense against pathogens and to the control and regulation of the local immune response.

2.4.2. Adaptive genetic diversity and genetic control strategies

Years of natural selection, under scavenging conditions, might have made village chickens robust and resistant to various diseases, especially those caused by bacteria, protozoa and other internal and external parasites. Generally, village chickens are known to have a better survival rate compared to commercial hybrid strains under village production conditions (Sonaiya *et al.*, 1999).

The study of genetic resistance in the chicken has progressed fairly fast due to their small size, rapid growth, short generation time and the availability of a number of well-defined inbred chicken lines (Wigley, 2004). Initial studies on genetic resistance centred mostly on resistance of various chicken breeds to fowl typhoid (*Salmonella gallinarum*) and pullorum disease (*Salmonella pullorum*) (Hutt & Crawford, 1960). Differences in disease resistance or susceptibility between strains of chickens have been observed in a number of population (Lambert, 1932; Bearse *et al.*, 1939; Abdelqader *et al.*, 2007). Differences in nematodes resistance were observed for *A. galli* (Abdelqader *et al.*, 2007) and resistance to viruses for avian influenza virus (Wang *et al.*, 2014).

Genetically determined diversity of the poultry immune system is considered the major cause for differences in resistance to diseases of infectious origin (Gavora, 1990; Hawken *et al.*, 1998). The release of the chicken genome in 2004 (ICGSC, 2004) has provided new possibilities to study the function of individual genes and gene networks in chicken to gain insight into their specific roles in chicken physiology. The functions of most of the chicken genes have been predicted based on sequence homology to genes of known function in other species (Hillier *et al.*, 2004). It is however, essential to obtain more detailed information about the expression of these genes in different tissues and under different conditions. Previous studies monitored the changes in gene expression that take place in host epithelial cells (Van Hemert *et al.*, 2007), lymphocytes (Sarson *et al.*, 2007), spleen tissues (Zhou & Lamont, 2007) and macrophages (Bliss *et al.*, 2005) post exposure to specific pathogens (Table 2.1).

Table 2.1: Gene expression change in different chicken cells in response to pathogens

Pathogen/molecule	Gene	Cell	Reference

Salmonella	Apolipoprotein B Cytochrome P450 70 kDa zeta-associated protein	Epithelial cells	Van Hemert *et al.*, 2007
Lipopolysaccharide stimulation	Heat-shock protein 70 Toll-like receptor 4 Caspase 3	Lymphocytes	Sarson *et al.*, 2007
Salmonella enterica Serovar enteritidis	Chemokine ah294 Chemokine ah221 T-cell surface glycoprotein CD28	Spleen tissues	Zhou & Lamont, 2007
E. coli and *Salmonella typhimurium*-derived lipopolysaccharide stimulation	Interleukin-1beta CXC chemokine K60 Chemokine ah189	Macrophage	Bliss *et al.*, 2005

2.5. Transcriptome analysis technologies

2.5.1. Methods for transcriptome analysis

The transcriptome is the complete set of transcripts and their quantity within a cell for a specific developmental stage or physiological condition (Jacquier, 2009). Although transcript sequencing has been possible for nearly 20 years, until recently it required the construction of cloned libraries. It has taken a few decades to determine the full-length gene structures of human, mouse and other species, including farm animals and important animal models (ICGSC, 2004; Warren *et al.*, 2010). There are various established methods that are used for transcriptome analysis. These methods have been improved over recent years from the use of traditional methods such as northern blotting, quantitative real-time polymerase chain reaction (qRT–PCR) or differential display to recently developed next generation RNA sequencing technologies (Wang *et al.*, 2009; Mutz *et al.*, 2013).

2.5.2 Traditional technologies for transcriptome analysis

The first candidate gene-based studies used Northern blot analysis (Alwine *et al.*, 1977). This is a low-throughput technique for RNA detection that involves the separation of cellular RNA

by size using gel electrophoresis followed by its transfer to a solid support (e.g. microcellulose or nylon membrane) where the presence and abundance of a specific RNA species of interest is inferred by hybridization of a complementary radioactively labelled nucleic acid probe (Morozova *et al.*, 2009).

Quantitative PCR (qPCR) is a technology utilized to measure gene expression in a more targeted manner. Quantitative PCR targeted expression is useful when specific genes or gene families are known to be involved in response. This technology may miss significant effects in biological processes (such as metabolic and signaling pathways and transcriptional networks across several pathways) because they are confined to the analysis of a limited number of selected genes of interest in a few samples (Subramanian *et al.*, 2005).

Complementary DNA microarray technology enables researchers to study many genes. The technique has rapidly evolved from its introduction in the mid- 1990's (Schena *et al.*, 1995) and it allows rapid analysis of thousands of genes simultaneously (Eisen & Brown, 1999). As a hybridization based technology, microarray analysis has weaknesses, especially the requirement for a prior knowledge of the sequence (Cassone *et al.*, 2007). An inherent limitation of the microarray is that the resulting transcriptome does not account for post-translational events like splicing. Moreover, although microarray rapidly generates numerous hypotheses, many of them could be fallen into false-positive error due to multiple testing error (Pounds, 2006). Furthermore, microarray technologies require high quality control and clone access and also there are high chances of cross-hybridization of probes across gene families.

Sequencing of RNA has long been recognized as an efficient method for gene discovery (Adams *et al.*, 1991) and remains the gold standard for annotation of both coding and noncoding genes (Haas *et al.*, 2012). Velculescu *et al.* (1995) described one of the first sequence-based methods used for gene expression profiling called serial analysis of gene expression (SAGE). Massively Parallel Signature Sequencing (MPSS) methods also measure short-tag sequences and facilitates the use of sanger sequencing for gene expression profiling (Hu & Polyak, 2006). Using these methods, expressed genes were represented by short tags, originally 14 base pairs in length but later improved to 21 bp (LongSAGE) (Saha *et al.*, 2002) that directly correlated quantitatively with gene expression levels. Originally, tags were concatenated, cloned and sequenced but the expense of sanger sequencing meant that the libraries created were rarely sequenced deep enough, normally yielding tens of thousands to a

few hundred thousand tags, to quantitatively evaluate low expressing transcript. Unlike SAGE, MPSS uses an *in-vitro* cloning process and hybridization-ligation based parallel 30 sequencing (Reinartz *et al.*, 2002; Meyers *et al.*, 2004).

2.5.2. Next-generation sequencing (NGS) method for transcriptome analysis

Transcriptome sequencing studies have evolved from determining the sequence of individual cDNA clones (Stone *et al.*, 1985) to more comprehensive attempts to construct cDNA-sequencing libraries representing the species transcriptome (Seki *et al.*, 2002). The advantages of new sequencing technologies are that no cloning is needed, allowing direct sequencing of cDNA fragments. RNA-Seq, is a high-throughput transcriptome analysis technology that allows the quantitative measurement of genes expressed within a specific sample with the ability to detect genes even at low expression levels (Marioni *et al.*, 2008). The use of next-generation sequencing promises to reduce or eliminate some of the weakness of microarrays. Next-generation sequencing (NGS) platforms can identify and quantify rare transcripts without prior knowledge of a particular gene and can provide information regarding alternative splicing and sequence variation in identified genes (Wang *et al.*, 2009). One can achieve within a short period of time reasonably complete coverage of transcriptomes (Wang *et al.*, 2009).

The declining cost of generating such data is transforming all fields of genetics (Lister *et al.*, 2009). Large-scale NGS is possible using platforms such as Roche 454 Life Sciences, Illumina, Life Technologies SOLiD, Helicos Biosciences, Complete Genomics (Metzker, 2010). The HiSeq 2500 sequencing platform from Illumina, for instance, is capable of generating up to 350 giga base pairs in one run that takes around eight days. One run on this platform has the capability to re-sequence the whole genome of nine individual chickens (or three individuals of human, cattle or swine) with coverage of 30 reads per nucleotide.

2.5.3. Bioinformatics pipelines for analysisng next generation sequencing transcriptome data

RNA-Seq experiment should be designed such that the measurements taken reflect as much as possible differences due to treatment and developmental stages. Technical and biological replications as well as positive and negative controls have to be included to reduce variability

in the measurements of treatment effects. Based on the sample size, including both technical and biological replicates in the design may be costly (Minoche *et al.*, 2011). As a result, these are suggestions that serve as standards for RNA-seq analysis (Minoche *et al.*, 2011). Using Illumina platforms, it is recommended to include phiX genome spike-in for each lane as a control (Zook *et al.*, 2012). The phiX spike-in across the lanes is used to make sure that the data produced for each run is accurately calibrated during data capture and analysis. Secondly, there is a suggestion that multiplexing large samples into single lanes should be used to remove the possible technical results of different lanes (McIntyre *et al.*, 2011b). There are different views on the amount of validation required for RNA-Seq experiments. While it is certainly true that some runs fail, the technical reproducibility of RNA-Seq experiments has led some to suggest that even RNA-Seq technical validation is not necessary once the sequencing instrument has been optimised.

There are various commercial and free software packages that can be used to analyse RNA-Seq data. Free programs play a crucial part in the analysis of RNA-Seq data as these programs adapted according to changes in technology, and are not limited by the need to wait for official release dates like commercialized programs. Bioconductor package (www.bioconductor.org) for analyzing RNA-Seq is developed using the R programming language (www.bioconductor.org; Gentleman *et al.*, 2004) and is usually of similar quality to that of commercial software. The DEGseq package (Wang *et al.*, 2010) and edgeR package (Robinson & Smyth, 2007; Robinson *et al.*, 2010a) are both from the Bioconductor suite (www.bioconductor.org). They are two examples of open source software that are freely available for use in analysing RNA-Seq data. Cufflinks (http://cufflinks.cbcb.umd.edu; Trapnell *et al.*, 2010) is an open source software which can be used to assemble transcripts, estimate their abundances, and test for differential expression and regulation in RNA-Seq samples. Cufflinks is particularly useful for researchers who are interested in alternative transcript or splice variants as it can identify novel transcripts and probabilistically assign reads to isoforms without the need for prior gene annotation knowledge.

CLC bio (http://www.clcbio.com) released the CLC Genomics Workbench as a commercial software package that integrates genomics (genome de novo and re-sequencing) and transcriptomics (RNA-Seq gene expression) in one environment. Another commercial software offering a web base service is called GenomeQuest. Its RNA-Seq workflow

(http://wiki.genomequest.com/index.php) accesses the databases of transcriptomes and genomes.

2.6. Conclusion

Village chickens are generally described as non-descript birds that have not been developed as a breed and with uncharacterized genetic attributes. They tend to be very robust and well adapted to harsh environmental conditions such as hot or cold weather, rain and periodic feed shortages. Agro-ecological zone is known to have a crucial role in defining scavenging chicken production systems. Transcriptome analysis present relaible tools that can be used to understand the immune response of chicken intestine to GIT parasites under different provinces. The challenges of doing transcriptome analysis in village chickens is the scarsity of information due to little to no studies carried out on the genetic resistance to gastrointestinal parasites in village chickens in South Africa. A good characterisation of the host transcriptome may however lead to a better understanding of the host biology and the development of new control strategies.

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Chapter 3:

A description of village chicken production systems and prevalence of gastrointestinal parasites: Case studies in Limpopo and KwaZulu-Natal provinces of South Africa

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Published in Onderstepoort Journal of Veterinary Research 2016, 83(1), a968

Abstract

The majority of rural households in developing countries own village chickens that are reared under traditional scavenging systems with few inputs and exposure to various parasitic infections. Understanding of the village chicken farming system and its influence on helminth infection is a prerequisite for optimal prevention and control strategies. This study investigated the village chicken production system and associated gastrointestinal parasites in 87 households from Limpopo (n = 39) and KwaZulu-Natal (n = 48) province of South Africa. A total of 191 village chicken faecal samples and 144 intestines were collected to determine the prevalence of gastrointestinal parasites in villages of Limpopo and KwaZulu-Natal s, respectively. The faecal floatation analysis of samples from Limpopo and KwaZulu-Natal province indicated infections by *Ascaridia galli* (18.77%), *Heterakis gallinarum* (15.56%) and

Capillaria species (4.00%); tapeworms *Choanotaenia infundibulum* (2.10%) and *Raillietina cesticillus* (6.00%) and *Eimeria* species (29.46%). Mixed infections were observed in five (4.90%) samples from Limpopo and only four (4.49%) from KwaZulu-Natal of which 1.12% were a mixture of *Choanotaenia infundibulum* and *Eimeria* species and 3.37% a combination of *Heterakis gallinarum* and *Eimeria* species. In Limpopo, 2.94% of the chickens were positive for *Heterakis gallinarum* and *Eimeria* species whilst 0.98% had *A. galli* and *Capillaria* species infections. Further investigation is needed to understand the impact of gastrointestinal parasites on village chicken health and production, and develop appropriate intervention and control strategies feasible for smallholder farmers.

Key words: Helminths; Village chickens; Smallholder farming systems; Faecal samples.

3.1. Introduction

Village chickens (*Gallus gallus domesticus*) are poultry species mostly owned by village communities in rural areas of Africa (Thekisoe *et al.*, 2004) and other developing countries (Muchadeyi *et al.*, 2004; Muhiye, 2007). They play a vital role through their contribution to the socio-economic and cultural lives of smallholder farmers (van Marle-Köster *et al.*, 2008; Nyoni & Masika, 2012). Village chickens can be used as tokens of appreciation for services rendered and are often given to visitors as gifts (Kusina & Kusina, 1999). Their role in national economies is through improved nutritional status and income of many smallholder farmers as well as landless and marginalized communities (Muchadeyi *et al.*, 2004; Tarwireyi & Fanadzo, 2013).

A majority of chicken populations in Africa are kept under traditional scavenging systems (McAinsh *et al.*, 2004; Mtileni *et al.*, 2009) that are often characterized by low productivity

and high mortality. Village chickens are left to scavenge to meet their nutritional needs (Muchadeyi *et al.*, 2004; Mwale & Masika, 2009) (Muchadeyi *et al.* 2004; Mwale & Masika 2009), which predisposes them to predators (Kusina & Kusina, 1999; Permin *et al.*, 2002), diseases and parasites (Swatson *et al.*, 2003) that coexist in the scavenging environment. Parasite infestation contributes to poor production and can cause mortality in severe cases (Soulsby, 1982).

Gastrointestinal parasites are the most prevalent parasites affecting the productivity of village chickens (Mwale & Masika, 2011). Their prevalence in village chickens has been studied in different countries and to a lesser extent in South Africa (Mukaratirwa & Khumalo, 2010; Mwale & Masika, 2011). Different species of endo-parasites have been identified (Permin *et al.*, 2002; Muhairwa *et al.*, 2007), including endo-parasites such as *Eimeria species* and helminthes (Norton & Ruff, 2003). Parasitic infection rates have been shown to differ among different production systems (Permin *et al.*, 1999) due to variations in environmental and management factors. Improved poultry management practices are responsible for the reduction in the incidence of parasitic infections (Puttalakshmamma *et al.*, 2008). Kaufmann (2011) indicated that village chickens do not only harbour a wide spectrum of helminths, but are also associated with relatively high intensity of infections such as *E. coli* compared to commercial chickens with more frequent incidences in free range than intensive systems (Permin *et al.*, 2006).

The smallholder farming sector of South Africa is similar to those in other African and developing countries which is characterized by low production inputs, exposure of chickens to diseases and parasites and compromised biosecurity and veterinary interventions (Acamovic *et al.*, 2005). The aim of the study was therefore to describe typical village chicken production systems in the Limpopo and KwaZulu-Natal provinces of South Africa and to determine the prevalence of gastrointestinal parasites in these communal low input-farming systems. Such information is considered important for the development and implementation of effective control programs.

3.2. Materials and methods

3.2.1. Study sites and animal populations

This study was conducted in 20 villages from two provinces of the Limpopo and KwaZulu-Natal provinces of South Africa (Figure 3.1). These provinces were targeted based on the existence of free-range village chicken production, the contrasting environmental conditions between them and because they are amongst the major poultry producers in South Africa. Mopani District in Limpopo is situated within the sub-tropical zone. It can be very hot in summer with average temperatures ranging from 20 to 43°C. Winters are mild during the day and cold during the nights with average temperatures ranging from 9 to 26°C. Vhembe District in Limpopo experiences a hot semi-arid climate with hot temperatures most of the year. Average annual precipitation amounts to 372 mm with extremely dry winter. uThukela District municipality in KwaZulu-Natal covers an area of approximately 11500 km². It experiences heavy snow on the mountains in winter. It is located in the western boundary of KwaZulu-Natal province. uMzinyathi District has a temperate climate. Frost occurs only in parts of uMzinyathi in winter. Rainfall varies from more than 800 mm in Endumeni and Umvoti, to less than 400 mm in parts of Msinga. Eighty-seven households were randomly selected from villages of the two provinces from November 2012 to February 2013. Information was gathered from veterinary extension officers on chicken ownership in the two provinces. Households in each village were then selected on the basis of the availability of free ranging chickens and the willingness of the chicken owners to participate in the study.

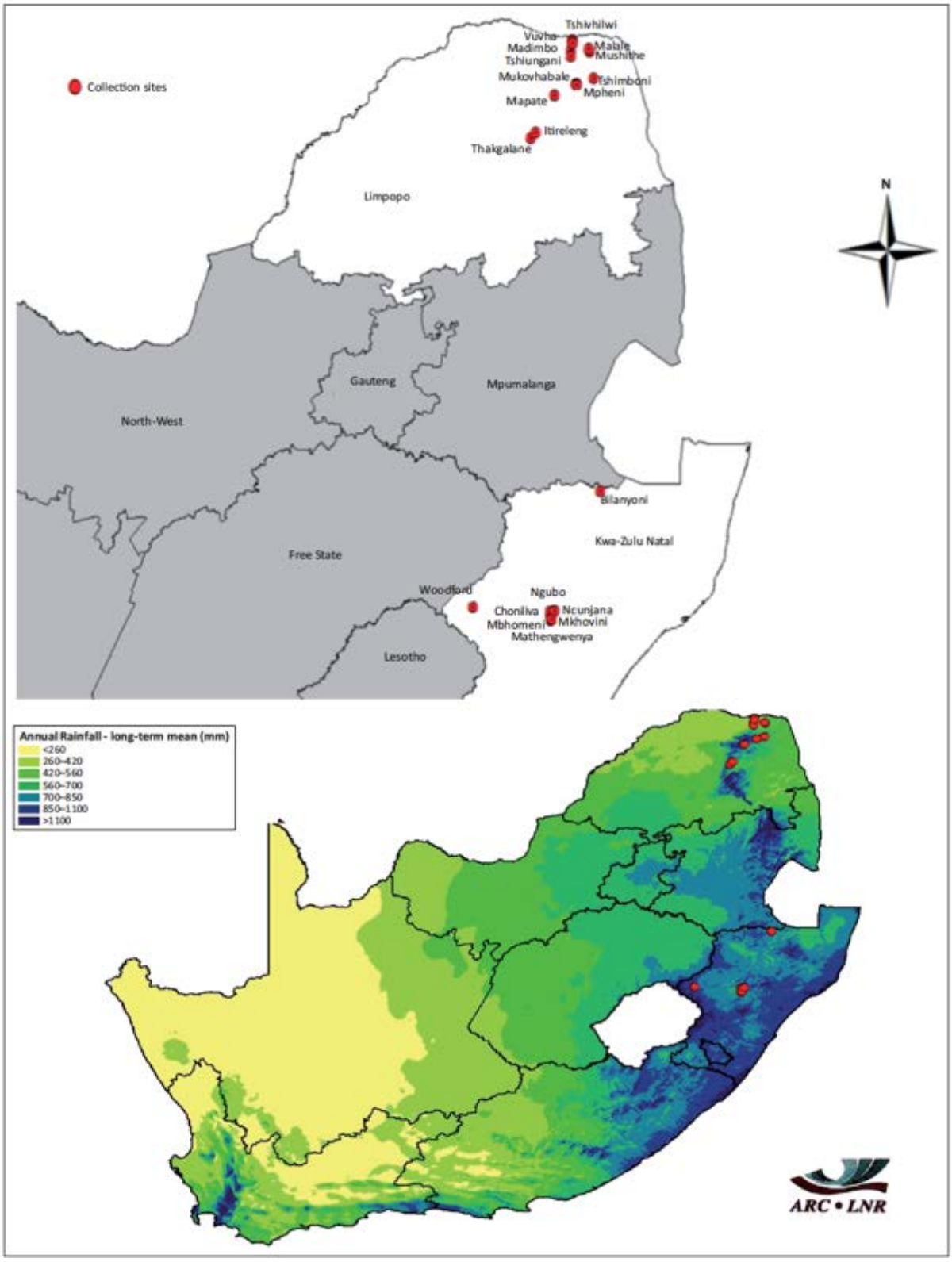

Figure 3.1: Map of South Africa showing sampled villages (Red dots) in Limpopo and KwaZulu-Natal provinces (Roy Williams, ARC-OVI)

3.2.2. *Questionnaire survey*

One on one interviews with farmers were conducted with assistance from the agricultural extension officers from the Department of Agriculture in Vhembe district, Limpopo and from community extension personnel of the Mdantsane Non-Governmental Organization in Tugela Ferry in KwaZulu-Natal between November 2012 and February 2013. Standardized questionnaires were administered to the eighty-seven randomly selected households from villages of Limpopo (n=39) and KwaZulu-Natal (n=48) provinces. The questionnaires were semi-structured with both closed and open-ended questions that were designed to capture information on the village chicken production systems with emphasis on the different livestock species kept by farmers, roles of village chickens, chicken nutrition, housing and health management and access of farmers to agricultural extension services. Information was also captured during these interviews on any chicken disease or clinical signs observed by farmers in their flocks. Farmers were then asked to rank the prevalent diseases or clinical signs in order of importance. Chicken production parameters that included number of eggs per clutch per hen, number of egg clutches per hen per annum and number of eggs that hatched per clutch were recorded. Farmers in both provinces did not keep farm records and as such data collected was based on farmer recall.

3.2.3. Sample collection and parasite identification

A total of 191 free-ranging village chicken faecal samples were collected from individual village chickens from the same households interviewed in Limpopo (102 faecal samples from 34 households) and KwaZulu-Natal provinces (89 faecal samples from 47 households). Freshly voided faecal samples were collected by following the chickens within the household, chicken pens and the surroundings. After collection, faecal samples were stored at 4 °C until further analysis to prevent the eggs of parasites such as *A. galli and Heterakis gallinarum* from hatching. The modified quantitative McMaster floatation technique was used to examine faecal samples (MAFF, 1986).

In addition to the faecal samples, 144 live matured chickens were purchased from the same villages in Limpopo (n = 99) and KwaZulu-Natal (n = 46). These chickens were slaughtered and the GIT were removed from the proventriculus to the cloaca after which each region was cut open by dissection following the World Association for the Advancement of Veterinary

Parasitology (W.A.A.V.P.) guidelines for evaluating the effectiveness of anthelmintics in chickens and turkey (Yazwinski *et al.*, 2003). All gastrointestinal parasites visible to the naked eye were recovered from the GIT using thumb forceps, washed and stored in 70% ethanol at ambient temperature awaiting parasite identification. Identification of each parasite was done based on the morphological parameters using the helminthological keys (Soulsby, 1982; Norton & Ruff, 2003).

3.2.4. Data analysis

Statistical analysis system (SAS) was used to analyze questionnaire-derived variables such as flock size, flock composition, diseases, disease clinical signs, internal parasites, external parasites, vaccination, treatment and access to veterinary services. Descriptive statistics using Generalized Linear Model procedures, SURVEYMEANS and SURVEYFREQ procedures were computed and presented as tables and graphs. The level of significance was considered at $P \leq 0.05$. For each province, the number or eggs per clutch per hen, number of egg clutches per hen per annum and number of eggs that hatched per clutch were calculated for clutch size, mean number of clutches and percentage hatchability respectively.

The prevalence of each recovered and identified GIT parasite was calculated as the number of chickens infested with that particular parasite species, divided by the total number of chickens sampled (Thrusfield, 1990). The prevalence of the gastrointestinal parasites was calculated per province using SAS (SAS, 2003). The mean intensity was determined by dividing the total number of recovered parasite of a particular species by the number of infected chickens with that parasite (Bush *et al.*, 1997). Abundance was calculated by dividing the number of parasites of a particular species by the total number of chickens examined (Bush *et al.*, 1997).

3.3. Results

3.3.1. Village chicken flock sizes and composition

A total of 858 and 1351 village chickens were reported by the village farmers in Limpopo and KwaZulu-Natal provinces respectively (Table 3.1). The least square means ± standard error (LSM±SE) of flock size per household was 22.03±2.85 for Limpopo province and 28.40±2.57 for KwaZulu-Natal province. Flock sizes varied between farms within provinces and about

44.68% of the farmers from both provinces had between 10 and 30 chickens. Twenty-seven percent of the farms owned between 30 to 50 chickens and 8.5% owned 50 to 100 chickens. Only 6.3% of the interviewed farmers had less than 10 chickens. Cock:hen ratios of 1:3 and 1:4 were observed in Limpopo and KwaZulu-Natal provinces respectively.

Table 3.1: Least squares means (LSM) ± standard error (SE) of the flock sizes and composition in Limpopo and KwaZulu-Natal provinces of South Africa

	Limpopo Province (N=39 households)		KwaZulu-Natal province (N=48 households)	
	Total number of chickens	LSM±SE	Total number of chickens	LSM±SE
Flock size	858	22.03±2.85	1351	28.40±2.57
Hens	374	9.59±1.51	638	13.50±1.36
Cocks	125	3.23±0.44	152	3.19±0.40
Chicks	359	9.21±1.08	561	11.71±1.62

3.3.2. Role of village chickens

Village chickens were predominantly kept for providing meat for household consumption (51% and 37%), selling (15% and 2%) and a combination of meat and selling (25.6% and 29.2%), of meat and eggs (5.1% and 4.2%) in Limpopo and KwaZulu-Natal provinces respectively as well as other functions including investment and rituals (Table 3.2). Most farmers indicated that they prefer village chicken meat as a supplement to their nutritional diets because it is tastier compared to intensively raised commercial breeds. Families also benefited indirectly from rearing village chickens through the use of manure for vegetable gardens. Farmers also indicated that chickens control weeds and insect pests by foraging.

Table 3.2: The percentage of farmers reporting the different roles of village chickens in the selected villages of Limpopo and KwaZulu-Natal province of South Africa

	Province	
	Limpopo	KwaZulu-Natal
Number of households	39	48
Meat	51.28	37.5
Selling	15.38	2.08
Investment	0	2.08
Meat and selling	25.64	29.17
Meat and eggs	5.13	4.17
Meat and investment	0	12.5
Meat, selling and rituals	2.56	10.42

3.3.3. Village chicken production systems

In addition to rearing village chickens, farmers in both provinces kept goats and cattle as well as sheep, pigs, and donkeys (Figure 3.2). Chickens were the predominant species in both provinces followed by cattle and goats. In Limpopo, 94.9% farmers provided chicken housing at night. Chicken structures in this province were made from locally available materials such paper boxes, scrap wood from discarded household furniture and wooden poles. Farmers in KwaZulu-Natal province did not provide housing for their chickens which will find shelter in trees and homestead kitchens. Village chickens in both provinces were left to scavenge around the homestead and the surrounding for feed and water during daytime. The typical diet for the village chickens consisted of locust, earthworm, grass, insects, disposed food and vegetables. Scavenging feed was supplemented with kitchen leftovers (16.72% and 4.59%) maize grain (28.73% and 52.26%), commercial feed (16.68% and 1.15%) and a combination of maize grain and husks and other leftovers (30.64% and 1.15%) in Limpopo and KwaZulu-Natal provinces respectively. Due to the nature of production system practiced, farmers did not have a well-organized and controlled chicken-breeding program. Broody hens would naturally incubate their own eggs, and none of the farmers practiced artificial incubation.

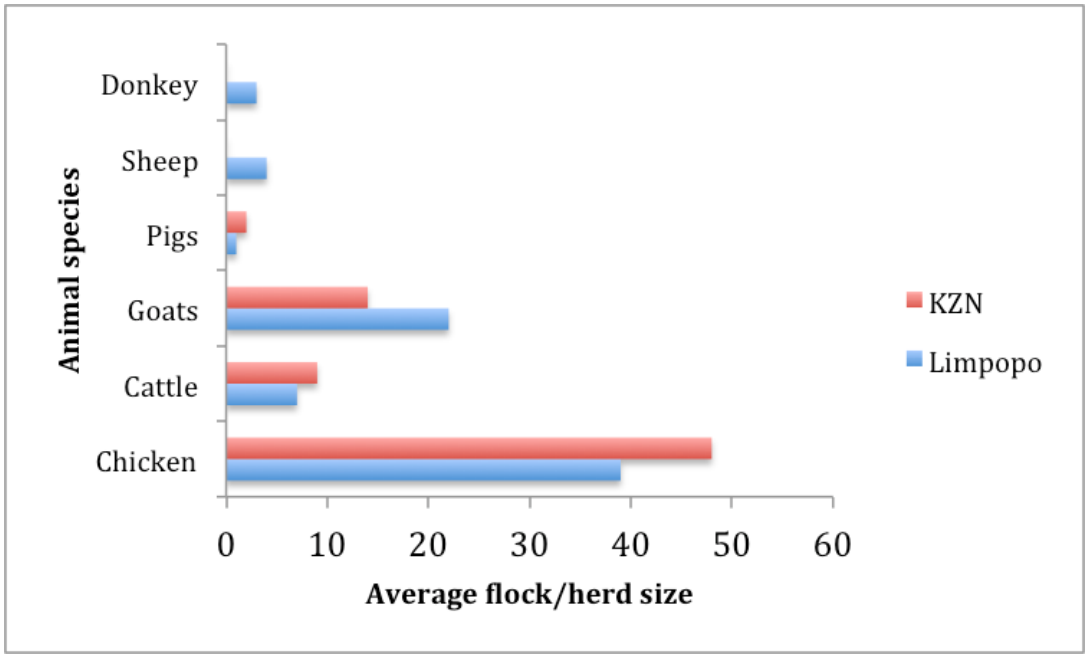

Figure 3.2: Livestock owned by farmers in the study areas of Limpopo and KwaZulu-Natal provinces. X-axis indicates the number of animals owned by farmer and y-axis shows the animal species owned

Hens laid an average of 3 clutches per year with an average of 12.08 and 13.64 eggs per clutch in Limpopo and KwaZulu-Natal provinces respectively. The average hatchability rate was 76.32% and 78.31% for Limpopo and KwaZulu-Natal provinces respectively. Farmers increased their flocks by sharing chickens (6.89% and 22.98%) amongst friends and relatives in Limpopo and KwaZulu-Natal provinces respectively.

Over 71.80% of the farmers reported that Newcastle disease (NCD) was the most important constraint that caused chicken mortality in both provinces. Twenty-three and forty-nine percent of the farms in Limpopo and KwaZulu-Natal provinces respectively were affected by NCD. Other diseases of importance were fowl pox and infectious bursal disease. The most predominant chicken disease clinical signs observed by farmers in descending order of importance in both provinces were generalized weakness (3.44% and 11.49), swollen eyes (6.89% and 6.10%), diarrhea (8.05% and 0%), coughing (5.75% and 1.15%) in Limpopo and KwaZulu-Natal, respectively. Other clinical signs included chickens being unable to walk (3.44% and 2.29%) and salivation (1.15% and 0%) in Limpopo and KwaZulu-Natal provinces, respectively. Of all farmers interviewed during this study, only 6.89% in Limpopo and 29.32%

in KwaZulu-Natal had observed gastrointestinal parasites on chicken droppings. A proportion of 11.49% and 66.23% of the farmers experienced the presence of external parasites on their chickens in Limpopo and KwaZulu-Natal provinces respectively.

3.3.4. Vaccination, treatment and access to veterinary interventions

Ninety four percent of the interviewed farmers in KwaZulu-Natal province used NOBILIS® ND CLONE 30 vaccine that was provided by Mdantsane Non-Governmental Organization to vaccinate their chickens against NCD. Other farmers in KwaZulu-Natal province used these vaccines to treat their chickens against diseases as they only administered it when chickens already developed clinical signs. The frequency of vaccination varied among farms depending on the knowledge they had and also on the clinical signs observed in the chicken flocks. Most of the farmers (52.10%) administer the ND clone vaccine once every two months whereas 14.58% used it every month, and 12.5% of the farmers used it once per annum. A total of 10.42% of the farmers used vaccination randomly or when need arose. Farmers in Limpopo province did not use any vaccination to manage the health of their animals.

In Limpopo province, 17 (43.59%) of the interviewed farmers used the ethno-veterinary medicine (EVM) such as aloe, garlic and hot chilli pepper for treating chicken diseases and parasites. Farmers implied to have more faith in the use of piperazine, Jeyes fluid, and laundry powders in the same order. None of the interviewed farmers in KwaZulu-Natal province used EVM and none of the farmers from both provinces used antibiotics or/and anthelmintic to treat diseases and parasites. It was also observed that all farmers (100%) in the surveyed villages of Limpopo province have never received extension support from the Department of Agriculture due to limited extension services available. In KwaZulu-Natal, a total of 47 farmers (97.92%) received veterinary interventions from a Non-Government Organization called Mdantshane.

3.3.5. Prevalence of the gastrointestinal parasites

Sixteen (15.73%) and 43 (42.16%) chicken faecal samples were positive for gastrointestinal parasites in KwaZulu-Natal and Limpopo provinces respectively. Six different parasitic species were detected, included nematodes *A. galli, H. gallinarum* and *capillaria* species, tapeworms *Choanotaenia infundibulum* and *Raillietina cesticillus* and the protozoan species, *Eimeria* were identified in both KwaZulu-Natal and Limpopo provinces (Table 3.3). Five (4.90%) samples

from Limpopo and four (4.49%) from KwaZulu-Natal had mixed infections in which the animal was infected by more than one species. *Ascaridia galli* was the most prevalent parasite at 17.65% in Limpopo, followed by *Eimeria* species (13.73%), *Heterakis gallinarum* (8.82%), *Raillietina cesticillus* (4.90%), *Capillaria* species (2.94%) and *Choanotaenia infundibulum* at 1.0%. The results from KwaZulu-Natal province indicated a lower prevalence of gastrointestinal parasites except for *Eimeria species*, that were observed at 15.6% (Table 3.3). The prevalence of *Heterakis gallinarum* was 6.74%, and that of *Ascaridia galli*, *Capillaria* species and *Raillietina cesticillus* were both 1.1% in the KwaZulu-Natal province. There were no trematodes observed in any of the samples.. Although variation in the prevalence of these parasites was observed between the two different provinces, they were not statistically significant.

Table 3.3: Prevalence (%), least squares means (LSM ± SE) and range of gastrointestinal parasites species from faecal samples from village chickens of Limpopo and KwaZulu-Natal provinces of South Africa

	Limpopo Province (N=102 Faecal samples)			Kwa-zulu Natal Province (N=89 Faecal samples)		
Parasite type	Prevalence	LSM±SE	Range	Prevalence	LSM±SE	Range
Nematode						
Ascaridia galli	17,65	0.18±0.04a	0-2600	1,12	0.011±0.011b	0-50
Heterakis gallinarum	8,82	0.09±0.03	0-1360	6,74	0.07±0.03	0-250
Capillaria species	2,94	0.03±0.02	0-200	0	0	0
Cestodes						
Choanotaenia infundibulum	0,98	0.01±0.01	0-150	1,12	0.01±0.01	0-50
Raillietina cesticillus	4,9	0.05±0.02a	0-200	0	0b	0
Protozoa						
Coccidia species	13,73	0.14±0.03	0=1500	15,73	0.16±0.04	0-750

Means with different superscript in the same row are significantly different (P <0.05); Figures in range columns represent the range for the actual values (untransformed data) of eggs per gram of faeces.

Twenty-nine (64.44%) of the 45 chickens slaughtered in KwaZulu-Natal, were positive for either one or two adult parasite species. Mixed infections of *Ascaridia galli* and tapeworm were

observed in 27.59% of the infected animals whilst the remaining 72.41% were positive for only one parasite species. In Limpopo province, 36 (36.36%) of the 99 slaughtered chickens were positive with at least one parasite species. Tapeworms were the most prevalent parasites at 75%, followed by *Ascaridia galli* at 52.78% and *Heterakis gallinrum* at 8.33% in this province. Mixed infections were observed in 15 (41.67%) of the intensines and (6.67%) of which *Ascaridia galli* and *Heterakis gallinurum* and 93.33% had *A. galli* and tapeworms. The species of tapeworm were not identified.

A total of 201 parasites from 36 positive chickens and 228 from 29 positive chickens were observed in Limpopo and KwaZulu-Natal provinces respectively (Table 3.4). The average number of worms recovered per animal was 5.9± 5.43 in Limpopo and 7±12.39 in KwaZulu-Natal province. The chickens that harboured more worms were observed in KwaZulu-Natal province where a total of 150 *A. galli* parasites/chicken were recovered.

Table 3.4: Total worm count and mean worm intensity for slaughtered free-range chickens from the selected villages in Limpopo and KwaZulu-Natal province of South Africa

	Total worm count (Mean worm intensity/bird)		
Parasite species	Limpopo Province	KwaZulu-Natal Province	Total per species
Number of chickens	36	29	65
Nematode			
Ascaridia galli	65(3.42)	166 (7.2)	231 (8.71)
Heterakis gallinarum	26 (8.67)	0 (0)	26
***Cestodes**			
Tapeworm	110 (4.07)	62 (4.13)	172
Total worm count per province	201	228	

*Cestodes were not identified to the species level.

The chicken that carried these 150 parasites was however not included in the statistical analysis as it was considered an outlier that was going to inflate the average number of worms per animal. In KwaZulu-Natal province, the average intensity of infection was highest for *A. galli* with average worm count of 7.2±13.76 per chicken. The highest mean abundance of infection

was seen in *A. galli* (3.61±10.28 worms/chicken) followed by tapeworm (1.35 ±2.13 worms/chicken) (Table 3.5). In Limpopo, the intensity was high for *Heterakis gallinarum* at 8.67 ±2.32 worms per chicken. Mean abundance of infection, was high at 1.11±3.27 worms per chicken for tapeworms.

Table 3.5: Mean worm abundance ± standard deviation (SD) from the intensines of slaughtered chickens from Limpopo and KwaZulu-Natal province of South Africa

Parasite species	Province	
	Limpopo (N = 99 chickens)	KwaZulu-Natal (N=46 chickens)
	Abundance ± SD	Abundance ± SD
Nematodes		
Ascaridia galli	0.66 ±1.65	3.61 ± 10.28
Heterakis gallinarum	0.25 ±2.05	0
***Cestodes**		
Tapeworm	1.11 ± 3.27	1.35 ±2.13

*Cestodes were not identified to the species level.

3.4. Discussions

This study reported the village chicken production systems and associated parasite infections in two provinces of South Africa. Village chicken production in South Africa is similar to most smallholder farming systems where the chickens are exposed to the harsh environmental and production challenges coupled with farmers having limited resources to manage their flocks (Acamovic *et al.*, 2005). An understanding of the dynamics of helminth infection within the context of the production systems and challenges is therefore crucial to the development and implementation of effective parasite control strategies. This study sampled from villages of Limpopo and KwaZulu-Natal provinces of South Africa, which are similar to villages in most African and other developing countries.

The proportion of hens was high in both provinces, which is in contrast to other studies that observed village chicken flocks being dominated by chicks (Maphosa *et al.*, 2004; Muchadeyi

et al., 2004). It was expected that high rates of helminth infections would be observed with increased flock sizes because of the increased number of animals per area unit that leads to larger amounts of faeces deposited on the ground and possibly increased infectivity per unit area (Permin & Hansen, 1998). The relatively low levels of helminth infections observed regardless of flock numbers in KwaZulu-Natal province could be attributed to the extensive management of these chickens and the hot and dry conditions during sampling, which negatively affects the development of parasite eggs into infective stages and their survival in the environment (Permin & Hansen, 1998). Farmers in both provinces practiced mixed livestock farming and owned goats, cattle, sheep and pigs in addition to chickens. The presence of other animal species in mixed livestock farming systems can however expose and increase the risk of parasite infestation in village chickens as other species could act as carriers of certain parasites (Permin & Hansen, 1998).

The majority of chickens in this study scavenged for their feed, an observation that was consistent with other studies (Muchadeyi *et al.*, 2007; Mwale & Masika, 2009). Whilst this is a viable option for resource-limited farmers, scavenging for feed results in poor quality nutrition and also exposes the chickens to predation, diseases and parasites (Acamovic *et al.*, 2005). Scavenging chickens are also exposed to the open air and environment, and have greater contact with host organisms such as insects and the earthworm where they can get infections. Insects and earthworms are intermediate hosts that may indirectly transmit the parasite eggs and infective stage of nematodes to chickens on consumption (Butcher & Miles, 2009). The prevalence of parasites in Limpopo province would probably be lower if the chicken housing was well managed with cleaning, removal of the droppings and application of disinfectant regularly (Pedersen 2002; Kusina & Kusina 2000). Farmers in the surveyed villages were also observed to exchange animals with their neighbors and nearby community members. According to the conventional commercial chicken management practices (Kitalyi, 1998), new and introduced stock needs to be isolated and monitored for a certain period of time so that the farmer does not introduce diseases and parasites to the farms. Such practices though challenging for smallholder communal farmers, can help in reducing the introduction and spread of new pathogens between neighboring farms and/or communities in village chicken and other livestock production systems.

The reported diseases and clinical signs were merely based on farmer's perceptions and their

limited knowledge of clinical signs. The majority of the farmers did not have access to veterinary extension services whilst those in KwaZulu-Natal province received extension services from a non-governmental organization working in that area. The administration of vaccines randomly without clear indications for the need for vaccinations and proper procedures being followed could result in poor response to vaccines.

The prevalence of *A. galli* in both provinces was higher than the 10%–14.5% reported in Kenya (Irungu *et al.*, 2004a). However, the prevalence of nematode infections (combination of *A. galli* and *Heterakis gallinarum* prevalences) was low in comparison with those observed in Zambian villages which revealed that 28.8% and 32.8% of the chickens were infected with *A. galli* and *Heterakis gallinarum* respectively (Phiri *et al.*, 2007), and those in Palestinian chickens where the prevalence *A. galli* and of *Heterakis gallinarum* was 75.6% and 68.9% respectively (Rayyan & Al-Hindi, 2010). In this study, variation in the prevalence of these parasites was observed between the two different provinces although they were not statistically significant. (Mukaratirwa & Khumalo, 2010) observed more parasite species and relatively higher prevalance of *A. galli, H. gallinarum* and *Capillaria* species in coastal KwaZulu-Natal than was observed in this study. The significantly lower prevalence of *A. galli* and *R. cesticillus* parasites in KwaZulu-Natal and the relatively lower prevalence of the other nematodes and tapeworms was probably because of the generally dry summer months in the sampled localities of Msinga in contrast to Limpopo province where it rained during the days of sampling. In both provinces, all parasitized chickens harboured 1 to 6 helminth species, which was comparable to a study by Mukaratirwa and Khumalo, (2010) but less than that from other studies that observed up to 7, 10 and 13 species of gastrointestinal helminths (Permin *et al.*, 2002; Phiri *et al.*, 2007). The level of mixed infection observed in this study was expected and are common in village chickens (Phiri *et al.*, 2007). The total worm burden observed in this study was low as compared to the reported 12.5 ± 5.8 worm/bird in native chicken of Jordan and 19.9 ± 4.3 in Lohmann LSL white (Abdelqader *et al.*, 2007).

No trematodes were observed in the faecal samples and GIT of the village chickens in this study which is concurrent with findings from previous studies in different populations (Abdelqader *et al.*, 2008; Mukaratirwa & Khumalo, 2010). However, this was in contrast with the study by Mwale & Masika (2011) who identified trematode *Postharmostomum gallum* and *Postharmostomum communtatum* Qolora by-sea and Nontshinga villages in Amathole district

Municipality, South Africa. According to Mwale & Masika (2011), trematodes require a wide range of intermediate hosts such as dragonflies and freshwater snails to complete their life cycle which may not be available in most production systems and as such are rare.

3.5. Conclusion

Overall the study described the chicken production systems of typical smallholder village chicken farming system. As observed in similar systems, village chickens contribute to the livelihood of the many families in marginalized communities of South Africa. The scavenging production system coupled with minimal management inputs and lack of knowledge on chicken health exposes the village chickens to different diseases and various internal and external parasites. Economically important parasites such as *A. galli* and *Eimeria* species were prevalent in both provinces with variations in worm burdens and infection intensity. Overall, this study presents gastrointestinal parasites as a problem affecting the village chickens of Limpopo and KwaZulu-Natal provinces. Prevention and control of parasites in these farming systems will be compromised by the mixed farming systems, the limited resources at the farmers' disposal for chicken management and the absence of biosecurity measures to avoid disease and parasite transmission within chickens and inter-species.

3.6. Conflicts of interest

The authors declare no conflict of interest.

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Chapter 4:

Population genetic structure of *Ascaridia galli* from extensively raised chickens of South Africa inferred using cytochrome c oxidase subunit 1 gene

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Short communication

Published in Veterinary Parasitology 2016, 216: 89-92

Abstract

Ascaridia galli is one of the most common nematode affecting chickens. This study characterized *A. galli* parasites collected from South African village chickens of Limpopo (n =18) and KwaZulu-Natal (n=22) provinces using the 510bp sequences of the cytochrome C oxidase subunit 1 gene of the mitochondrial DNA. Fourteen and 12 polymorphic sites were observed for Limpopo and KwaZulu-Natal sequences, respectively. Six haplotypes were observed in total. Haplotype diversity was high and ranged from 0.749 for Limpopo province to 0.758 for KwaZulu-Natal province isolates. There was no genetic differentiation between *A. galli* from Limpopo and KwaZulu-Natal provinces. The six South African haplotypes were unique compared to those published in the GeneBank sampled from Hy-line chickens raised under organic farming in Denmark. The utility of cytochrome C oxidase subunit 1 gene as a potential genetic marker for studying *A. galli* in village chicken populations is presented.

Keywords: *Ascaridia galli;* cytochrome c oxidase subunit 1; genetic diversity; village chickens

4.1. Background

Ascaridia species are among the most prevalent and pathogenic parasitic nematodes found in domestic and wild birds that have a worldwide distribution (Abdelqader *et al.*, 2008). *Ascaridia galli* damages the intestinal mucosa leading to blood loss and secondary infection and occasionally the obstruction of the small intestines of chickens that occurs due to high worm burdens (Soulsby, 1982). Information on the genetic diversity and population structure may be of importance to understanding the genetic properties influencing pathogenicity and response to treatment regimes of any disease pathogen, as well as in tracing of the escalating spread of drug resistance among parasitic nematodes (Kaplan, 2004).

Mitochondrial DNA sequences have been useful markers in studies of genetic variability and population structure in livestock (Muchadeyi *et al.*, 2008) and animal pathogens (Walker *et al.*, 2007). Since mitochondrial genomes evolve 5–10 times faster than nuclear genomes (Brown *et al.*, 1982) probably due to lack of replication repair mechanism (Clayton, 1982), they are suitable for discriminating closely related organisms (Le *et al.*, 2001; Kaplan, 2004) especially

at the species and sub-species levels (Galtier *et al.*, 2009). Networks analysis of mtDNA sequences has been widely used in phylo-geographic analysis (Bandelt *et al.*, 1999).

The majority of South African chickens are raised extensively by smallholder farmers under low-input scavenging systems and chickens are therefore exposed to parasites and other disease pathogens during scavenging. The type and level of parasitic infections vary across provinces, which make it imperative, for disease control, to characterize parasites from different productions. Traditional control of gastrointestinal nematode infections depends on the use of anthelmintics (Woolaston & Baker, 1996), which are often unavailable in low input production systems. Genetic control strategies that depend on the use of natural resistance and tolerance of host chicken populations to prevailing parasites could be an alternative for disease control in village chicken production systems (Lamont, 1998). However, the success of genetic control strategies depends on a good understanding of the pathogens. This study used *Cox*1 gene of the mitochondrial DNA as a genetic marker to investigate the genetic variability of *A. galli* parasites collected from village chickens in South Africa.

4.2. Methods

A total of 144 non-descript village chickens kept by smallholder communal farmers were sampled from two geographically distinct provinces of Limpopo (n = 99) and KwaZulu-Natal (n = 45) in South Africa. The distance between the villages were between 25 and 160 km and 20 and 140 km in Limpopo and KwaZulu-Natal provinces, respectively. Chickens were slaughtered by cervical disarticulation and the gastrointestinal tracts were removed according to the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines (Yazwinski *et al.*, 2003). Briefly, the whole intestine was removed and placed in a tray. It was then opened in a longitudinal section with a pair of scissors from the gizzard to the cloaca. The intestinal contents were recovered and gastrointestinal parasites visible with a naked eye were identified based on the morphological parameters using the helminthological keys (Soulsby, 1982).

DNA was extracted from adult *A. galli* worms using the QIAamp blood and tissue kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was performed using gene-specific primers (G*Cox1*F4F, 5-ATTATTACTGCTCATGCTATTTTGATG and G*Cox*14R, 5-

CAAAACAAATGTTGATAAATCAAAGG) (Katakam *et al.*, 2010). PCR amplicons were directly sequenced using the same primers on ABI3500 XL genetic analyzer at Inqaba-Biotec, Pretoria (South Africa). Sequence data as assembled and edited using GAP4 of the Staden package (Version 1.6.0) (Staden and Beal, 2000) to obtain the 510 bp consensus sequence. Sequences were aligned with the available sequences of related genera downloaded from GenBank (Supplementary Figure S1) using ClustalX version 2.1 (Larkin *et al.*, 2007). The haplotype sequences produced in this study were deposited into GenBank with these accession numbers: KT388435 to KT388440.

The *Cox*1 gene sequences diversity indices were determined using DnaSP software version 5.10.01 (Rozas *et al.*, 2003). The level of differentiation within and amongst populations was estimated using analysis of molecular variance (AMOVA) implemented in ARLEQUIN v3.5 (Excoffier & Lischer, 2010). Gene flow between populations was estimated based on the F_{ST} derivative of migration (Nm=(1/1+2N*m*) over 1000 simulations using DnaSP 5.10.01 (Wright, 1951; Rozas *et al.*, 2003). The Tajima's D (Tajima, 1989) and Fu's Fs tests (Fu, 1997) were also performed using DnaSP 5.10.01 for mismatch distributions of exponential population growth.

The forty sequences from Limpopo and KwaZulu-Natal provinces together with four partial *Cox*1 *A. galli* sequences from the GeneBank were aligned and trimmed to produce overlapping sequences that were 393bp long. NETWORK 4.1.0.8 (Fluxus Technology Ltd.) was used to generate intra-species median-joining networks and investigate the possible evolutionary relationships among all sequences of each identified haplotype (Bandelt *et al.*, 1999).

4.3. Results and discussion

Twenty-nine (64.44%) of the 45 chickens slaughtered in KwaZulu-Natal province and 36 (36.36%) of the 99 chickens slaughtered in Limpopo province were positive for one or two adult parasite species. A total of 201 and 228 parasites were recovered from Limpopo and KwaZulu-Natal provinces, respectively. In Limpopo province, tapeworms had the highest prevalence of 54%, followed by *A. galli* and *Heterakis gallinarum* at 32% and 13%, respectively. The prevalence of *A. galli* was 72% and that of tapeworm 27% in KwaZulu-Natal province. The average intensity of infection for *A. galli* in KwaZulu-Natal province was highest

with average worm count of 7.2±13.76 per chicken whilst that for Limpopo was 3.42 ± 2.34. A detailed analysis of prevalence and worm burdens in the two provinces was presented by Malatji et al. (2015). Overall, 40 *A. galli* parasite worms sampled from chickens from Limpopo (n =18 chickens) and KwaZulu-Natal provinces (n= 22 chickens) were sequenced.

The 40 sequences were closely related to *A. galli* mitochondrion, complete genome (Accession no. JX624728), *A. galli* haplotype I (Accession no. GU138668), *Ascaridia galli* haplotype II (Accession no. GU138669), *A. galli* haplotype III (Accession no. GU138670), and *A. galli* partial *Cox*1 gene (FM178545) with nucleotide identity ranging from 97% - 99%. Mitochondrial DNA polymorphisms were observed amongst the samples from different geographical localities as illustrated in Supplementary Figure S2.

Fourteen polymorphic positions were observed, which defined 6 haplotypes (Table 4.1). Haplotype diversity (Hd) of the two populations was moderate for both provinces and ranged from 0.749 for Limpopo province to 0.758 for KwaZulu-Natal province (Table 4.1). The average nucleotide diversity pi (π) was 0.013 with an average number of nucleotide differences of k = 5.213 between populations. Haplotype diversity was higher compared to that observed in other *Ascaris* of pigs and humans. A lower haplotype diversity ranging from 0.138 – 0.605 was reported for *Ascaris lumbricoides* and from 0.553 – 0.741 for *Ascaris suum* in China (Zhou *et al.*, 2011).

Table 4.1: Sequence diversity indices of *Cox*1 gene of mtDNA in Limpopo and KZN *A. galli* parasite populations

Population	N	S	H	Hd	π	k
Limpopo	18	14	6	0.749	0.013	5.150
KZN	22	12	4	0.758	0.014	5.307

N: Number of sequence used; S: Number of segregation site; H: Number of haplotypes; Hd: Haplotype diversity π: Nucleotide diversity k: Average number of differences.

The AMOVA analysis indicated no significant genetic differentiation among the populations (P-value = 0.584). The genetic variation within and among populations was 0.379 and 0.006, respectively. The genetic differentiation was mainly observed within populations. A low

fixation index (F_{ST}) value of 0.017 (P-value = 0.584) was observed that indicated no differentiation between the overall population and its subpopulations. Absence of population substructure may be an indicator of common maternal origin of parasites from different provinces. This observation was different from what was observed with Swedish and Danish *A. galli* populations that were moderately sub-structured according to farms and geographical areas (Höglund *et al.*, 2012) despite the higher geographic distances between the South African provinces sampled (> 700km) compared to that between Swedish farms that ranged from approximately 125 km to 324 km. However, genetic diversity within population was higher in the current study than reported in previous studies where most of the genetic variation between the nematodes was contained within individual Jönköping and Danish hens (Höglund *et al.*, 2012).

The *A. galli* sequences had a positive Fu's Fs value of 0.625 (P = 0.016) and Tajima's D value of 1.842 (P > 0.05). Mismatch distribution analysis of the complete datasets revealed the presence of a multi-peak (Figure 4.1), which suggests that there was no rapid expansion event that occurred in the South African's *A. galli* population demographic history and a gene flow (*Nm)* value of -30.53 was observed. The large and negative *Nm* value in this study indicated less gene flow among the *A. galli* populations from the two provinces over time suggesting less movement of chickens between provinces. Höglund *et al.* (2012) observed a relatively high gene flow amongst Swedish *A. galli* isolates in contrast to our findings. The positive values from Tajima's D test signify that *A. galli* might not have experienced population expansion in the past. The Tajima's D and mismatch distribution analysis confirmed that *A. galli* from the two provinces were genetically similar and could not be considered as distinct populations. The high level of genetic similarity between the KwaZulu-Natal and Limpopo populations could therefore be due to other factors other than gene flow such as overlapping selection pressures and common founder effects between populations.

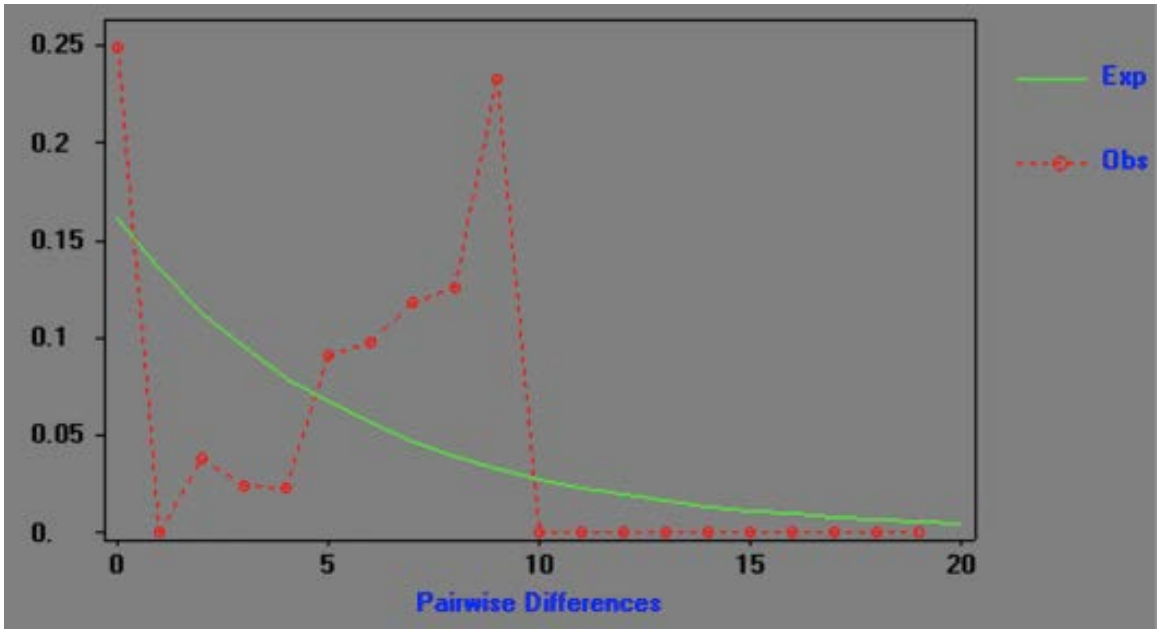

Figure 4.1: Mismatch-distribution to test the expansion of 40 *A. galli* isolates. The number of nucleotide differences between pairs of sequences is indicated along the X-axis, and their frequency along the Y-axis. The straight line represents the expected values and the dotted line represents the observed values

Four of the six haplotypes were shared between the two provinces (Figure 4.2). Katakam *et al.* (2010) reported the presence of three *A. galli* haplotypes (I, II and III). Network analysis revealed that the six haplotypes observed in this study clustered separately from each other and were novel in comparison to those found in Genbank. Four South African haplotypes (V, VI, VII and VIII) were shared between isolates from Limpopo and KwaZulu-Natal provinces whilst haplotypes IV and IX were restricted to Limpopo province. Haplotype VIII had the highest frequencies of 14 *A. galli* isolates followed by haplotype V (n=13), VI (n=6) and VII (n=5) whereas haplotype IV and IX consisted of only one individual each (Figure 4.2).

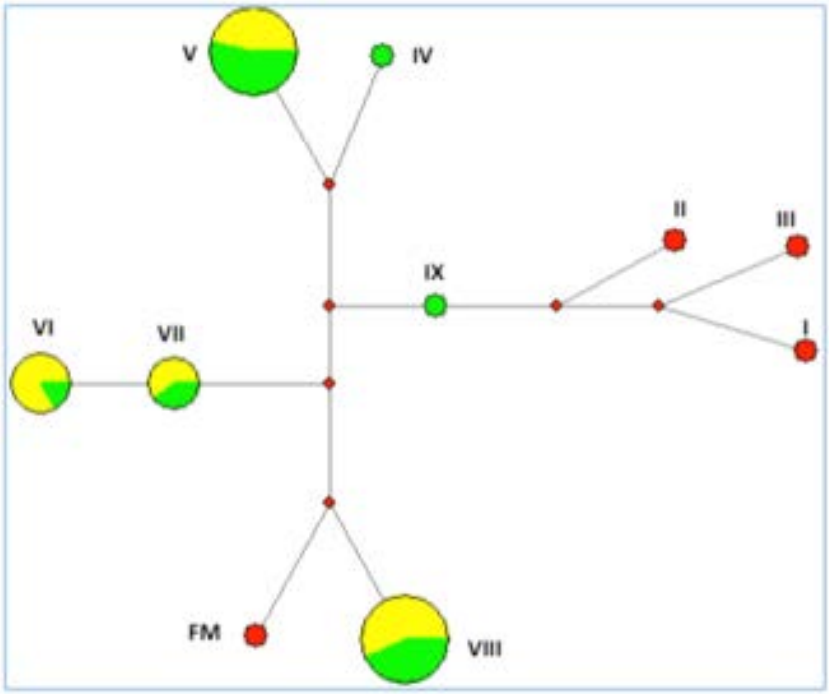

Figure 4.2: Median-joining networks depicting the genetic relationships among cytochrome c oxidase subunit 1 of A. galli. (mtDNA). The sizes of the circles and colored segments are proportional to the haplotype frequencies in the datasets. Green denotes KwaZulu-Natal, yellow refers to Limpopo and Red stands for Genebank sequences. Red diamonds are median vectors used in connecting indirectly related haplotypes.

This study presents the first attempt to genetically characterize *A. galli* parasites from village chicken populations of South Africa. The genetic similarity amongst the South African isolates could be useful in implementing helminths management and control strategies between South African provinces. The haplotypes observed in South Africa were different from those from Denmark, which suggests that unique *A. galli* control strategies might be necessary rather than replicating control regimes from other areas. Further characterization of the worms is required particularly focusing on pathogenicity profiles.

Competing interests

The authors declare that they have no competing interests.

Author Contributions

FCM (ARC-Biotechnology Platform) was the project leader. All authors were responsible for experimental and project design. DPM. (ARC-Biotechnology Platform; University of Pretoria) performed all the experiments, analysed the data and drafted the manuscript. EVM (University of Pretoria) and AMT (ARC-Onderstepoort Veterinary Institute) made conceptual contributions.

Acknowledgements

This study was funded jointly by the Agricultural Research Council-BTP and the NRF under the Zambia/South Africa bilateral Research Program. Ms Maltji received an NRF-DST Professional Development Program research fellowship and University of Pretoria postgraduate support bursary.

Ethical standards

Ethical approval for the study was obtained from the Animal Ethics Committee from the University of Pretoria (EC090-13) and Agricultural Research Council-Animal Production Institute (APIEC13/004).

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Chapter 5:

Transcriptome analysis of the small intestine of village chickens from *Ascaridia galli* infected environment

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Submitted for publication in Veterinary Research

Abstract

Nematodes of the genus *Ascaridia* are known to infect many species of birds and result in fatal diseases. *Ascaridia galli* damages the intestinal mucosa of chickens leading to blood loss, secondary infection and occasionally the obstruction of small intestines due to high worm burden. Analysis of differences in gene expression profiles associated with infestation and pathology status can provide an understanding into the molecular basis of the host immune response. This study aimed at investigating the gene expression profiles in chickens from two different provinces of South Africa that are naturally infected by the *A. galli* parasite. Moreover, the study investigated gene expression profile of *A. galli* infected duodenum, jejunum and ileum tissues. The small intestine from non-infected chickens did not show any presence of parasite and did not display detectable histological changes whereas the naturally *A. galli* infected intestines displayed hypertrophy of the intestinal villi with accumulation of inflammatory cells and necrosis of the crypt of Lieberkühn gland. Total RNA isolated from small intestines of infected and non-infected village chickens was sequenced using Illumina HiSeq technology to generate up to 23,856,130 reads. An average of 85.25% of trimmed and quality controlled reads were mapped to the reference chicken genome (*gallus.galgal4.74*) using open-access Tophat software. Cuffdiff was then used to analyze differentially expressed genes (DEG) in infected versus non-infected intestines. Between any two-way comparisons of the intestines, 277 and 190 transcripts were significantly expressed in Limpopo and KwaZulu-Natal chickens, respectively. Gene ontology analysis of the DEGs revealed an enrichment of genes proposed to function in immune response, defense response, inflammatory response and cell signaling genes. T cell receptor signaling pathways and arachidonic acid metabolism pathways were among the most significantly impacted pathways.

Key words: Nematode infections, chickens, transcriptomics

5.1. Introduction

The chicken is an important species in animal agriculture. It is a historically important model in genetics, developmental biology, and immunology, and the first farm species to have its genome fully sequenced in 2004 (ICGSC, 2004). Chickens are a major source of animal protein

around the world. In South Africa, indigenous chickens are raised by smallholder farmers under low-input production systems and they play an important role in alleviating poverty as they are predominantly kept for providing meat for household consumption and selling to generate some income (Mtileni *et al.*, 2009). Village chickens are left to scavenge to meet their nutritional needs (Mtileni *et al.*, 2009) which predisposes them to predators (Kusina & Kusina, 1999; Pedersen, 2002), diseases and parasites (Swatson *et al.*, 2003) that coexist in the scavenging environment. Parasite infestation contributes to poor production and can cause mortality in severe cases (Gauly *et al.*, 2005; Permin *et al.*, 2006; Daş *et al.*, 2010). Infection with *A. galli,* may directly contribute to economic losses due to higher feed conversion rates or reduced weight gain and decreased egg production (Permin & Ranvig, 2001; Skallerup *et al.*, 2005). *Ascaridia galli* has been identified in South African village chickens (Mukaratirwa & Khumalo, 2010; Mwale & Masika, 2011) and is known to damage the intestinal mucosa leading to blood loss, secondary infections and occasionally the obstruction of small intestine of poultry due to high worm burden (Soulsby, 1982).

The gastrointestinal tract is the largest interface between an animal's internal milieu and its exterior environment and is a major immune organ that represents one of the main combat zone in an animal's defense against invading pathogens (Kogut & Swaggerty, 2012). This is due to the large surface area, the nutrient rich luminal environment and the requirement for a thin lining permeable to nutrients (Freeman et al 2012). Through specialized receptors, the small intestine is able to sense and actively respond to changes in its environment. The intestine, as a first barrier, is in contact with commensal and pathogenic microorganisms (Abreu, 2010). The response to changes allow the intestine to contribute to the defense against pathogens and to the control and regulation of the local immune response. The intestinal epithelium is a sensor of the luminal environment, not only controlling digestive, absorptive, and secretory functions, but also relaying information to the mucosal immune, vascular and nervous systems (Pluske *et al.*, 1997). The intestinal epithelium as a critical component of a communications network that is essential for transmitting signals generated in response to infection with pathogens to cells of the innate and acquired immune systems in the underlying intestinal mucosa.

The normal habitat of the parasitic stages of *A. galli* is in the small intestine of poultry (Soulsby, 1982). The life cycle of *A. galli* is direct and involves a single host (Permin *et al.*, 1997). Worms that are sexually matured live in the lumen of the small intestine, whereas eggs containing

infective stage larvae (L3) develop in the surroundings (Permin *et al.*, 1997). Larvae moult inside the eggs until they become infective (L3), they do not hatch in the environment. After ingestion, the infective eggs are mechanically transported to the proventricalus and gizzard and further down to the duodenum where they hatch within the first 24 hours. The larvae burrow into the mucosal layer of the small intestine to enter the histotrophic phase following hatching (Ackert, 1931); Luna-Olivares *et al.*, 2012). The duration of the histotrophic phase is 3 to 54 days before the larvae return to the intestinal lumen where they reach final maturity (Permin & Hansen, 1998). After the histotrophic phase, the mature worms settle down in the lumen of duodenum where they live and feed on ingesta. It has been reported by (Ikeme, 1971a) that adult worms when present in large numbers, migrate up and down the intestinal lumen.

Identification of tissue-specific expression signatures has both theoretical and practical implications toward understanding host–parasite interactions. Gene expression is influenced by infection and can be measured on multiple tissues to give insight into the regulation of genes and pathways during infection. A study that compared small intestine (jejunum) with colon samples using deep sequencing of the intestinal mRNAs in mouse suggested region-specific expression patterns (Klostermeier *et al.*, 2011). Moreover, studies using microarray suggested that there is a clear difference between the gene expression patterns of proximal colon tissues and distal colorectal tissues in human healthy individuals (Glebov *et al.*, 2003; LaPointe *et al.*, 2008). Whole transcriptome comparison between the abomasum versus small intestine also identified five protein-coding genes that were uniquely expressed in one of the tissues. Another study observed a difference in expression patterns in different sections on porcine intestine suggesting significant variations in gene expression profiles that may control cellular gastrointestinal development (Freeman *et al.*, 2012). Differential gene expression analysis has been applied to the spleen of *A. galli* infected chicken (Dalgaard *et al.*, 2015) but limited studies has been done in the different sections of the small intestine of infected chickens. Previous studies support the variation in gene expression and therefore immune response mechanisims amongs tissues and tissue segment when infected by pathogens.

Traditional control of gastrointestinal nematode infections depends on the use of anthelmintics (Katakam *et al.*, 2010) and previously, *A. galli* control has been based on synthetic anthelmintics. These three anthelmintic drug classes are available to treat GINs, the imidazothiazoles, benzimidazoles and macrolides (Bowman, 2003). Concerns have been raised

on parasite drug resistance and left-over residues in food products which call for alternative disease control strategies such as genetic resistance (Sangster, 1999; Jackson & Miller, 2006). Genetic control strategies have been suggested as alternatives for disease and parasite control in chicken production systems (Lamont, 1998). Local immune homeostasis in the intestine is critical for both host health and commensal survival and at the same time is required to provide useful defense against harmful pathogens (Hill & Artis, 2009; Hooper & Macpherson, 2010). The gastrointestinal immune system of chickens is consequently highly specialized and composed of cellular and molecular components with complex functional and regulatory features (Hill & Artis, 2009; Hooper & Macpherson, 2010). Reduced penetration of parasitic larvae and persistence of developmentally arrested larvae, decreased worm fecundity, accelerated worm expulsion, and reduced pathology are all factors that contribute to the ability of chicken hosts to fighting infections. Considerable evidence supports the idea that traits associated with parasite resistance are under host genetic control (Conesa *et al.*, 2005). Genetic resistance to infection is likely as a result of heritable genes or genomic loci that have a direct or indirect role in expression of molecules that appropriately regulate host immunity to control infection and limit pathology. Variants in these genes and/or genomic loci can be applied to breeding programs to enhance parasite resistance.

Next generation sequencing technologies and bioinformatics have been established for analysis of host and pathogen genomes and quantification of transcriptomes, to produce insight in to the interactions between the host and pathogen (Li *et al.*, 2011; Li & Schroeder, 2012). RNA sequencing (RNA-seq) is a high-throughput method developed for profiling transcriptomes that is cost-economic, time saving and can generate transcriptome data for non-model species for which incomplete genome information is available (Wang *et al.*, 2009; McIntyre *et al.*, 2011). The approach allows gene expression profiling that in contrast to array-based techniques, allows for the characterization of both known and unknown gene transcripts. Furthermore, data outputs is based on read counts instead of microarray hybridization intensity signals, which renders the technology feasible in studying variations in transcript sequences (Mortazavi *et al.*, 2008).

Gene expression profiles aid to identify pathways regulated in response to infection. Investigation into differentially expressed genes can allow investigators to decipher gene expression profiles for responding to pathogen infections. Zulkifli *et al.* (2000), laid the

foundation for comprehensive genomics and transcriptomics studies in this species. Expression of immune related genes has been shown to be heritable in chicken. Parental chickens with high and low expression levels of immune related genes were shown to produce chicks with similarly increased or decreased expression levels of the same genes (Swaggerty *et al.*, 2008). The aim of this work was to investigate the gene expression profiles of small intestines of South African village chickens in response to *A. galli* infection. Furthurmore, the three segments of the small intestine (duodenum, jejunum and ileum) were investigated independently in order to analyze variations of gene expression that could play a role in the site-specific functionalities of the gastro-intestinal tract. The end goal was to make inferences on genes, pathways and biological processes that play a role in village chickens from gastrointestinal nematodes infected areas.

5.2. Materials and Methods

5.2.1. Chicken populations and intestinal tissue sample selection

A total of 144 chickens were purchased from villages in Limpopo (Limp) (99) and KwaZulu-Natal (KZN) (45) provinces. These provinces represent different agro-ecological zones of South Africa as Limpopo province is more of a dry are and KZN province been mild. The chickens were reared under scavenging system with no feed supplements and housing provided. The 144 chickens were slaughtered by cervical disarticulation. Of these 144 chickens, 42 were positive for *A. galli* infections, 19 and 23 from Limpopo and KwaZulu-Natal provinces respectively with worm burdens ranging from 1 to 150 worms per chicken. Two adult non-descript chickens of mixed gender each positive for *A. galli* and two negative for the parasites were selected from each province for histology and transcriptome analysis. Worm burdens were 8 and 14 for KZN (KZN4) and Limpopo (Limp5) *A. galli* positive chicken respectively. The time of infection is not well known, however, the worms collected coresponded to adult stage of parasite development. Chicken KZN1 and Limp39 were the chickens negative for *A. galli* infections from KwaZulu-Natal and Limpopo provinces respectively. The *A. galli* parasites that were recovered from Limpopo and KZN chickens indicated no significant genetic differentiation (Chapter 4) indicating that the parasite sharing some ancestral maternal lineages was infecting the chickens from the two provinces. From

each of the selected chickens, 1.5–2 cm long jejunum, ileum and duodenum tissues were collected for histological examination. Each sample was opened along the mesenteric border and placed in tissue cassettes with wet filter papers to avoid curling and folding of the tissue. Ethical approval for the study was obtained from the Animal Ethics Committee from the University of Pretoria (EC090-13) and Agricultural Research Council-Animal Production Institute (APIEC13/004).

5.2.2 Histology

The intestinal tissue samples were fixed in 4% neutral buffered formaldehyde for 24 h and transferred to 70% ethanol. The samples were then processed conventionally, post-trimmed and embedded in paraffin. A 3_µm thick slide was made from the intestinal sample, mounted on conventional glass slides and stained with haematoxylin and eosin (HE) for overall evaluation. From every chicken two transverse sections of each intestinal sample were evaluated using light microscopy.

5.2.3. RNA isolation from small intestine tissue

Three 60 mg tissues were cut from each of the jejunum, ileum and the duodenum of the adult infected and non-infected chickens from KZN and Limpopo provinces. The three tissues from each section (jejunum, ileum and duodenum) of animal intestine were combined and crushed together resulting in three pooled samples per chicken from which total RNA was isolated using RNeasy Mini Kit (Qiagen), according to instructions of the manufacturer. Briefly, after homogenisation, insoluble material was removed from the homogenate by centrifugation at 12,000 ×g for 10 min at 4 °C. RNA was eluted with 50 µl RNase-free water or Elution Buffer and stored at -80°C. This was followed by a subsequent sample purification using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. To remove contamination from genomic DNA, the RNA sample was DNase-treated using RNase-free DNase I (Qiagen). RNA concentration and quality were detected by measuring absorbance at 260nm and A260/A280 ratio using NanoDrop (Thermo Scientific). The absence of RNA degradation was assessed by electrophoresis of 1µg of RNA on a 1.8% agarose gel. Distinct 28S and 18S bands were visualized for all RNA extractions with no severe degradation detected. The 12 RNA samples were stored in small aliquots at -80°C until further use.

5.2.4. RNA Sequencing using Illumina HiSeq SBS technology

The TrueSeq RNA Sample Preparation kit (Illumina, San Diego, CA) was used for the preparations of the mRNA library preparations using 10 µg of total RNA for each sample following the manufacture's protocol. Finally, TruSeq universal adapters were ligated to the cDNA fragments, and PCR was performed to produce the final sequencing library. The sequencing library was constructed from the twelve samples using TruSeq Stranded mRNA sample preparation kit (Illumina, San Diego, CA, USA). RNA was fragmented and randomly primed for reverse transcription to generate double stranded cDNA fragments. Gel electrophoresis was used to assess quantity and quality of starting material. The cDNA was colligated and nebulized and then fragmented after which, adapters were ligated to both ends of the fragmented nucleic acid. The fragments were hybridized to a flow cell, which extended hybridized template or performed bridge amplification. This generated thousands copies of single-stranded DNA and prepares flow cell for sequencing using Illumina HiSeq 2500 at the Agricultural Research Council Biotechnology Platform of, South Africa.

5.2.5. Quality control of sequence data

Quality control was first performed on the primary sequencing data produced by Illumina HiSeq 2500. Sequencing adapters were trimmed off from the reads. For each of the sequencing reads, low quality bases with quality score greater than 20 as determined by phred (Ewing & Green, 1998; Ewing *et al.*, 1998) along 95% of the read length were also trimmed using CLC Bio workbench version 6.5 (CLC Bio, Aarhus, Denmark). Reads that were >20 bp long on both sides of paired-end format were kept for further downstream analysis and were classified as high-quality reads.

5.2.6. Mapping of RNA-Seq reads to reference genome

Quality controlled reads were aligned to the *gallus.galgal 4.74* reference genome using TopHat v2.0.11 (Trapnell *et al.*, 2009). TopHat use Bowtie v2.1.0 (Langmead *et al.*, 2009) to perform the alignment based on quality information accompanying each read, an initial alignment to the reference genome using default parameters. TopHat allowed a maximum of two mismatches when mapping reads to the reference and binary alignment map (BAM) files

containing mapped and unmapped reads with a maping report were produced.

5.2.7. Data preparation for transcript assembly and differential gene expression

Reads were assembled into transcripts using the default parameters of Cufflinks (Trapnell *et al.*, 2012). The abundance of assembled transcripts was estimated and reported as fragments per kilobase of exon per million fragments mapped (FPKM), with confidence intervals estimated for each FPKM. The differentially expressed genes ratios were tested for statistical significance using Bayesian statistics as described recently (Bullard *et al.*, 2010). The significance scores were corrected for multiple testing using the Benjamini-Hochberg correction (Benjamini & Hochberg, 1995). Differentially expressed genes were defined, as those with absolute value of log2 fold change higher than two and *P-value* was less than 0.05. Cufflinks constructed a minimum set of transcripts that bests described the reads in the generated dataset. It also used the normalized RNA-Seq fragment counts to measure the relative abundances of transcripts. The unit of measurement used was the FPKM. Confidence intervals for FPKM estimates were calculated using a Bayesian inference method (Jiang & Wong, 2009).

Cuffdiff was used to find significant changes in transcript expression, splicing, coding output and promoter use (Trapnell *et al.*, 2012). It used the Cufflinks transcript quantification module to calculate transcript/gene expression levels and test them for significant changes. Cuffdiff used the reference transcripts as a gene transfer format (GTF) file and the three sequence alignment map (SAM) or binary version of SAM (BAM) files containing the fragment alignments of the three biological replicates of each sample three small intestine. To analyse whether genes were ubiquitously expressed among the targeted tissues, a venn diagram was plotted by using Venny, a freely accessible (http://bioinfogp.cnb.csic.es/tools/venny/index. Html) interactive tool for the comparison of lists. The multidimensional scaling plots (MDS), which measure the similarity of the samples and project it into two dimensions, were performed to measure the relationship between duodenum, jejunum and ileum based on multidimensional scaling, using cummeRbund package in the R statistical environment (Trapnell *et al.*, 2012). Principal component analysis (PCA) was also used for clustering and exploring the relationships between conditions. Graphs were generated by using cummeRbund and the ggplot2 package (Wickham, 2009). Visualisation of results was also undertaken using the

cummeRbund package in R (Trapnell *et al.*, 2012).

5.2.8. Functional annotation and pathway analysis

For each differentially expressed gene, GO annotation was done by using blast2go (Liu *et al.*, 2011), which uses a blast algorithm to assign GO terms to sequences based on similarity. This was done to identify the key pathways and interactions that might be relevant for the response of intestinal tissues to *A. galli* infection and to annotate significant interactions with metabolic pathways. The DEGs between infected and non-infected intestinal tissues were classified into the categories of biological process, molecular function and cellular components. Bar graphs illustrating similarities and differences between GO terms was plotted using WEGO web-based tool (Ye *et al.*, 2006).

5b.2.9. Validation of RNA-seq results with RT-qPCR

Real time quantitative PCR (RT-qPCR) was performed using QuantiTect SYBR Green kit (Qiagen Inc., Valencia, CA) to test the validity of specific gene transcription, RNA-seq data and variations in gene expression among individuals (Redmond *et al.*, 2010). The RNA used for qRT-PCR was prepared in the same way as the total RNA extraction and DNase I treatment described above. Five genes were selected based on their functions in immune response and significance within the RNA-seq results. Primer pairs were designed using Primer3 (Rozen & Skaletsky, 2000) and are primer sequences are listed in Table 5.1. A reference gene (*βeta-actin*) was used as endogenous control for normalization of the real-time PCR analysis. Reverse transcription (RT) of RNA was achieved using iScript Advanced first strand cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) in a total reaction volume of 20 µl following the manufacturer's instructions. Quantitative PCR was performed in triplicate for each cDNA sample on Roche LightCycler® 96 System (Roche Applied Science, US) using KAPA SYBR FAST Universal qPCR kit (KAPA Biosystems, SA) according to the manufacturers instructions. Threshold lines were adjusted to intersect amplification lines in the linear portion of the amplification curve and cycles to threshold (Ct) were recorded. Standard curves for each gene and the reference gene were constructed using serial dilutions. The relative gene expression level estimated using the comparative cycle threshold (CT) method (Livak & Schmittgen, 2001; Pfaffl, 2004). The method calculates the ratio of relative expression from

the efficiency (E) and threshold cycle (C_t) values of an unknown sample versus a control sample.

Table 5.1: Primer sequences of selected differentially expressed genes used for RT-qPCR. *βeta-actin* was used as a reference gene for normalization of the RT-qPCR analysis

Primer name	Forward	Reverse
FABP1	GGGAAGAGTGTGAGATGGA	CCCCTTCAAGTTAGCAACCA
APOA4	GAACTAACACGCGCGCTAATT	TTCAGGTCTTCATGCGCTTCT
DRD4	TGGGCTCCAGACTGTCTCTT	GATCTTGGCTCGCTTCTGAC
BLB2	CCGCAGCGTTCTTCTTCTAC	CCGGTTGTAGATTTGCCTGT
MR1	CACGTGTTTGGAGTTTGTGG	TCATCCCCAGGA AATAATGC
*ßeta-Actin**	ACGTCTCACTGGATTTCGAGCAGG	TGCATCCTGTCAGCAATGCCAG

Gene used as reference is marked *, Primers were designed using Primer3 program

5.3. Results

5.3.1. Histology

The three anatomical sections of the intestine of uninfected chickens had no evidence of histopathologic necrosis. There was no histological difference between the infected duodenum, jejunum and ileum of chickens from Limpopo and KZN provinces (Figure 5.1, micrograph A, B and C). Intestines from *A. galli* positive duodenum, jejunum and ileum presented necrosis and hypertrophy of the intestinal villi with accumulation of inflammatory cells, and necrosis of the crypt of Lieberkühn gland and necrosis in the mucosa as shown in Figure 5.1, micrograph D, E and F. Lymphocytes and macrophages were observed in the infected chickens.

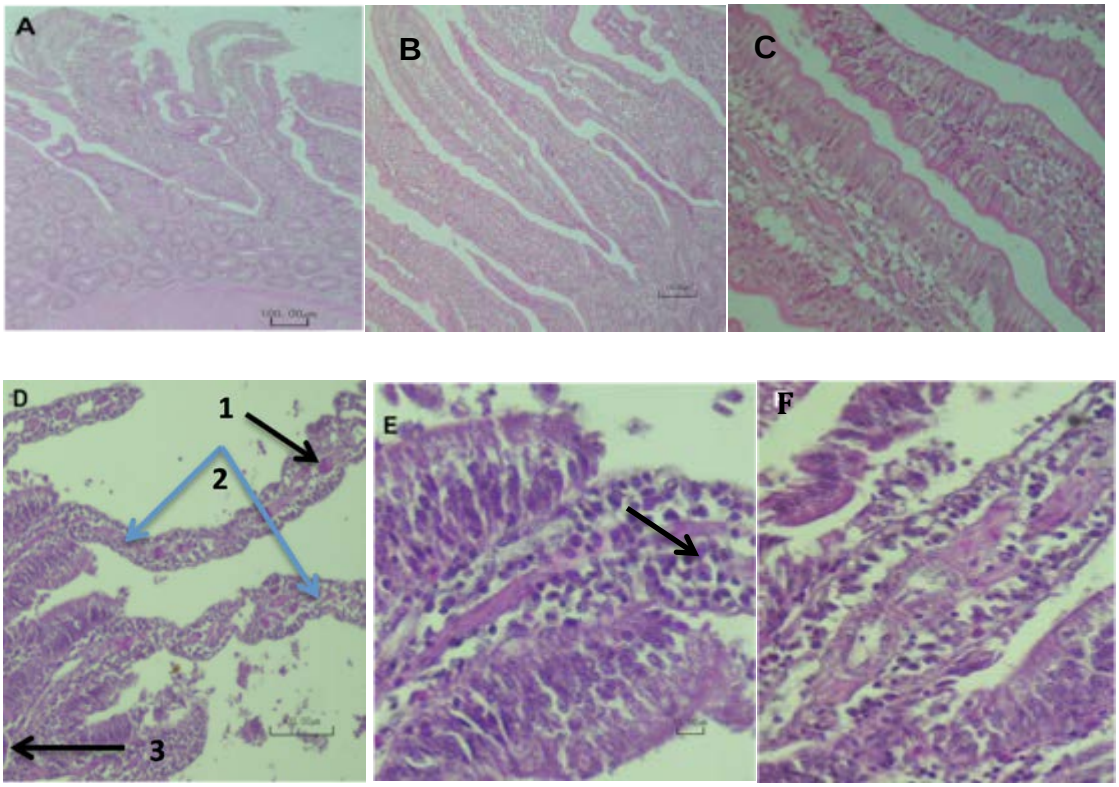

Figure 5.1: Photomicrograph of a section of non-infected duodenum (A), jejunum (B) and ileum (C). *A. galli* infested duodenum (D) jejunum (E) and ileum (F). The arrow in D1 shows macrophage, D2 shows villus, D3 shows crypt of lieberkühn, E shows lymphocytes and F shows intestinal villi. (Scale bar = 100 µm, x20)

5.3.2. RNA sequencing and read mapping

The number of reads generated and mapped to the chicken reference genome per sample are indicated in Table 5.2. At least 3,958,329 reads (125bp paired-end) were generated per tissue sample. An average of 85.25% reads were mapped to the reference chicken genome (*gallus.galgal4.74*).

Table 5.2: Mapping statistics of RNA-seq reads from the small intestines of Limpopo and KZN chickens

Province	Sample	Input reads	Mapped reads	Multiple alignments	Mapped %
KZN	1 Duodenum_N	3975932	3424380	274661 (8.0%)	86,10
KZN	1 Jejunum_N	3908924	3382905	231499 (6.8%)	86,50
KZN	1 Ileum_N	3985426	3553114	258992 (7.3%)	89,20
KZN	4 Duodenum_P	3986510	3515810	869970 (24.7%)	88,20
KZN	4 Jejunum_P	3970936	3364737	990410 (29.4%)	84,70
KZN	4 leum_P	3968942	3304257	1278564 (38.7%)	83,30
KZN	1 (N)*	23740564	20753238	274661 (7.5)	87.40
KZN	4 (P)*	23852776	20385341	869970 (30.6)	85.50
Limpopo	5 Duodenum_P	3994946	3214396	472546 (7.3%)	80,50
Limpopo	5 Jejunum_P	3958329	3234689	261153 (8.1%)	81,70
Limpopo	5 Ileum_P	3974790	3202224	231450 (7.2%)	80,60
Limpopo	39 Duodenum_N	3946073	3378801	208579 (6.5%)	85,60
Limpopo	39 Jejunum_N	3963238	3515159	221744 (6.6%)	88,70
Limpopo	39 Ileum_N	3984576	3451642	192259 (5.5%)	86,60
Limpopo	5 (P)*	23856130	19332316	472546 (77.5	81,00
Limpopo	39 (N)*	23787774	20726265	208579 (6.1)	87.10

*= Whole organism N=Negative; P=Positive

5.3.3. Differentially expressed genes

Differential gene expression (DEG) for the whole small intestine and three regions of the small intestines was calculated using the ratio of *A. galli* infested versus normal FPKM values for every gene. Genes with expression level fold change between two conditions of more than 2,

p<0.05 and FDR< 0.05 were considered differentially expressed genes. A total of 55,014 genes were identified from the four samples (whole organism) in this study. Of these, 277 (0.50%) and 190 (0.35%) genes were differentially regulated in the samples representing Limpopo and KZN provinces respectively. Comparisons were made between negative and positive chickens from Limpopo (LIM_N/P comparison) and KwaZulu-Natal (KZN_N/P comparison). Among the differentially expressed genes in the LIM_N/P comparison 124 (44.77%) were up regulated and 153 (55.23%) down regulated. A total of 34 differentially expressed genes were shared between KZN_N/P and LIM_N/P comparisons (Figure 5.2).

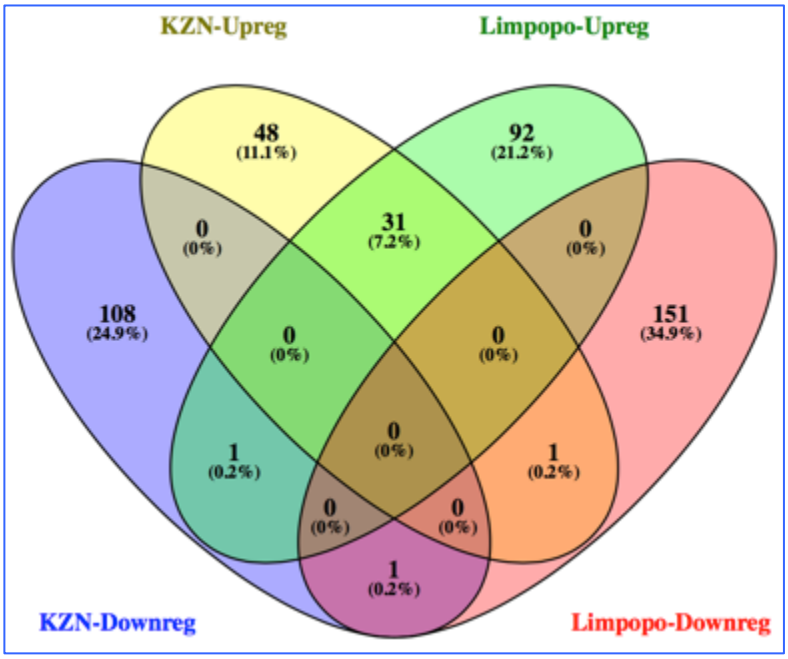

Figure 5.2: Overlapping differentially expressed genes (up or down-regulated) between KZN-negative compared to positive and Limpopo-negative compared to positive

In KZN_N/P comparison, 80 (42.11%) were up regulated and 110 (57.89%) down regulated. There were 156 and 243 differentially expressed genes that were only present in the KZN_N/P comparison verses LIM_N/P comparison, respectively. Some of the genes identified in LIM_N/P comparison are listed in Table 5.3a.

Table 5.3a: Differential gene expression between Limpopo village chicken intestines that were negative and positive for *A. galli* parasites

Gene name	Seq. Description	RPKM_N	RPKM_P	log2(fold_change)	p_value
Limpopo up-regulated					
		5.74292	337.971	5.87897	0.00055
	senescence-associated protein	1944.51	178020.0	6.51649	5,00E-05
GOLGA4	low quality protein: golgin subfamily a member 4	1944.51	178020.0	6.51649	0.0057
	hypothetical protein CP8484711_2381, partial	4.46432	125.568	4.81388	0.00125
SYNPO2	synaptopodin- partial	1.16224	21.6793	4.22134	0.0041
	phosphatidylinositol - bisphosphate 5-phosphatase a-like	2.37946	39.1956	4.04198	0.0041
TLR7	tlr7	0.636696	8.0061	4.25142	0.0026
HPS5	hermansky-pudlak syndrome 5 protein	10.2695	124.333	3.59778	0.001
	PREDICTED: spidroin-1-like, neurofilament medium polypeptide isoform x1	86.7866	800.611	3.20556	5,00E-05
IL21R	interleukin-21 receptor	1.05924	9.72641	3.19887	0.0033
	envelope partial, pol partial	28.3597	218.111	2.94315	0.0001
IL20RA	Interleukin-20 receptor subunit alpha	5.68072	41.4622	2.86765	4.2073
	transitional endoplasmic reticulum partial, low quality protein: syncytin-1-like	33.9977	164.989	2.27886	0.0001
HSP30C	heat shock protein 30c-like	1.40715	9.57623	2.76668	0.006
HSPB7	heat shock protein beta-7	3.05223	10.6764	1.80649	0.0014
Limpopo down regulated					
	hypothetical protein A306_09416	56.8579	2.14685	-4.72707	5,00E-05
LOC100857191	c-c motif chemokine 21	420.328	16.2807	-4.69028	0.002
CXCR4	c-x-c chemokine receptor type partial	35.5861	2.67279	-3.7349	5,00E-05
	cd209 antigen-like protein a	48.9896	3.73815	-3.71208	0.0005
	class i histocompatibility f10 alpha chain-like isoform x1	368.999	29.9511	-3.62294	5,00E-05

	cyclic amp-dependent transcription factor atf-3 isoform x2	26.7937	2.29904	-3.54279	5,00E-05
HSP105	heat shock protein 105 kda isoform x2	47.0572	4.5253	-3.37833	5,00E-05
	tyrosine-protein phosphatase non-receptor type 7	111.45	14.4115	-2.95111	5,00E-05
SLAMF1	signaling lymphocytic activation molecule	88.0741	16.2089	-2.44193	0.0039
	t-cell surface glycoprotein cd3 epsilon chain	94.6376	20.5752	-2.20151	0.0015

P- value of < 0.05 is significant

In Table 5.3b, some of the genes that were regulated in the presence of *A. galli* in KZN_N/P comparison are listed.

Table 5.3b: Differential gene expression between KZN village chicken intestines that were negative and positive for *A. galli* parasites

Gene Name	Seq. Description	RPKM_N	RPKM_P	log2(fold_change)	p_value
KZN up-regulated					
ATF7IP	activating transcription factor 7-interacting protein 1 isoform x1	3.2018	179.746	5.81093	5,00E-05
	collagen alpha-1 chain-like	98.7552	4324.89	5.45266	0.0002
LOC419333	formin-f- partial	1.35691	24.1984	4.15651	0.0033
HPS5	hermansky-pudlak syndrome 5 protein	13.1854	293.446	4.47608	5,00E-05
CSF2RB	cytokine receptor common subunit beta- partial	0.471599	7.52509	3.99608	5,00E-05
TRAF3IP3	traf3-interacting jnk-activating modulator isoform x1	0.759631	8.20497	3.43313	0.00335

	Cytochrome P450 2h1-like isoform x1	4.29065	35.8769	3.06378	5,00E-05
	rna-binding protein 25 isoform x2	5.68072	41.4622	2.86765	5,00E-05
FABP5	fatty acid-binding epidermal	12.8834	92.782	2.84834	0.0027
KZN Down-regulated					
DAK	bifunctional atp-dependent dihydroxyacetone kinase fad-amp lyase	2148.25	82.2961	-4.7062	5,00E-05
APOA	apolipoprotein a-iv	784.167	33.1607	-4.56361	0.0015
	cgmp-dependent protein kinase 1-like	925.85	47.8782	-4.27334	0.0029
	sperm-associated antigen 5	88.3557	8.22701	-3.42488	0.00465
DPEP1	dipeptidase 1	171.901	17.6539	-3.28352	5,00E-05
	maltase- partial	311.361	32.8363	-3.24522	0.0033
	solute carrier family 26 member 6	130.194	16.6938	-2.96328	5,00E-05
	testican-2	304.962	39.9486	-2.93241	0.00015
HSP70	Heat shock protein 70	76.4445	9.57394	-2.99723	0.00015
	interleukin- partial	175.379	17.7204	-3.30699	5,00E-05

P- value of < 0.05 is significant

Transcriptome analysis of the three regions of the small intestine from Limpopo chickens identified 76, 99 and 78 DEGs in the duodenum, jejunum and ileum sections respectively. In tissues from KwaZulu-Natal, 33, 15 and 18 DEGs were identified in the duodenum, jejunum and ileum sections of the intestine respectively (Table 5.4).

Table 5.4: Gene expression statistics for comparisons between negative and *A. galli* positve sections of the small intestine of village chickens from KZN and Limpopo provinces

Province	Limpopo			KZN		
Section of the intestine	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
Significantly up & down regulated genes	76	99	78	33	102	158
Significantly up-regulated genes p<0.05	37	58	34	15	41	16
Significantly down-regulated genes p<0.05	39	41	44	18	61	142
Non-significant genes p>0.05	34976	34953	34974	35019	34950	34894
Number of Sequences	123	64	78	32	79	123
Sequences with blast hits e-value ≤1E−10	91	41	40	20	51	91
Sequences without blast hits	32	23	38	12	28	32

A total of 31 genes were co-expressed in all the sections of the small intestines collected from Limpopo and 10 genes where common in the three sections collected from KZN. Three (3) genes were differentially expressed in all the analysed samples (Figure 5.3). Only 16 genes were exclusively expressed in duodenum while 47 and 25 genes could be detected only in the jejunum and ileum of chickens from Limpopo. In chickens from KwaZulu-Natal, 15 genes were exclusively expressed in the duodenum while 50 and 137 genes could be detected only in the jejunum and ileum respectively (Figure 5.3). Some annotated genes showed similar patterns of expression between different sections of the intestine, which included c-c motif chemokine ligand 21 that was down-regulated in duodenum and ileum of chickens from Limpopo. A similar pattern of expression was also observed with tyrosine-protein kinase jack3.

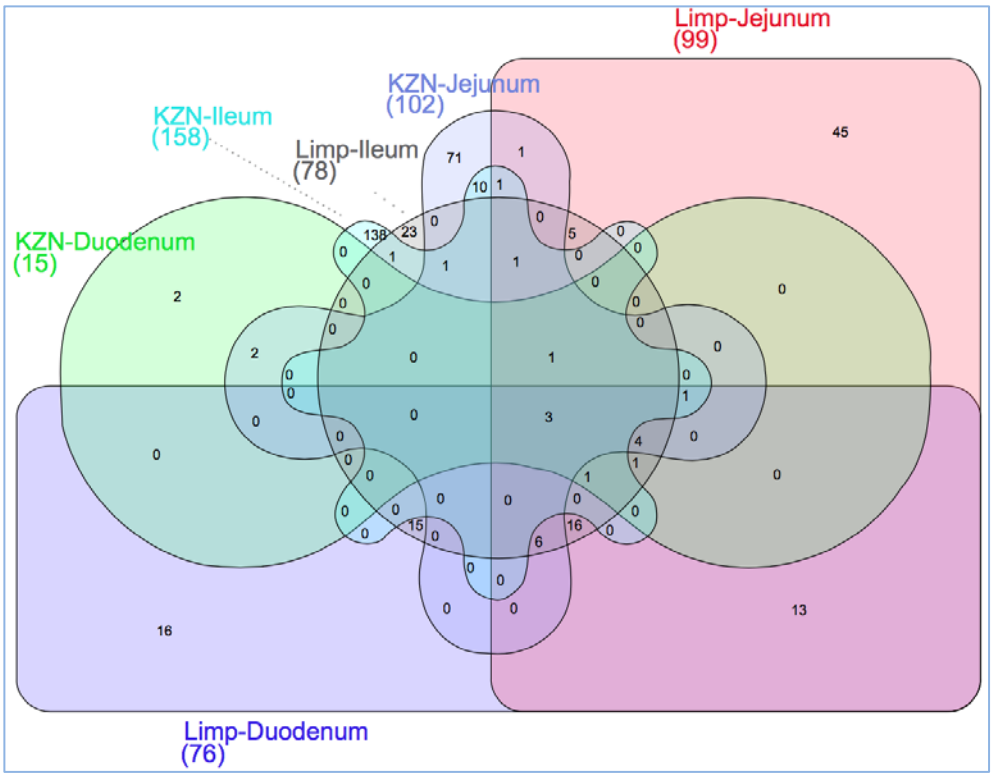

Figure 5.3: Venn diagram of the differentially expressed genes along the 3 different tissues (duodenum, jejunum, ileum) infested with *A. galli* parasites. Venn diagram was plotted by using and interactiVenn

5b.3.4. Multidimensional scaling plots

Investigation of the MDS plot produced an apparent structure within the data that is consistent with tissue segments. The plot revealed tissue dissimilarity of genes expression patterns between tissues by clearly separating duodenum, jejunum and ileum segments. This was observed in both Limpopo and KZN provinces (Figure 5.4a(I) and 4b(I). In agreement with MDS plot, PCA plot based on principal components 1 and 2 resulted in a distinct separation based on tissue segments (Figure 5.4a[I] and 4b[II]).

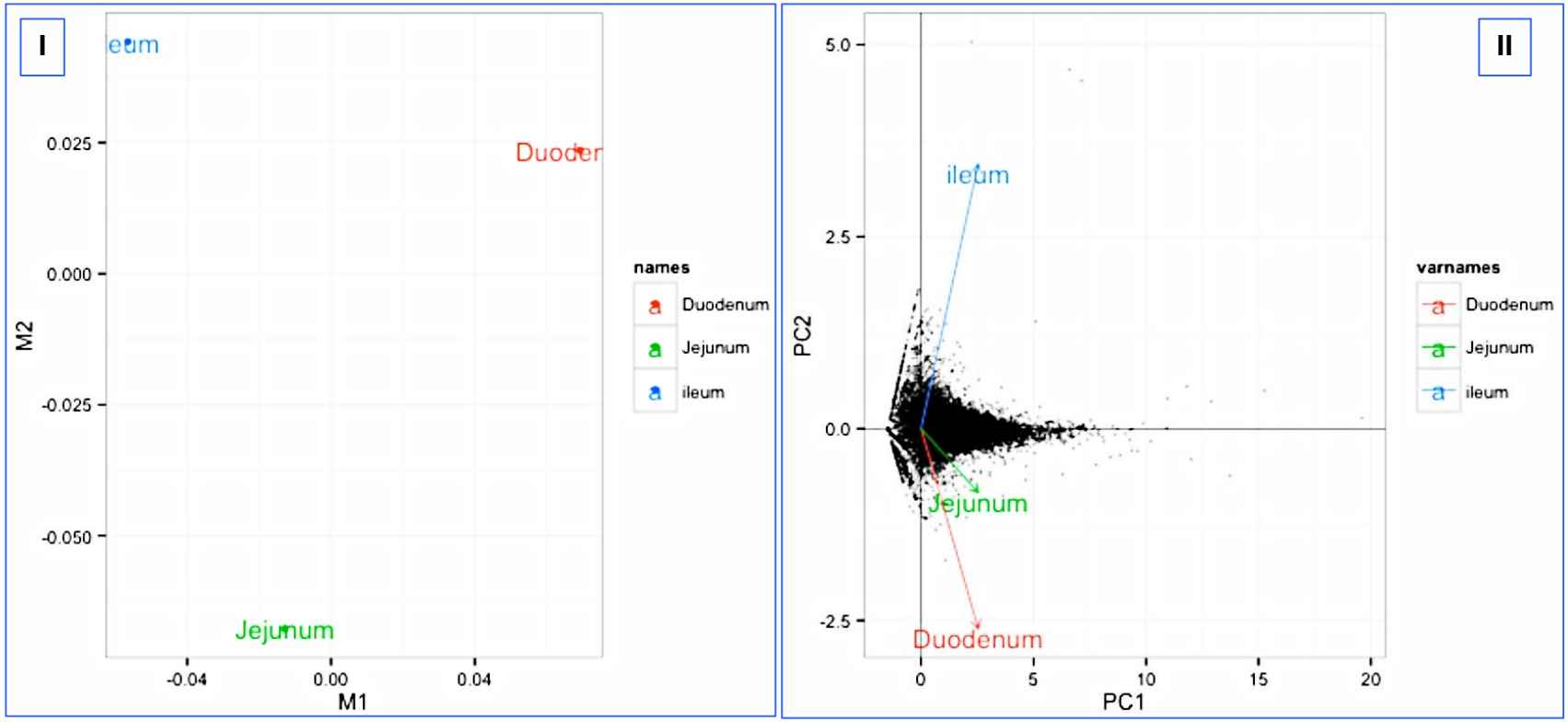

Figure 5.4a: Multidimensional scaling analysis (I) and principal component analysis (II) using expression of all genes for tissues from KwaZulu-Natal. Plots were performed through the use of cummeRbund package in the R statistical environment

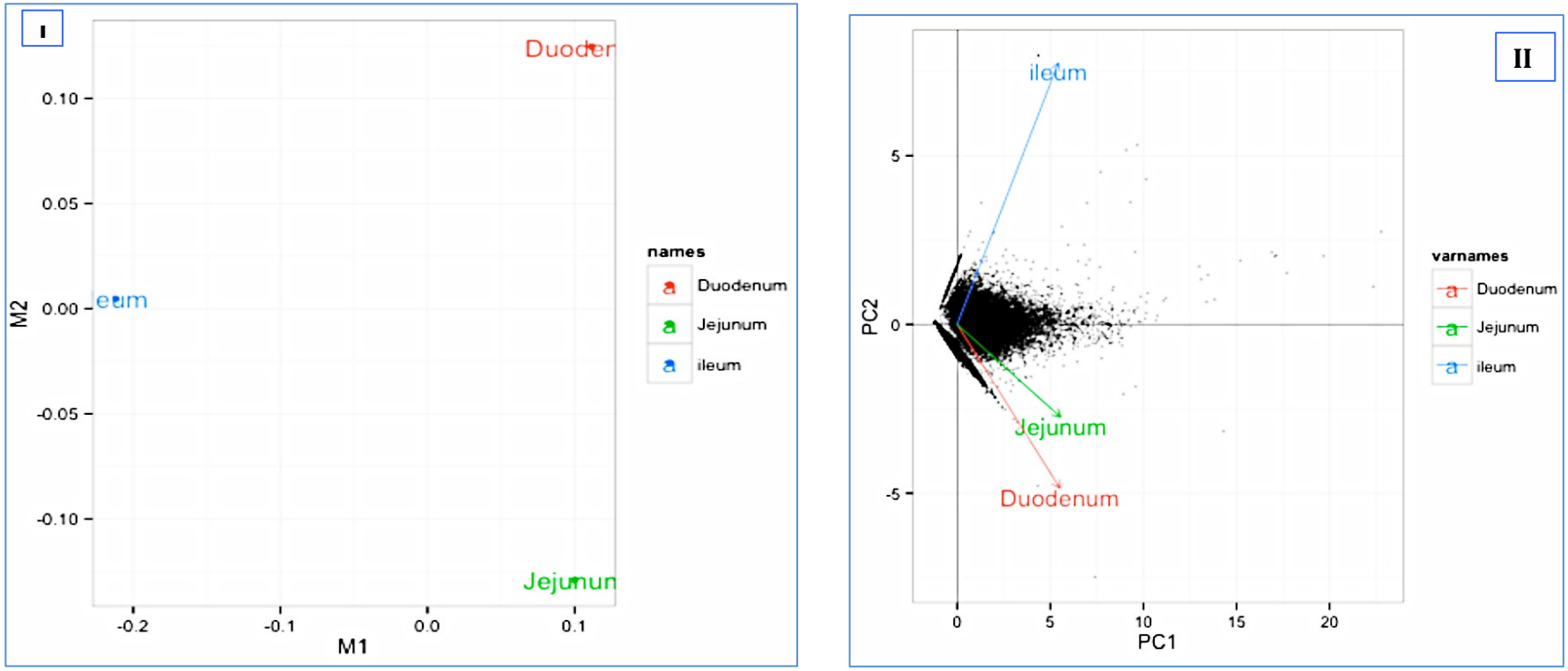

Figure 5.4b: Multidimensional scaling analysis (I) and principal component analysis (II) using expression of all genes of tissue from Limpopo. Plots were performed through the use of cummeRbund package in the R statistical environment

5.3.4. Blast hits and Gene Ontology (GO) analyses

A strong homology (E-value < 1.0E-10) was observed for the annotated genes with the sequences hits in the Universal Protein Resource (Uniprotkb), MGI, RGD, ZFIN and FB database, respectively. The abundace of the annotated transcripts matched *Gallus gallus* in the databeses (Figure 5.5). Other transcripts correspond to the species such as meleagri gallopavo and buceros rhinoceros in the non-redundant GenBank database. This trend was consistent across all samples indicative of proper assembly and annotation of the sequences of village chickens intestinal tissue transcriptome generated in this study.

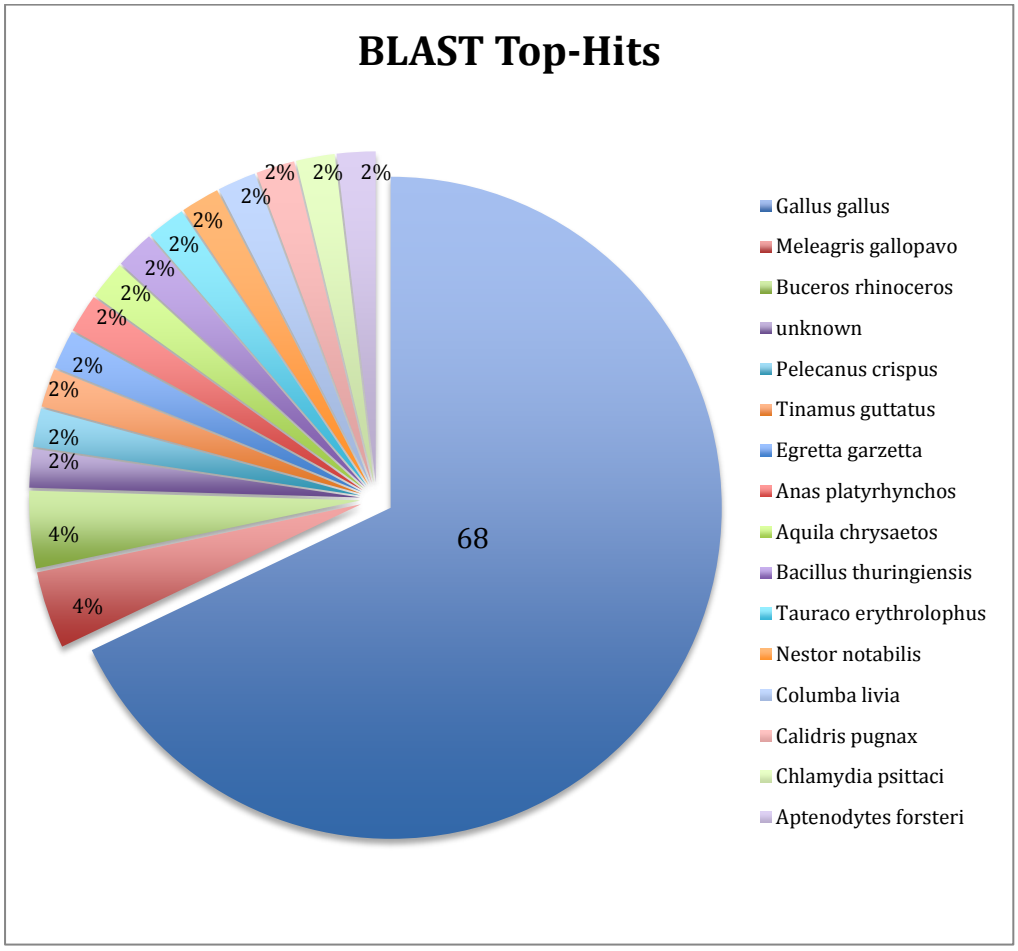

Figure 5.5: Blast top hit of the annotated transcripts using E-value < 1.0E-10 revealing matches with protein sequences deposited in the non-redundant GenBank database

Over-representation of GO terms was determined using Fisher's exact test and filtered using a multiple correction control for false discovery rate (FDR) < 5%. GO terms associated with the

190 and 277 differentially expressed genes in KZN_N/P and LIM_N/P comparisons were analyzed. In general, fewer significantly enriched GO terms were found in the KZN-N/P compared to the Limpopo-N/P comparison. GO terms at level 2 were used as representatives (Figure 5.6a). Of these GO terms, biological processes such as immune response (GO:0006955), response to stimulus (GO:0050896), immune system process (GO:0002376) and signaling (GO:0023052) were enriched. Binding GO such as dopamine receptor activity (GO:0001591) and ATP binding (GO:0005524) along with cellular processes such as cellular response to stimulus (GO:0051716) and cellular response to stress (GO:0033554) had the highest percentage of transcripts involved.

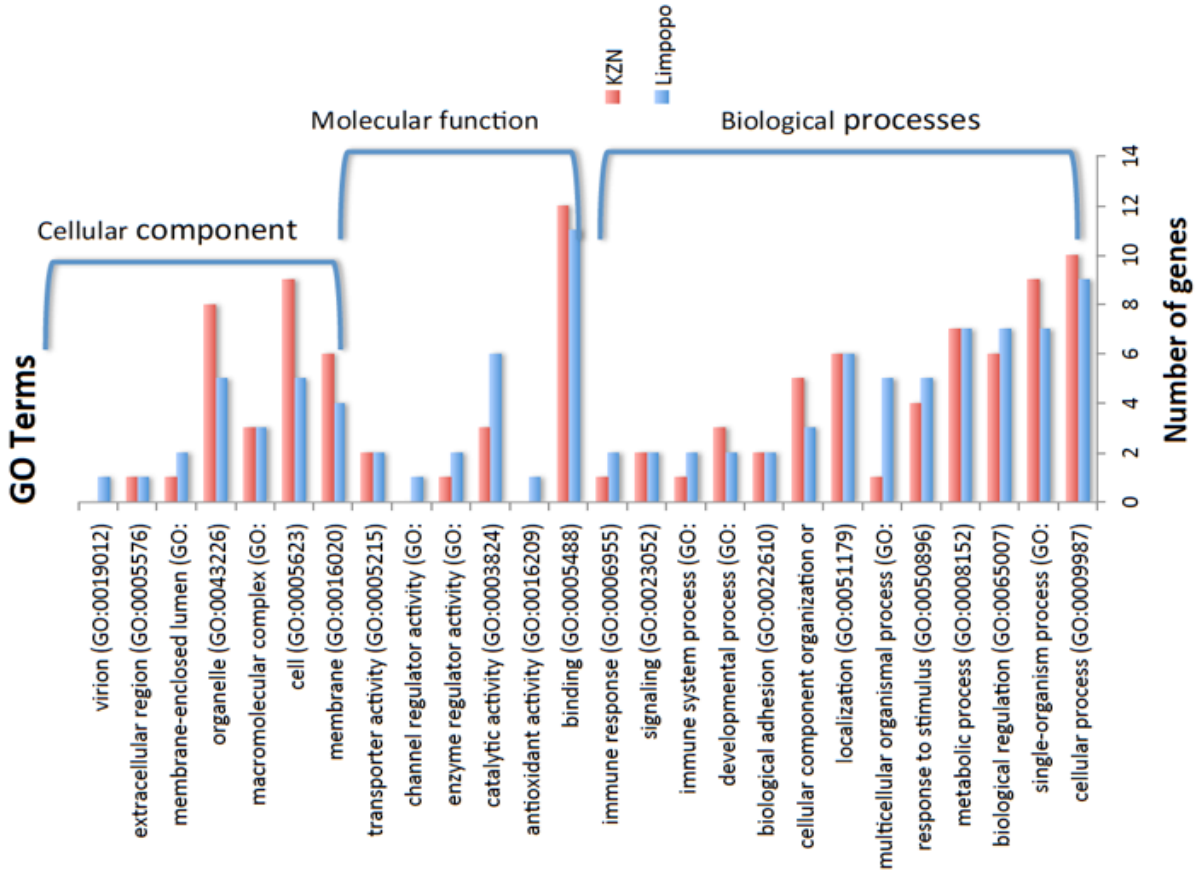

Figure 5.6a: Enriched gene ontology terms between *A. galli* infected and control small intestine tissue from Limpopo and KZN provinces. The x-axis indicates the number of genes in a category. The y-axis indicates the percentage of a specific category of genes in that main category

The most predominant biological functions were common amongst the different anatomical segments of the small intestine. The first five predominant biological processes observed for tissue samples from Limpopo provinces were cellular process, biological regulation, metabolic processes, pigmentation and response to stimulus (Figure 5.6b). The GO profiles were similar between Limpopo and KZN provinces with the exceptation of biological regulation that was not in the top three of the biological process term in KwaZulu-Natal. Immune response terms that included immune system process, response to stimuli and cell killing were also observed in the presence of *A. galli* infection (Figure 5.6b).

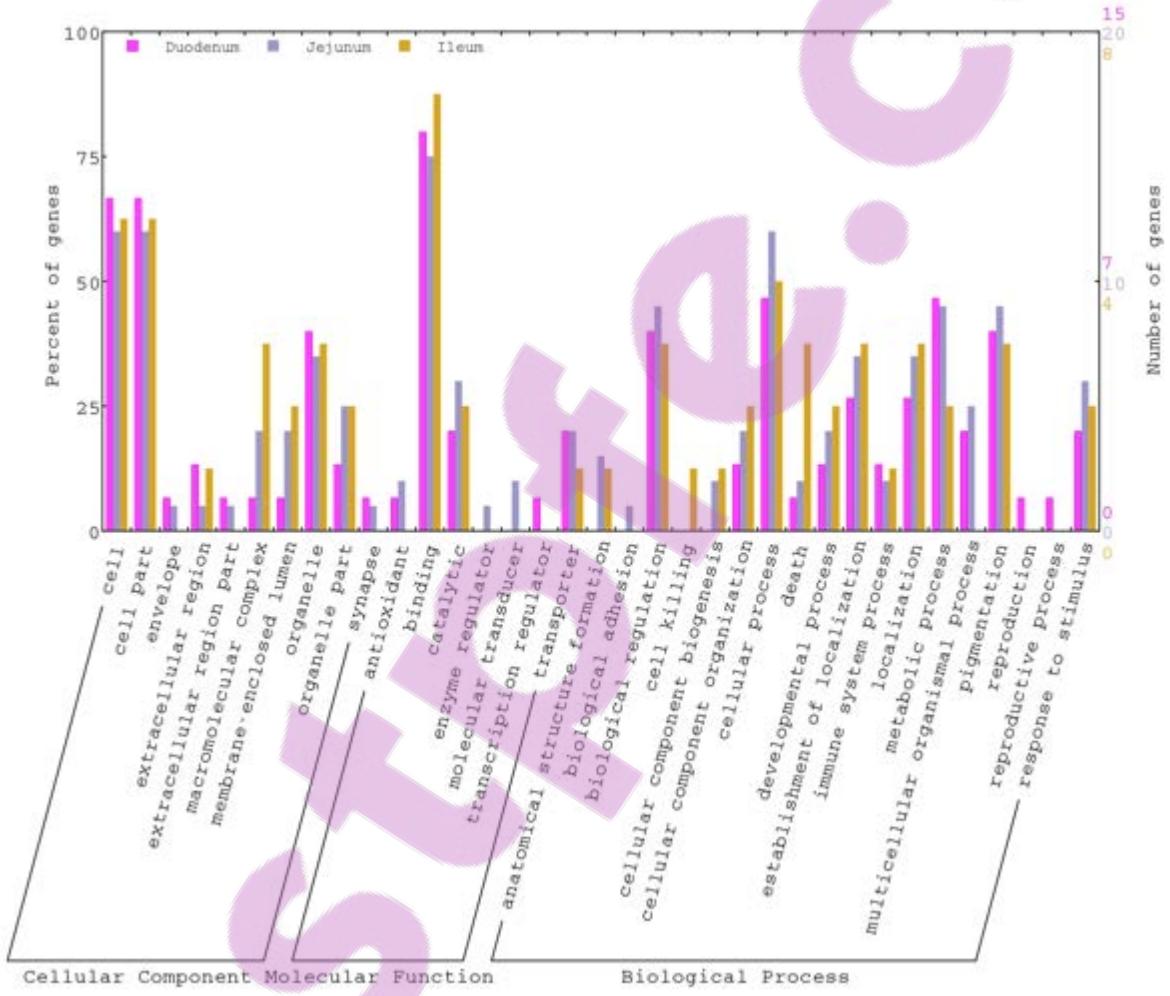

Figure 5.6b: GO analysis was performed to describe the properties of DEGs in duodenum, jejunum and ileum of *A. galli* infested Limpopo chickens. The right y-axis indicates the number of genes in a category. The left y-axis indicates the percentage of a specific category of genes in that main category

5.3.5. Biological pathways associated with chicken's response to A. galli infections

To gain an understanding into pathways involved in the village chickens reared in *A. galli* prevalent area, gene annotation was conducted using enzyme code and enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The top 5 KEGG pathways associated with most abundant differentially up-regulated (Figure 5.7a) genes in infected and non-infected chickens from Limpopo included T cell receptor signaling pathway, Drug metabolism - cytochrome P450, Metabolism of xenobiotics by cytochrome P450, histidine metabolism and arachidonic acid metabolism. The impacted T cell receptor signaling pathway involved tyrosine-protein kinase btk, tyrosine-protein kinase csk and tyrosine-protein kinase jak3 genes that play a major role in immune response. In KZN chickens, pathways impacted included arachidonic acid (Figure 5.8), methane metabolism, glycerolipid metabolism, purine metabolism, glutathione metabolism and cyanoamino acid metabolism. Arachidonic acid and purine metabolism were observed to have impacted tissues from both provinces. However, genes involved in these pathways were different for the two provinces.

Pathway analysis was also undertaken using all the up and down regulated genes from the three sections of the small intestine (Figure 5.7b). Genes up-regulated in the ileum of KZN chickens impacted the most pathways. In Limpopo, the jejunum had the most impacted pathways. Other pathways were impacted in only one or two sections of the intestine. In Limpopo, T cell receptor pathway was impacted in the duodenum and ileum and not the jejunum. Most of the pathways had only one gene involved except for purine metabolism that had four genes involved, followed by Tryptophan metabolism and Pyrimidine metabolism with two genes each. The Linoleic acid metabolism, Arachidonic acid metabolism, Metabolism of xenobiotics by cytochrome P450, Drug metabolism - cytochrome P450 and Purine metabolism pathways were commonly impacted in both provinces. The common pathways were however not impacted in the same sections of the intestine.

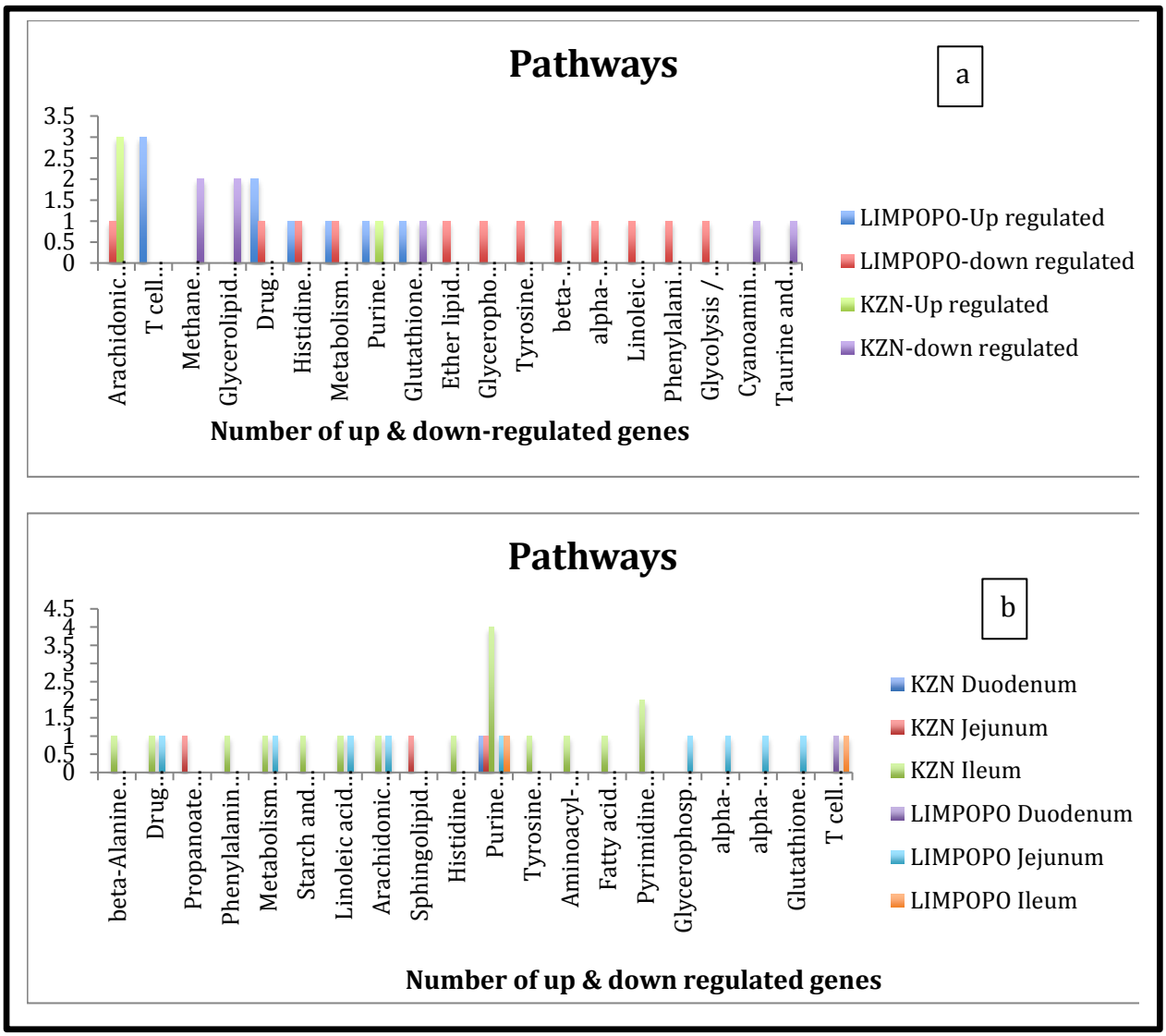

Figure 5.7: KEGG pathways enriched in differentially expressed genes in Limp_N/P and KZN__N/P (a). KEGG pathways enriched in differentially expressed genes along sections of the intestine in KZN (b). The x-axis indicates the number of sequences involved and the y-axis indicates the pathways impacted

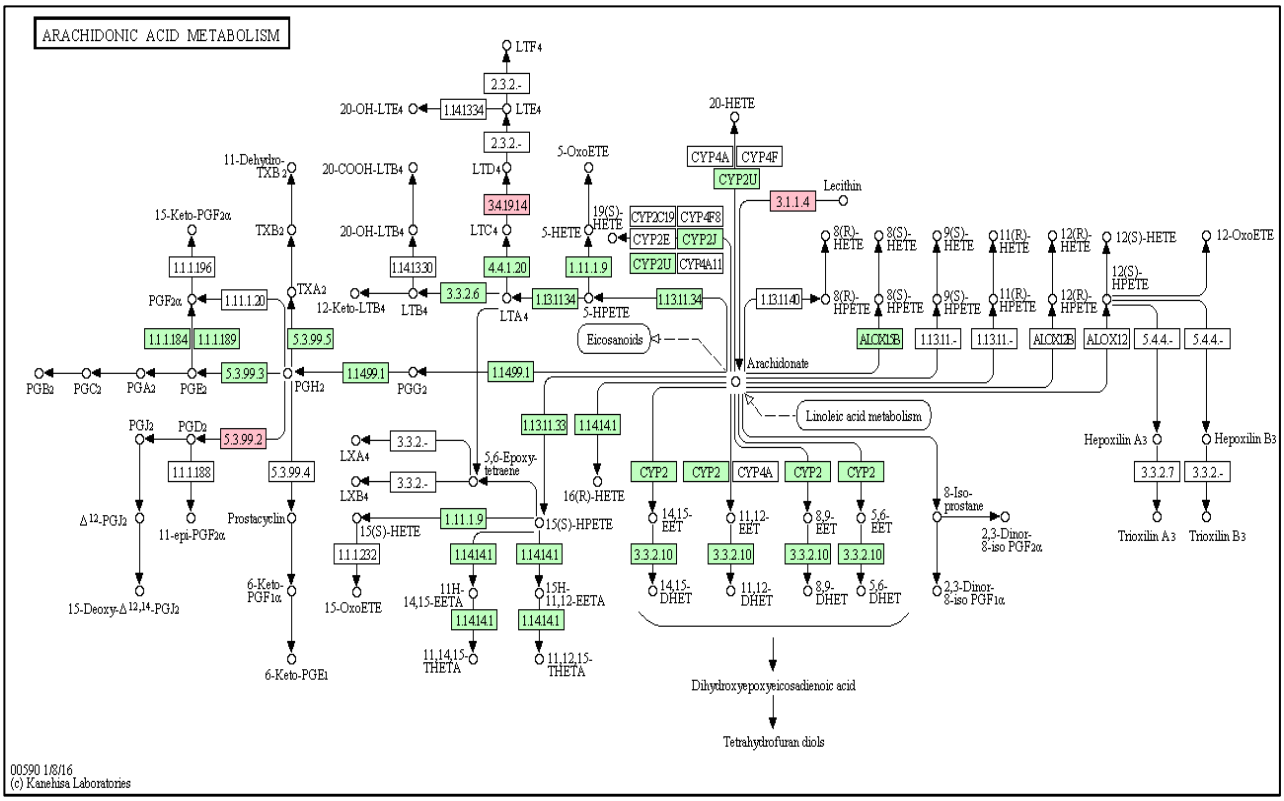

Figure 5.8: Impacted arachidonic acid metabolism pathway. Genes involved in the pathway are shown in pink colour

5b.3.8. Validation of RNA-seq results using RT-qPCR

RT-qPCR was performed to validate the RNA-seq results with reference to *βeta-actin* control gene. Genes were selected based on their functions in immune response and significance within the RNA-seq findings. Of all the five genes tested, *MR1* gene was not differentially expressed in the RT-qPCR results (Table 5.5). However, the other genes were consistent between RNA-seq and RT-qPCR results.

Table 5.5: Comparison of gene expression levels (fold changes) between RNA-seq and qRT-PCR. *βeta-actin* was used as a reference gene for normalization of the RT-qPCR analysis

Comparison	Method	*APOA4*	*MR1*	*FAB1*	*DRD*	*BLB2*
Duodenum N/P**	RNA-seq			2.85		
	RT-qPCR			0.57		
Jejunum N/P	RNA-seq	3.11			2.41	
	RT-qPCR	2.04			0.46	
Ileum N/P	RNA-seq		1.58	2.61		1.43
	RT-qPCR		0.78*	1.79		0.73

**N=Negative; P=Positive; *Represents gene that was not significantly differentially expressed in RT-qPCR results

5.4. Discussion

Nematodes of the genus Ascaridia are known to infect many species of birds causing fatal diseases. It is responsible for economic losses due to the growth and weight reductions it causes in its hosts (Gauly *et al.*, 2005). The small intestine is a principal target organ of *A. galli* infection (Taylor *et al.*, 2007; Katakam *et al.*, 2010; Luna-Olivares *et al.*, 2012). Earlier studies suggested that the preference for the small intestine by these parasites is to complement their physiological osmotic feeding nature where nutrients exist in dissolved form (Smyth, 1976). Previous studies (Fatihu *et al.*, 1990; Luna-Olivares *et al.*, 2012) showed that most of the helminth parasites were restricted to the small intestine, particularly the duodenum where there is optimum concentration of saline and glucose.

The histopathology of the three anatomical sections of the infected small intestines of chickens from Limpopo and KZN showed signs of damage that are assumed to be caused by *A. galli* parasites. Even though the number of recovered worms differed amongst the chickens from the two provinces, the observed histopathology signs were the same. A study by Idi (2004) found that larvae of *A. galli* were localized deeply among the intestinal villi and penetrated the

intestinal Lieberkühn glands. On the other hand Lapage (1956) and Kaufmann (1996) described that the newly hatched larvae live in the contents of the small intestine, but later bury their head in the intestinal crypts, and afterwards leave the intestinal mucosa to live in the intestinal lumen as adults. Larvae recovery from the intestinal wall was reported to be highest from the anterior part of the jejunum, whilst larvae was found in the posterior part of the jejunum day 7 post infection (Ferdushy *et al.*, 2013). Such findings imply different roles of the different sections in the life cycle of *A. galli*. Soulsby (1982) reported that in many cases, the intestinal mucosa reveal inflammatory lesions and focal hemorrhages caused by the burrowing of parasites. Another study conducted by Luna-Olivares and colleagues (Luna-Olivares *et al.*, 2012), observed the highest number of the larvae located in the crypts, followed by the transitional zone, and the villus zone of chicken jejunum. Necrosis of the crypt of Lieberkühn gland and intestinal villus in this study, was therefore expected. This implied that *A. galli* parasites from the two provinces imposed the same effect on the small intestine regardless of environmental differences.

This study further investigated the genes and pathways affected in the presence of *A. galli* parasites and whether the genomic response was the same between chickens from KwaZulu-Natal and Limpopo province. Illumina HiSeq 2500 generated an average of 3.97E+06 paired-end reads per sample, which provided sufficient sequence coverage for transcriptome profiling (Sultan *et al.*, 2008). The average percentage (80.60-89.20%) of reads that mapped to the *gallus.galgal 4.74* reference genome was within RNA-Seq quality standards (Mortazavi *et al.*, 2008) for downstream analysis. The availability of a high quality, annotated reference genome in this case, ensured a high degree of mapping.

Differences in gene expression associated with infestation or histopathology status provides understanding into the molecular genetic architecture of host response to infection. Understanding the role of genes involved in village chickens from the low-input extensive production system is essential to development of proper management and control of *A. galli* in chicken. The recognition of invaded microorganisms can cause the changes in the gene expression levels in particular host cells, and these changes in gene expression level can be different between susceptible and resistant chickens. Genes with significant expressional changes are associated with the control of immune response such as initiation, regulation and termination (Gibson & Fuller, 2000). To identify and understand the role of these genes is the

key to unveiling the mechanism of resistance of parasites in poultry.

Generally, the transcriptome of the studied small intestines from village chickens raised in *A. galli* persistant environment was altered. The exact time of infection is not established as the chickens were naturally infected with the *A. galli* parasites, however, the worms collected from the small intestines were adult mature worms insinuating that the infection was at the late-stage. Genes were identified as defferentially expressed between the infected versus non-infected, indicating altered gene expression patterns after infection. The small intestines of chickens from Limpopo and KZN chickens affected by *A. galli* infection however, differed in the quantitative composition of the genes differentially expressed. The differences in the number of differentially expressed genes implies differences in the genetic mechanisms in which a village chicken from different provinces respond to infection with implications of more genes and pathways involved in Limpopo versus those from KwaZulu-Natal province. We hypothesized in this study that environmental conditions might have played a role in the differences in regulation of immune response genes. Although the chickens responded with the same histopathology reaction, the genes differentially expressed were different between chickens from different provinces. There were therefore differences in the genetic mechanisms used by chickens from different provinces to resist gastro-intestinal nematode infections.

Complementary to the whole organism analysis, this study provided a finer resolution of the molecular processes happening in the different sections of the small intestine. Danos (2013) observed the strong correlation between libraries prepared from pooled RNA samples (pooling RNA from 3 biological replicates) and average of 3 libraries prepared separately from RNA of 3 biological replicates. Due to financial constraint and computational resources, we decided to use “pooled” RNA approach to reduce the cost of sequencing however; the results were validated with RT-qPCR method. Results revealed that these sections differed in the quantitative composition of the genes expressed in response to *A. galli* infection. The results indicated that the duodenum, jejunum and ileum displayed distinct transcriptome characteristics with differences in gene transcription required for the response of *A. galli* infection. This support the hypothesis that different regions of the intestine are impacted differently during the nematode invation due to the different functions they play. However, the expression levels between the sections of the small intestine didn’t follow any obvious pattern between the different segments and both provinces For example, the number of DEGs in

Limpopo was higher in the jejunum followed by ileum and duodenum whilst in KZN, the ileum had the highest DEGs followed by jejunum and duodenum. It was however, not surprising that ileum had the largest or second largest number of expressed genes in KZN and Limpopo provinces respectively. The ileum is the lower small intestine that is known to be associated with increased immune surveillance. In a study by (Freeman *et al.*, 2012), the largest cluster of genes that contained many genes associated with the immune system were observed to be expresses two- to three-fold higher expression in the ileum relative to the other regions. Uniquely expressed genes in the different tissues could provide insight on the unique attributes of each tissue segments and how it facilitate parasite colonization. Interference with parasite recognition of these signals could thus provide a basis for strategies to control parasitism that could prevent inflammation and immune modulating cascades induced by developing parasitic nematodes (Li *et al.*, 2012).

Several genes involved in immune response were observed to be regulated in this study. Of the top six DEGs in KZN chickens, HPS5 and TRAF3IP3 are known to be involved in immune response (Smith *et al.*, 2011; Connell *et al.*, 2012), however, CSF2RB, collagen alpha- 1 chain-like, ATF7IP and LOC41933 genes haven't been identified in response to nematode infection in chickens or any other livestock animals. Further studies need to be conducted to investigate their role in response to *A. galli* infection in a more controlled study. The chicken MHC (B complex) has been implicated with conferring either resistance or susceptibility to a variety of viral, parasitic, and bacterial diseases (Lamont, 1998). The DEG analysis indicated that *MHC class II m alpha chain precursor* was up regulated whilst *MHC class II partial* down regulated in chickens from Limpopo region. *Class I histocompatibility f10 alpha chain-like isoform x1&2* were down regulated in Limpopo. The MHC is a group of genes present in all vertebrates that plays a major role in immune response mechanisms. The chicken MHC was first reported by Briles and colleagues as a highly polymorphic erythrocyte antigen or blood group system (Briles *et al.*, 1950). It consists of three distinct regions, each of which codes for specific gene products. Various MHC haplotypes have been associated with resistance to *A. galli* (Schou *et al.*, 2010). The MHC molecules are known to play important roles in the regulation of the immune response by communicating among different cellular components of the immune system: T cells, B cells, and antigen-presenting cells (Lamont, 1998). This group of genes is also responsible for training the leukocytes to prevent self-identification and autoimmunity. These antigens were also found to influence a variety of biological functions including

coccidiosis, therefore their expression in response to *A. galli* in this study was remarkable. Knowledge of MHC of indegenous chickens would add to an improved undestanding of machanisms underlying natural disease resistance.

Pro-inflammatory cytokines genes were observed as another set of genes that were regulated in this study. The expression of pro-inflammatory cytokine genes are activated by TLRs to directly control parasite replication (Gazzinelli & Denkers, 2006). *Interleukin-20 receptor subunit alpha and interleukin-21 receptor* were up-regulated in Limpopo chickens whereas *interleukin- partial* was down-regulated in KZN. Interleukin-22 is a member of the interleukin 10 related cytokine family (Zenewicz *et al.*, 2008). The interleukin-22 receptor is highly expressed within tissues, such as epithelial cells of the gastrointestinal tract. In a more direct manner, interleukin-22 protects tissues from damage during inflammation. It allows for maintenance of epithelial barriers, such as those of the gastrointestinal tract during inflammation (Radaeva *et al.*, 2004). In a study by Dalgaard *et al.* (2015), an increased expression of pro-inflammatory cytokines were observed in spleen from *A. galli* infected chickens where the larvae re-enter the intestinal lumen. Cytokines are cell-signaling molecules of the immune system involved in regulating pro- and anti-inflammatory responses and recruitment of leukocytes to sites of infection (Kogut *et al.*, 2001). They can be released through complement receptor-mediated signaling or by pathogens through a wide array of pattern recognition receptors (PRR) (Lacy & Stow, 2011). Pro-inflammatory cytokines, such as *interleukin-1β* and *interleukin-6* help in the destruction of pathogens, while anti-inflammatory cytokines, such as *interleukin-10*, help to protect the host against excessive inflammation. In chickens the function of IL-10 appears to be conserved as it acts as an anti-inflammatory cytokine and down-regulates the effects of the Th1 cytokine Interferon gamma (Rothwell *et al.*, 2004). The studies, which were performed on *A. galli*-infected chicken, demonstrated systemic and local increase in *interleukin -4* and *interleukin -13* mRNA expression in splenic and ileal tissues (Degen *et al.*, 2005; Kaiser, 2007). The cytokines *interleukin -4* and *interleukin -13* have an essential role in the immune response to intestinal nematode infections (Herbert *et al.*, 2009). Even though the gene family was regulated in this study, these specific genes were not differentially expressed in Limpopo and KZN chickens. Because pro-inflammatory cytokines are also expected to be regulated when there's inflammation or infection, therefore a controlled study is needed as a baseline.

Different families of pattern recognition receptors (PRRs) have been identified (Mair *et al.*, 2004) including among other receptors, toll-like receptors (TLRs) (O'Neill, 2006). Pattern-recognition receptors expression in immune cells have been well described (Werling & Jungi, 2003; Alvarez *et al.*, 2008), but expression of these receptors in the intestine is less well documented. *TLRs* play a central role in the initiation of immune responses against a number of pathogens (Ingham *et al.*, 2008). They recognize various pathogen-associated molecular patterns (PAMPs) from parasites (Kawai & Akira, 2001). This study identified *TLR7* to be up-regulated (4.2 fold increase) in infected chicken from Limpopo (Limp5). *TLR7* is commonly involved in pathogen reconition which explains why it was regulated in chickens from *A. galli* parasite environment. This member of *TLRs* was also observed in a separate study (Ingham *et al.*, 2008) that showed transcript level changes in resistant sheep in response to *Haemonchus contortus*. Furthermore, genes representing soluble pattern recognition receptors and T cell signature cytokines were differentially expressed ($P < 0.05$) after the *A. galli* infection compared to the control group (Dalgaard *et al.*, 2015). Due to expression of this gene, it might be important for the host immunity in the Limpopo chickens. However, this gene family has not been identified in infected chicken from (KZN4). It's importance in regards to response to *A. galli* infection needs furthur investigation. The T cell response observed may reflect greater activity of regulatory or helper T cells, both of which play a crucial role in intestinal homeostasis. TLRs have shown expression changes due to pathogen challenge in multiple tissues (Abasht *et al.*, 2008; Lu *et al.*, 2009; Ferdushy *et al.*, 2013). Thirteen mammalian TLRs (1-13) have been reported in literature with at least one ligand that has been identified for each different TLR except TLR10 (Ackert, 1923; Temperley *et al.*, 2008; Li *et al.*, 2012). Previous studies on the identification of avian TLRs were primarily dependent on bioinformatics (in silico) approaches (Yilmaz *et al.*, 2005; Temperley *et al.*, 2008). Limited information is available regarding avian TLRs' functions and mechanisms of regulation; therefore, this study provides information in understanding the involvement of avian TLRs in immune response to nematodes.

Cytochrome P450s (*CYPs*) are a large superfamily of enzymes found in almost all living organisms (Stear *et al.*, 2001). *Cytochrome P450 2h1-like isoform x1* was down regulated in infected KwaZulu-Natal chickens and not defferentially expressed in the chickens from Limpopo region. In a study on malabsorbtion syndrome, which is a model for intestinal disturbances in young chickens (van Hemert *et al.*, 2004), *Cytochrome P450* and

apolipoprotein B showed differences in regulation among lines. In this study, *Apolipoprotein a-iv* was down regulated in chickens from KZN. Down regulation of *apolipoprotein B* and *cytochrome P450* in intestinal epithelium was also reported as a response to pro inflammatory cytokines (Besnard *et al.*, 2002; Darmawi *et al.*, 2013). Therefore, the down-regulation of *apolipoprotein* and *cytochrome P450* might be a response to intrusion in the small intestine. *Apolipoprotein* has been reported to be an endogenous anti-inflammatory protein (Vowinkel *et al.*, 2004).

The *CXC chemokine receptor type partial* and *CXC chemokine 21* were down regulated in small intestines of *A. galli* infected chicken from Limpopo province. Similarly, *CXC chemokines IL-8* and *K60* were also up regulated in the jejunum of *Salmonella serovar Typhimurium* infected chicken (Withanage *et al.*, 2004). It was also reported that CXCL10 gene expression was significantly up regulated in H5N1-infected ferret lungs (Swaggerty *et al.*, 2006). A Th2 polarised cytokine response was reported in the jejunum and spleen of *A. galli*-infected chickens (Kaiser, 2007; Pleidrup *et al.*, 2014). An increased expression of the Th2 signature cytokine IL-13 in the spleen of *A. galli*-infected chickens was observed. *CXC activates natural killer cells* and is thought to play a role in the temporal development of innate and adaptive immunity (Neville *et al.*, 1997) and as suggested by results from this study, have a major role in the defense of chickens from *A. galli.* This gene family also showed to trigger necrosis (Sato et al., 2008), which is what we found in histology results.

Stress related proteins such as *heat shock protein 30c-like* and *heat shock protein beta-7* were up regulated and *heat shock protein 105 partial* down regulated in Limp5. *Heat shock protein 30c-like* belongs to the small heat shock protein 20 family and is involved in stress response. *Heat shock protein 70,* which can also play a significant role in heat stress, went under significant change as it was significantly down-regulated in infected chicken from KZN4. This protein was also expressed in other studies (Kim *et al.*, 2014). When a host encounters heat stress, the expression of highly conserved heat-shock protein will be changed in different tissues and cells to protect the body from excessive inflammation (Burdon, 1986); (Sun *et al.*, 2015). Further studies will be required to evaluate the role of these cytokines in the host response to *A. galli.*

Notably, this study revealed several genes that were specific to different segments of the intestine that might be important for immune response. Mast cells are key effector cells in

mediating worm expulsion from the small intestine. Darmawi *et al.* (2013) investigated the three tissue layers namely mucosa, submucosa and tunica muscularis/serosa in the jejunal of both normal and *A. galli* infected chicken groups and observed the excretion of mass cells to have been differentially increased in the different layers of the jejunum with large numbers of mast cells observed in the mucosa. The number of mucosal mast cells in healthy individuals were on the other hand stable while fewer mast cells manifested in the submucosa and tunica muscularis/serosa respectively (Darmawi *et al.*, 2013). In another study, an increased count of mast cells was also observed in the abomasum of immunized sheep which may indicate the role that mast cells play in orchestrating the protective immune response by producing cytokine and chemokines that recruit other cells to the site of infection and direct the acquired immune response (Bowdridge, 2009). In this study, the down-regulation of mast cell protease 1a-like in jejunum of Limpopo chicken may signalize a down-regulation of the inflammation response following a successful defense response. This phenomenon support that the mast cells play an important role for controlling of *A. galli* infection (Darmawi *et al.*, 2013).

Mitogen-activated protein kinases (MAPK) are differentially activated by cytokines, hormones and growth factors. Previous studies have shown that MAPK modules may be activated by a variety of stress stimuli including wounding, DNA damage and bacterial products such as lipopolysaccharides. Protein kinases covalently attach phosphate to the side chain of tyrosine, serine or threonine of specific proteins. Some cellular activities such as gene expression, metabolism, cytoskeletal functions and other cellular regulatory events are controlled by the activation of MAPK in response to these stimuli. In this study, protein kinase c inhibitor aswz variant 5 were up-regulated in the jejunum and ileum of Limpopo chickens. The high expression of pattern-recognition receptors (PRRs) was also observed in the jejunum and ileum which are the distal segments of the intestine. This observation suggest that the immune system of the different anatomic sections of the small intestine develops differently .

Some biological functions related to nutrient metabolism were found to be differently enriched between tissue segments, which is in line to the distinct anatomical intestinal segments are possibly involved in different physiological functions. In a study conducted along the small intestine of pigs, nutrient metabolism was more enriched in duodenum and jejunum relative to the ileum (Mach *et al.*, 2014). While the expression of *fatty acid-binding protein* (*FABP*) was consistent in the jejunum of Limpopo and KZN chickens (Fold change= 2.6), the expression

of this gene was highest in the duodenum with fold change value of 5.9. This gene was reported elsewhere to be involved in metabolic regulation (Besnard *et al.*, 2002). In humans, the expression of *FABP2* has been used as plasma marker for the detection of injury along the duodenal to colonic axes (Besnard *et al.*, 2002).

Plotting the MDS results highlighted that all three targeted sections of the intestine separated well into three distinct groups, which was consistent with the PCA results. Taken together therefore, the MDS and PCA suggest that there were significant biological dissimilarities amongst segments, which may be a result of the tissue's degree of specialization or number of different cell types. This study was consistent with the study by (Mach *et al.*, 2014) that investigated gene expression patterns along the proximal-distal axis of the porcine small intestine (duodenum, jejunum and ileum) an observed an obvious structure within the data that is consistent with tissue segment.

Blast2GO top-hit species distribution revealed significant BLASTx matches (E-value < 1.0E-10) with chicken protein sequences deposited in the non-redundant GenBank database. Furthermore, there were also a number of sequences similar to non-avian organisms, corresponding to prokaryotic and eukaryotic key proteins of cell function, for example transcription, translation and elongation. The genomes of these species *e.g* bacillus thuringiensis and chlamydia psittaci are completely sequenced and available at the common databases, which explain the sequence similarities. However, the percentage of sequence similarity was low at 2%.

To gain understanding of ontology categories resulting from interactions between chicken and *A. galli* infection, it was helpful to first have knowledge of gene expression at the global level. The analysis of enriched GO terms enabled the discovery of significant gene categories that could have been overlooked by evaluating individual genes. The enriched GO terms could aid in interpreting the dominant functions controlled by differentially expressed genes. The higher number of identified GO terms was positively correlated to the number of differentially expressed genes in all comparisons. This trend was observed both in up and down regulated genes. The enriched biological processes such as response to stimulus, immune system process and signaling are involved in immune response and could be associated with the resistance or tolerance of chickens to *A. galli* parasites. Further studies are needed to verify the biological implications of these changes and if they are associated with resistance to *A. galli* parasites.

The study also gave insight into biological pathways that are interfered with in the presence of nematode infections. The approach used helps in the interpretation of molecular mechanisms underlying protective immunity and host resistance to *A. galli* parasitic infection. Arachidonic acid metabolism was amongst the pathways significantly impacted in *A. galli* infected chickens from KwaZulu-Natal. Arachidonic acid is capable of modifying or regulating one or more immune functions and may serve as a potent inhibitor for Type 1 helper T cell (Th1) response (Freeman *et al.*, 2012). It is one of the crucial polyunsaturated fatty acids associated with membrane phospholipids which when liberated from the plasma membrane, can be oxidized to a variety of eicosanoids. Eicosanoids act in signaling molecules and stimulating a variety of responses in their target cells including immune response (Peters-Golden *et al.*, 2005). Worm killing activities of arachidonic acid have previously been demonstrated in a study conducted in mice where a single oral dose of arachidonic acid led to a significant reduction of total worm burden of Schistosoma. Arachidonic acid-mediated parasite killing is suggested to be due to excessive activation of parasite neutral sphingomyelinase, leading to sphingomyelin hydrolysis into ceramide and phosphorylcholine (El Ridi *et al.*, 2010). In a study conducted in cattle, dietary supplementation with polyunsaturated fatty acids resulted in a 24% reduction in egg per gram in calves that were infected with *Ostertagia ostertagi* and *Cooperia oncophora* (Muturi *et al.*, 2005). This acid metabolism pathway was reported to be activated in cattle in response to gastrointestinal nematodes (Li *et al.*, 2012). The importance of the activation of arachidonic acid metabolism pathway in the presence of *A. galli* in village chickens needs further investigation.

Another pathway mostly impacted is T cell receptor signaling pathway. The ability of nematodes to destabilize the TLR signalling pathway has been reported previously (Kane *et al.*, 2004). Together, our results suggest that T-cell receptor signaling pathway may play an important role in invoking effective host immune responses and in the development of host resistance. The future work should focus on the mechanistic link between TLR signalling pathway and the development of host resistance to gastro-intestinal nematode infection in chickens.

5.5. Conclusion

In summary, the study successfully identified and analyzed DEGs from small intestine collected from village chickens that were naturally infected with *A. galli* using RNA-seq technology. There were noteworthy differences in the mechanisms of host resistant to infection between chickens from different provinces. To identify and understand the role of induced genes is the key to unveiling the mechanism of resistance of parasites in poultry. The data presented in the study could find application in development of genomic markers for use in selection and breeding of chickens against pathogens. Understanding the molecular mechanisms that contribute to protective immunity, immune suppression, pathology, and host resistance will have a significant impact on alternative disease/pathogen control strategies.

5.6. References

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Chapter 6

Critical review and discussion

6.1. Critical review and discussion

Recent developments in molecular and genetic technologies have opened up new avenues to investigate and understand the influence of environment and production systems on a number of difficult and costly to measure traits in livestock and poultry. Traits such as resistance to diseases are good examples (van Marle-Köster *et al.*, 2015). In poultry, genetic resistance studies have reported different chicken genotypes to differ in their response to helminth infections (Permin & Ranvig, 2001; Gauly *et al.*, 2002; Kaufmann *et al.*, 2011). Genetic

differences disease resistance and other adaptation traits may be used to select for more suitable breeds for village chicken production systems, characterised by poor biosecurity (Kaufmann *et al.*, 2011) and compromised disease control programs.

This study focused on village chickens due to their significant contribution to the livelihood of rural communities of South Africa as a source of income, improving food security and quality protein supply. Village chickens are also an important element in diversifying agricultural production (Mtileni *et al.*, 2009). Despite their importance, village chickens are raised under extensive production system where they are exposed to harsh weather conditions, diseases and disease pathogens and are at increased risk of infection. The scavenging system exposes village chickens to gastrointestinal nematodes resulting in high prevalence of parasitic infections and diseases. *Ascaridia galli* parasite is the most prevalent parasite of village chickens and is of major concern due to its association with production losses, severe health effects, economic impact (Irungu *et al.*, 2004a; Kaufmann & Gauly, 2009).

Understanding the genetics of parasite infection and host resistance is key to successful development of genetic control strategies that depend on the natural resistance of chicken populations to parasites. The aim of this study was to employe high throughput cutting edge genomic technologies to investigate genes associated with resistance to gastro-intestinal parasites in village chicken populations. Firstly, the study characterised the village chicken production systems in the villages of Limpopo and KwaZulu-Natal (KZN) provinces by collecting baseline information on the production system and related gastrointestinal parasites. Limpopo and KZN provinces were selected for this study due to their contrast agro-ecological zones and high number of village chickens. Limpopo province is traditionally a dry area compared to KZN province which is wet. However, during sampling the conditions were dry in KZN and wet in Limpopo province, the deviations of which were noted and posed limitations for the study in that atypical climatic conditions were experienced during the study period.

The survey of production system results emphasised an important role of village chickens from villages of Limpopo and KZN provinces in the livelihood of the farmers. Majority of the farmers kept chickens mainly for meat consumption and income. Although some farmers also owned cattle, sheep, pigs and goats, the numbers of these livestock were relatively low compared to chickens hence chickens were regarded as most important. Most farmers reared their chickens extensively with minimum or no veterinary intervention, feed supplementation

and/or housing. Despite the fact that village chickens are adapted to these harsh environmental stresses, their productivity is inefficient, characterized by low productive performance and high productive wastage (Pedersen, 2002). The presence of gastro-intestinal parasites which was evident in this study can be attributed to the poor biosecurity in free range systems that presents favorable conditions for helminth infections and exposes chickens to high risk of infection. The extensive production systems harbour parasite eggs that are able to remain viable for months under cool and moist conditions and chickens get re-infested over and over again from the same source. Under these extensive village chicken production system, chickens are exposed to the extreme environment and are in constant contact with parasite eggs and intermediate hosts completing the life cycle of helminthes. It was mentioned in other studies that parasitic infection rates differ among certain production systems (Permin *et al.*, 1999) due to the different environmental conditions and chicken management systems. This could have been the case in this study whereby the hot and dry conditions in KZN province might explain the lower parasite infection levels compared to Limpopo province, which was wet during sampling time. Differences in biosecurity and management might also explain the observed differences in levels of parasite infestations. The number of mixed parasite infections was however the same in both provinces. Whether the environmental conditions play a role in the number of mixed parasite infections or not could not be ascertained within the scope of this project. The hypothesis that environmental conditions influence type and level of infestation could be tested by simulating environmental conditions from different agro-ecological zones in a more controlled study. Due to the challenges in implementing good and conventional biosecurity practices in the villages, it was concluded in this study to focus on alternative practices that avoid exposure of village chickens to pathogens and assist the smallholder farms to reach optimal production. Some form of housing to prevent scavenging, strict disinfection of the house, improved nutrition and training farmers to contribute to giving practical solutions could be better avenues in assisting the farmers. Furthurmore, the understanding of the production system practice by farmers was seen as crucial in the development of holistic breeding and improvement program and strategies. Adaptation traits like diseases or parasites resistance should be given priority when developing breeding objectives particularly for low-input extensive production systems.

The second experimental study investigated the genetic diversity and population structure of South African *A. galli* parasites sampled from the production systems of KwaZulu-Natal and

Limpopo provinces. Sequencing of the mitochondrial DNA made it possible to characterize and compare the *A. galli* parasite populations from the two different provinces. Other markers such as first and second internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA (rDNA) have shown to provide reliable genetic markers for the identification of nematode species (Gasser & Newton, 2000). Another mitochondrial gene, nicotinamide dehydrogenase (nad1 and nad4), have proven useful as genetic marker for species-specific differentiation. Sequence data for the nad1 and nad4 gene provided novel insights into the genetic relationships among several major lineages of nematodes such as *A. galli*, *A. columbae* and *Ascaridia spp* (GHL-2012) (LIU 2013). Applying PCR-RFLP on the *Cox*1 gene of the mitochondrial DNA used three different types of *A. galli* cohorts (Katakam *et al.*, 2010). The mitochondrial DNA is maternally inherited in *A. galli* as in other parasites (Nejsum *et al.*, 2008) and is therefore inheritated as a haplotype. Future work should use the combination of *Cox*1 gene along with the ITS-1, ITS-2, nad1 and nad4 to improve resolutions of haplotypes particularly in closely related populations.

For this study, F_{ST} revealed no significant variation between the two provinces, which may be a sign of common maternal origin. There was evidence of limited gene flow experienced among the populations implying less movement and sharing of chickens between farms and provinces. Limited gene flow was expected considering the long geographic distances between the provinces. The *Nm*, F_{ST}, Tajima's D and mismatch distribution all confirmed high genetic diversity and genetic similarities of *A. galli* from the two provinces. It was therefore concluded in this study that the *A. galli* from the two provinces were from the same genetic population. Genetic similarities between *A. galli* from limpopo and KZN were contrary to the production system survey results that showed some level of differences between chicken production systems practiced in the two provinces (Malatji *et al.*, 2016b). The study didn't support the hypothesis that genetic properties of the *A. galli* parasite from two province varies due to the contrasting production systems and environmental conditions. It was suggested that the genetic similarity of *A. galli* population would probably be due to overlapping selection pressures between the two provinces such as substandard shelter, poor husbandry, poor and fluctuating nutrition. The future studies should investigate whether the removal of these potential selection pressures will affect the genetic make-up of *A. galli* parasite population in the different agro-ecological regions.

A total of six *A. galli* haplotypes were identified, which were novel relative to those in literature (Höglund *et al.*, 2012). However, more samples from the Limpopo and KZN provinces need to be investigated. Because the South African *A. galli* population clustered separate from the Danish population using median-joining networks, the control strategies for the different populations will also differ. However, *A. galli* parasites from other provinces of South Africa need to be investigated in order to reach full conclusions of genetic make-up of the South African population. Overall, the findings of this experiment gave insight on the genetic structure of the South African *A. galli* parasites. These results would contribute to management and implementation of control strategies specifically for *A. galli* parasites. Particulary for this study, they would inform interpretations on how village chickens from different farming systems would respond to gatsrointestinal parasite infection.

With the knowledge gained from the first objectives, the third experiments investigated if the *A. galli* parasite populations would trigger the same histopathologic response on the small intestine of chickens from the two provinces. Pathology differences were evident between infected and uninfected chickens from both provinces. The impact of the parasite on the histology of small intestine was however the same for the intestines from KwaZulu-Natal and Limpopo provinces which could be attributed to the genetic similarity of the *A. galli* parasites. An investigatation into the correlation between the parasites load, body weight and the histological effects was beyond the scope of this study but would give insight onto the extend of an impact of low/high worm burden on the body weight and small intestine of the host. Permin & Ranvig (2001) indicated that a low number of adult worms in the intestine together with an appropriate feeding would protect the hosts from developing clinical signs against *A. galli*.

Chapter 5 investigated the gene expression profiles and genetic mechanisms involved in fighting infections in *A. galli* prevalent areas using RNA-seq approach. The RNA-seq provided the advantage of sequencing whole transcripts, not limiting itself to preselected genes as with microarray based analysis (Trapnell *et al.*, 2010). This approach enabled us to uncover the transcriptional networks and the genetic mechanisms that were implicated in resistance of village chickens to *A. galli* infection. For RNA-Seq analysis, the FPKM values of the transcripts were log-transformed using log2 to approach normal distribution and to partition the DEGs into up- and downregulated groups. Log2 was used because a two-fold increase in

expression has a log2(fold change) value of +1, while a two-fold decrease in expression has a log2(fold change) value of -1. Also, 2-fold is likely high enough to avoid most spurious changes in gene expression. This criteria was also used in previous expression studies (Chapple *et al.*, 2013; Li *et al.*, 2011).

Gene expression profiles differed between chickens from the two provinces. It could however, not be concluded whether the differences were exclusively due to agro-ecologocal zone effects. It was challenging during this study to control or account for all variables that might have played a role in gene expression profiles. The different production systems practised within the provinces is one such variable. Also, chickens that were used were naturally infected with *A.galli* parasites, which have a potential to trigger secondary infections. Another variable that couldn't be accounted for was the exact age of the chickens used. It has been reported that the age of an animal can influence gene expression as the function of reproduction is prioritized over the expression of immunity in adult animals whereas young animals may give priority to the development and expression of immunity (Coop & Kyriazakis, 1999; Kaufmann *et al.*, 2011). Information about the expression of genes in small intestine, under different conditions and different production systems generated in this study form basis of selection of animals with the right genotypes for enviroments infested by *A. galli* nematodes.

A total of 277 and 190 DEGs were reported. Immune family genes such as TLR and cytokines were regulated in chickens reared in *A. galli* infected areas. Similar gene families have been reported in previous studies from which they were considered to have a role in combating diseases (Swaggerty *et al.*, 2006; Lu *et al.*, 2009). MHC group of genes were also reported in this study and play a vital role in immune response mechanism (Lamont *et al.*, 2002). Understanding the role of these genes in host resistance to *A. galli* in village chickens is particularly important in developing effective disease management and control strategies. Analysis of the enriched GO terms gave a reasonably better insight of the underlying biological processes in this experiment. It was concluded in this study that chickens from different provinces used different mechanisms to resist nematodes infection. The RNA-Seq approach enabled unveiling of the transcriptional networks and the genetic machanisms involved in village chickens raised in *A. galli* environment some of which were supported by literature.

As a complementary analysis to the analysis conducted using the whole organ, we also sought to investigate the gene expression profile of the different segments (duedunum, jejunum and

ileum) of the small intestine. This study analysed transcriptome data from the three sections to identify genes whose tissue-specific enrichment might be connected to the resistance phenotype. To our knowledge, this is the first study that reported a detailed analysis of the genome-wide gene expression patterns in the different anatomical sections of the small intestines in village chickens. The gene expression profiles were different amongst the duodenum, jejunum and ileum across provinces. These three regions differed in the number of DEGs ileum appearing as the key transcriptional section of immune response genes. The results suggests that each region of the intestine functions differently in response to nematode infection, implying significant variations in gene expression profiles that may control cellular gastrointestinal reactions.

Important genes such as *CLDN10* were detected in jejunum, and would not have been detected using the whole small intestine based analysis perfomed. This gene showed high expression level (5.3 fold increase), suggesting that it was missing from the whole small intestine data set not because of insufficient coverage, but probably due to the interference of signals in pooled data. A previous study that looked at the importance of tissue specificity on RNA-seq experiments suggested that using of whole organ lead to false negatives for genes that are strongly expressed in specific organs (Johnson *et al.*, 2013). If genes are expressed in many different sections within the composite structure, then signals of gene expression from the different organs may interfere with each other.

The results differed with those observed by Ingham *et al.* (2014), where no single body tissue emerged as the key site of transcription of putative insecticide resistance genes. Nonetheless, these authors as in other studies indicated that the use of RNA extracted from the whole organism to identify candidate insecticide resistance genes has a risk of missing candidates genes if the expression of these genes is restricted to certain tissues or regions (Ingham *et al.*, 2014). For example, if expression of a gene is restricted to an organ that contributes only a small proportion of mRNA to the total RNA pool, or differential expression occurs in only one tissue, even large differences in expression between a resistant and susceptible population may not be detectable.

GO enrichment analyses implied that tissue-specific genes were enriched with GO terms corresponding to the biological functions of tissues from which they originated. This experiment provided a transcriptome understanding of how natural selection has shaped the

patterns of variation in the genome of South African village chickens. In general, our results form a basis for more detailed future investigation of the role of the observed genes and pathways impacted by the DEGs in the regulation of host-*A.galli* interactions. This information can direct the development of effective parasite and parasitic disease control strategies in poultry.

The enrichment of four of the five genes in specific small intestine segments was confirmed by RT-qPCR. The other gene (*MT1*) did now show any significant change for RT-qPCR. However, the direction of change and magnitude of *MT1* gene was consistent between the two methodologies (down-regulation or up-regulation). A much greater over expression was observed by RNA-seq than for qPCR in all five genes. This kind of differences between RNA-seq and RT-qPCR is frequently observed (Li *et al.*, 2010; Kocmarek *et al.*, 2014) probably due to the sensitivity of RNA-seq method over RT-qPCR method.

6.2. Future studies

This study was conducted on villages chickens that were reared in low-input farming system. Due to the nature of this production system, it was not possible to control for other variables which among others included the time of infection, diseases, previous parasite infections and other infections. Therefore, a more controlled study is needed in the future in order to eliminate those variables.

The focus of this study was only on two provinces of South Africa. Further work on characterizing production systems in the smallholder farms from other provinces need to be conducted. Furthermore, the diversity and population structure of *A. galli* parasites from other provinces can contribute to a complete representation of a South African *A. galli* population. Moreover, additional controlled experiments are needed to study the effect of environment on *A. galli* parasites.

Gene expression in the current research was investigated using the most cost effective, quantitatively accurate and comprehensive available, RNA-seq technology (Morozova *et al.*, 2009; Wang *et al.*, 2009). This approach has revolutionized the study on gene expression as it identified genes that show low expression levels (Morozova *et al.*, 2009; Wang *et al.*, 2009).

In the past years, microarrays were used and they relied on specific probe sequences to capture transcript expression levels. This approach wasn't adding any value to the chicken genome, that remains not fully annotated with unknown function. The future studies using chickens from other provinces should continue utilizing RNA-seq approach so as to fill up the gaps in the chicken genome (van den Berg *et al.*, 2010).

This study investigated a global gene expression profile of the small intenstines after *A. galli* infection. An additional analysis focused on the different sections of the small intestine. Our study suggests that it is imperative to conduct a comprehensive study by investigating the different regions of the small intestine separately (Ingham *et al.*, 2014). Future studies should avoid analysing the whole organ. Additionall studies will be necessary to ascertain the transcriptional dynamic occurring in the small intestine in response to *A. galli* infection over time.

Mixed parasite infections are common particularly in village chickens (Phiri *et al.*, 2007; Mukaratirwa & Khumalo, 2010). There is therefore value in conducting gene expression profile analysis of village chickens infected with more than one parasites species. Detailed information about the immune response and underlying mechanisms to mixed parasite infections may be used in the development of biomarkers for selection for mixed parasite infections.

Furthur research using genome-wide association studies (GWAS) would complement RNA-seq analysis and assist in evaluating genetic differences for various traits like resistance to disease and/or parasites. GWAS utilize information on genetic markers or single nucleotide polymorphisms (SNP) evenly spaced across the genome to determine associations with a trait of interest (Goddard & Hayes, 2009). GWAS and transcriptome data will be useful in identifying SNP markers, candidate genes and biological pathways associated with diseases and/or parasites.

6.3. Conclusions

This study, showed that *A. galli* is the most prevalent parasite in the villages of South Africa. It reduces productivity which will negatively impact on the role of village chickesn for food security and income generation for the rural household. The *A. galli* parasite from the two

studied provinces appeared to come from the same lineage and imposed the same histopathologic changes. The gene expression profiles and impacted pathways were different between chickens from different provinces and amongst the different segments of the small intenstine. Overall the study presented a comprehensive analysis of the village chicken production system in South Africa and how it would impact on nematode infection as well as the genetic mechanisms village chickens use to fight and resist infection. This study form basis for the identification of biomarkers for use in selection and breeding for nematode resistance as well as development of other parasite control strategies.

6.4. References

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Chapter 7

Addendum

List of software used for bioinformatics analysis- Chapter 5

Software	Version	URL/Reference
Blast2GO	2.6.0	http://www.blast2go.com/b2glaunch/start-blast2go (Conesa *et al.*, 2005)
Bowtie	0.12.7	http://bowtie-bio.sourceforge.net/index.shtml/ (Langmead *et al.*, 2009)
CLC-bio	5.5.1	www.clcbio.com/
Cuffdiff	2.0.2	http://cufflinks.cbcb.umd.edu / (Trapnell *et al.*, 2012b)
Cufflinks	2.0.2	http://cufflinks.cbcb.umd.edu/ (Trapnell *et al.*, 2012b)
Edge R	1.6.15	http://bioc.ism.ac.jp/2.6/bioc/html/edgeR.html/ (Robinson *et al.*, 2010b)
cummeRbund package in R		http://bioconductor.org/packages/release/bioc/html/cummeRbund.html (Trapnell et al 2012).
FASTQC	0.5.0	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (Andrews, 2010)
FASTX-Toolkit	0.0.13	http://hannonlab.cshl.edu/fastx_toolkit/download.html (Gordon, 2011)
Ggplot2	0.9.1	http://cran.r-project.org/web/packages/ggplot2/ (Wickham, 2009)
IGV	2.1	http://www.broadinstitute.org/igv/ (Robinson *et al.*, 2011)
Interproscan	2012	http://www.ebi.ac.uk/Tools/pfa/iprscan/ (Hunter *et al.*, 2009)

KEGG	1-10-12	http://www.genome.jp/kegg/pathway.html (Kanehisa *et al.*, 2004)
Primer-blast	3.1	http://www.ncbi.nlm.nih.gov/tools/primer-blast/
Samtools	0.1.18	http://samtools.sourceforge.net (Li *et al.*, 2009)
Tophat	1.4.1	http://tophat.cbcb.umd.edu/ (Trapnell *et al.*, 2012b)
NETWORK	4.1.0.8	http://mbe.oxfordjournals.org/content/16/1/37.full.pdf+html (Bandelt, Foster et al. 1999)
DnaSP software	5.10.01	http://www.ub.edu/dnasp/
ClustalX	2.1	http://clustalx.software.informer.com/2.1/
BioEdit program	7.0.5.2	http://www.mbio.ncsu.edu/bioedit/page2.html
Staden package	1.6.0	http://staden.sourceforge.net
MEGA	5	Tamura et al., 2011
WEGO web-based tool		Ye et al 2006
Venny, and interactive tool	2.1	http://bioinfogp.cnb.csic.es/tools/venny/

List of databases used for transcript annotation- Chapter 5

Database	**URL**
Basic local alignment search tool (BLAST)	(http://www.ncbi.nlm.nih.gov/blast/Blast.cgi
Universal Protein Resource (Uniprotkb)	http://www.uniprot.org
MGI	http://www.informatics.jax.org/expression.shtml

RGD	http://rgd.mcw.edu
ZFIN	http://zfin.org
swiss-prot	www.ebi.ac.uk/swissprot/

List of formula used - Chapter 4

Analysis	**Formula**
Nucleotide diversity	$\pi = n/(n-1)\Sigma xixj\pi ij$ or $\pi = \Sigma \pi ij/nc$
Haplotype diversity	$h = 2n(1-\Sigma xi2)/(2n-1)$
Gene flow	(Nm=(1/1+2Nm)

Additional tables - Chapter 4

Molecular analyses of variance for the two *A. galli* populations

Source of variation	d.f.*	Sum of squares	Variance	% variance
Among populations	1	0.251	0.00646	1.704
Within populations	38	14.650	0.37246	98.295
Total	39	14.651	0.37892	

*d.f.: degrees of freedom

Estimates of evolutionary divergence between the Cox1 gene sequences. The number of base differences per sequence from analysis between sequences is shown. All results are based on the pairwise analysis of 25 sequences. There were a total of 393 positions in the final dataset. Evolution analyses were conducted in MEGA5 (Tamura *et al.*, 2011)

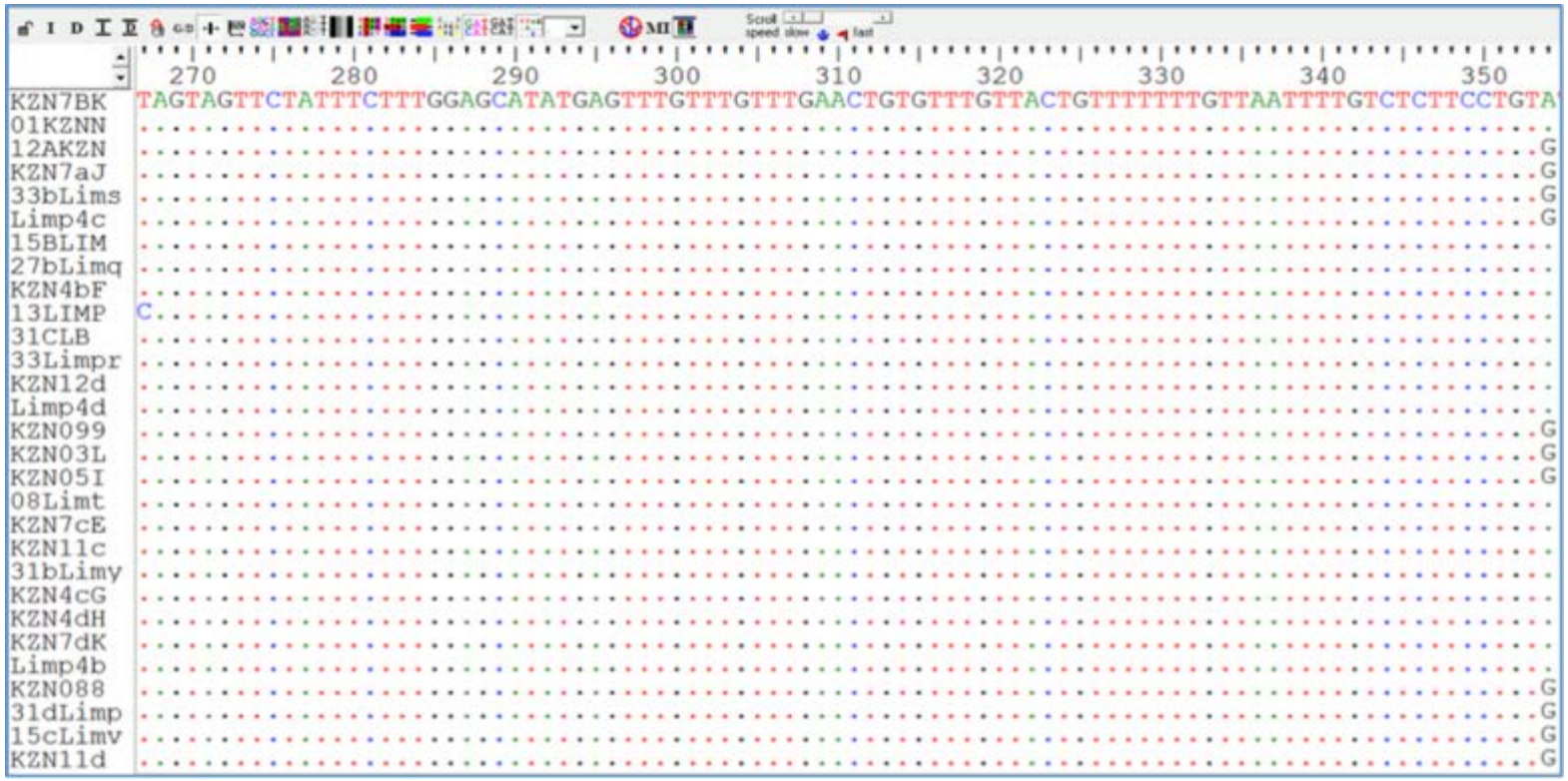
	1	2	3	4	5	6	7	8	9
1. Haplotype VIII									
2. Haplotype IX	0,008								
3. GUI38669 II	0,026	0,029							
4. Haplotype IV	0,010	0,008	0,031						
5. GUI38670 III	0,015	0,008	0,031	0,015					
6. Haplotype V	0,013	0,010	0,034	0,008	0,018				
7. Haplotype VIII	0,015	0,018	0,031	0,021	0,021	0,023			
8. Haplotype VI	0,005	0,013	0,031	0,015	0,021	0,018	0,021		
9. GUI38668 I	0,147	0,147	0,161	0,151	0,157	0,155	0,150	0,154	

Haplotype names and accession numbers of *A. galli* cox1 gene sequences of mtDNA used in this study

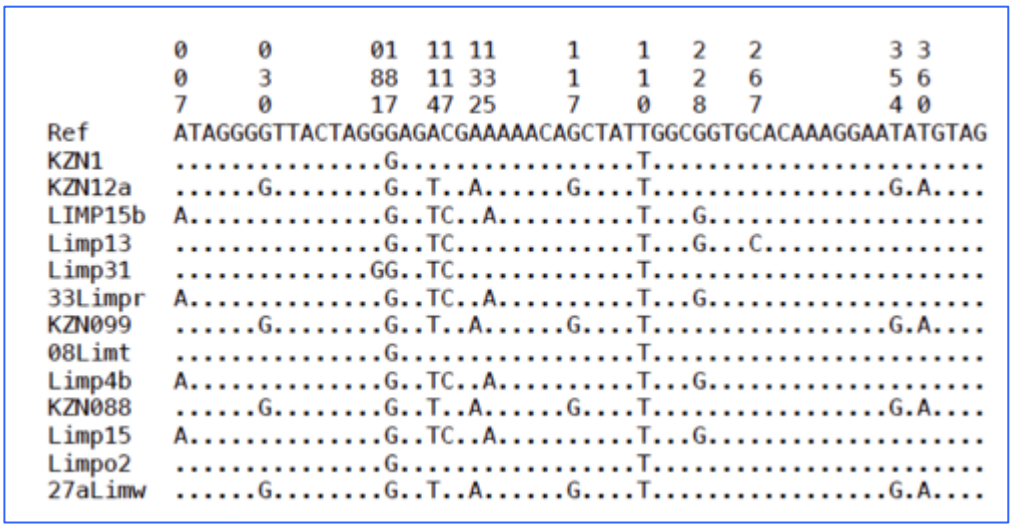
Haplotype	**Genebank accession number**	**Reference**
Haplotype 1	GU138668	(Katakam *et al.*, 2010)
Haplotype 1I	GU138669	(Katakam, Nejsum et al. 2010)
Haplotype 1II	GU138670	(Katakam, Nejsum et al. 2010)
A.galli mtDNA partial COXI gene	FM178545	NCBI-unpublished
Haplotype IV	KT388435	This study
Haplotype V	KT388436	This study
Haplotype V1	KT388437	This study
Haplotype V1I	KT388438	This study
Haplotype VIII	KT388439	This study
Haplotype IX	KT388440	This study

Additional figures - Chapter 4

Supplementary figure 1

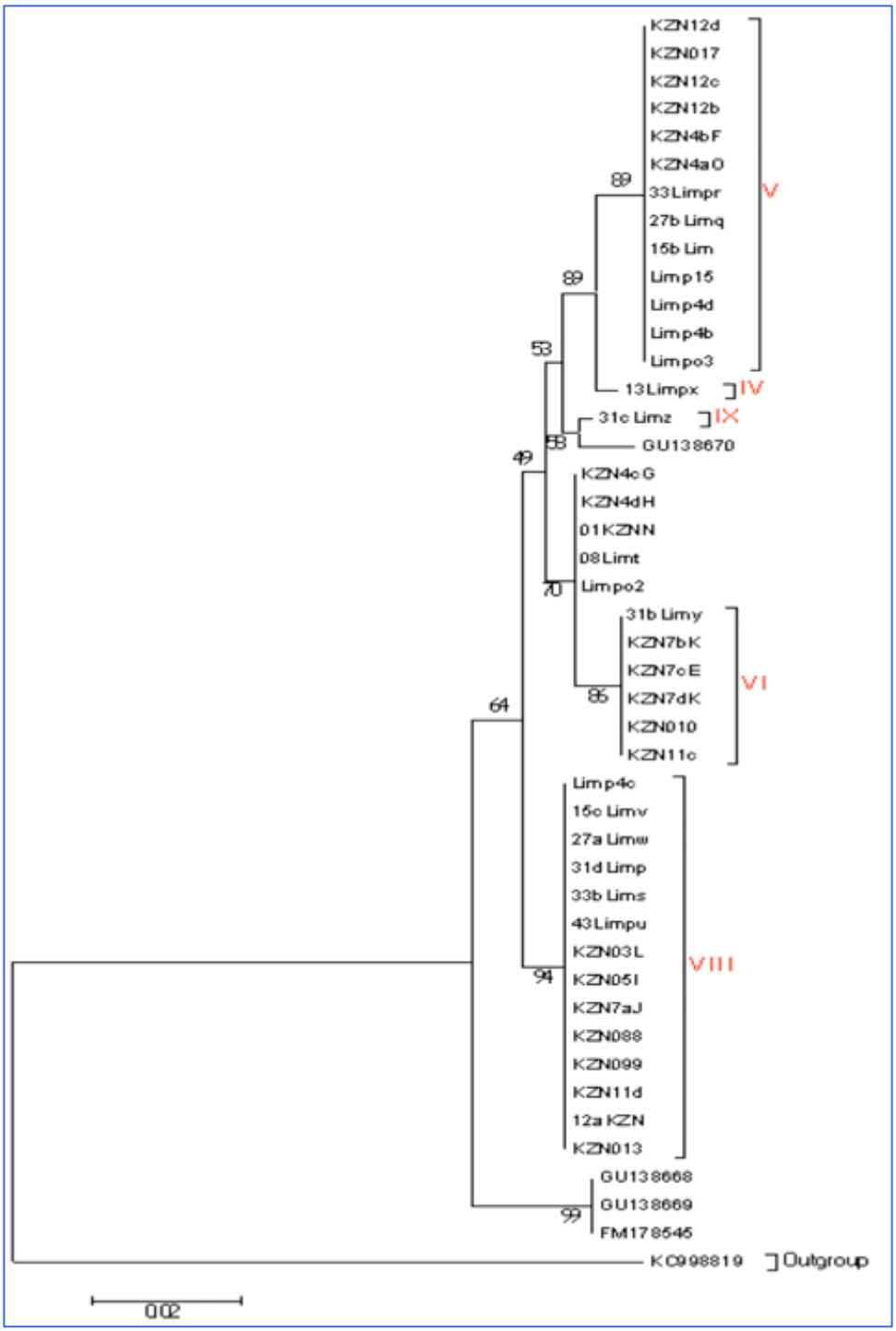


A comparison of A. galli sequences of from Limpopo and KZN provinces with 3 different *A. galli* haplotype sequences obtained from the NCBI database (reference of accession number, GU138668, GU138669 and GU138670) from 220bp to 390 bp



```
          0     0       01  11 11     1    1   2   2         3 3
          0     3       88  11 33     1    1   2   6         5 6
          7     0       17  47 25     7    0   8   7         4 0
Ref       ATAGGGGTTACTAGGGAGACGAAAAACAGCTATTGGCGGTGCACAAAGGAATATGTAG
KZN1      ..............G.................T.......................
KZN12a    ......G........G..T..A......G....T..................G.A....
LIMP15b   A.............G..TC..A..........T...G...................
Limp13    ..............G..TC.............T...G...C..............
Limp31    ..............GG..TC.............T.....................
33Limpr   A.............G..TC..A..........T...G...................
KZN099    ......G........G..T..A......G....T..................G.A....
08Limt    ..............G.................T.....................
Limp4b    A.............G..TC..A..........T...G...................
KZN088    ......G........G..T..A......G....T..................G.A....
Limp15    A.............G..TC..A..........T...G...................
Limpo2    ..............G.................T.....................
27aLimw   ......G........G..T..A......G....T..................G.A....
```

Nucleotide polymorphisms observed in Cox1 domain of 40 A. galli. Vertically oriented numbers indicate the site position and the sequences shown are only the variable sites. Dots (.) indicate identity with the reference sequence (GUI38668)



Neighbor-joining tree, with the Kimura two-parameter distance showing the phylogenetic relationship between samples derived from Limpopo and KZN provinces including 4 GeneBank sequences. Ascaris suum was used as an outgroup. Numbers below/above the branch indicate bootstrap support from 1000 replications