Genomic markers associated with immune traits in Sasso chickens raised in Ethiopia

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DECLARATION

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I declare that the above thesis is my work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references. I further declare that I have not previously submitted this work, or part of it, for any degree or examination in any other higher education institution.

SIGNATURE:  **DATE**: 18 May 2023

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ABSTRACT

Newcastle disease virus (NDV) is one of the highly contagious avian pathogens that threaten poultry producers based in endemic zones as a result of its epidemic potential. Selection for antibody (Ab) response has potential to effectively improve the resistance of disease in chickens. However, the molecular basis of the variation among chickens in Ab response to NDV remains unclear. This study aimed to identify the genes modulating Ab response to a viral pathogen such as NDV while under outdoor conditions. A genome-wide association study (GWAS) was conducted on Sasso T451A chickens that were naturally exposed to infectious diseases to identify regions associated with Ab response to NDV. Phenotypic and immune data from 1022 chickens in two batches (507 in batch four and 515 in batch five) and genotyping from 935 chickens (2,676,181 single nucleotide polymorphisms(SNP)) were used for association analysis. BioMart data mining as well as variant effect predictor tools were used to annotate SNPs and candidate genes, respectively. The results revealed that batch four compared to batch five chickens showed a stronger Ab response at 56 days and lower Ab response at 112 days old. A total of five significant SNPs (rs733628728, rs316795557 (*FOXP2*), rs313761644 (*CEP170B*) and two unnamed) were significantly ($p \leq 3.92E\text{-}7$) associated with chicken antibody response to ND. These SNPs present on chromosomes 1, 5 and 13, are in genomes regions including several genes with roles in the regulation of the immune response. The results of this study pave the path for more investigation into the host immune response to NDV.

Keywords: Antibody response, Genome-wide linkage analysis, Newcastle disease, Sasso T451A, Vaccine challenges

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LIST OF ABBREVIATIONS

Ab	Antibody
bp	Base pair
BWG	Body weight gain
CSA	Central Statistical Agency
CTLGH	Centre for Tropical Livestock Genetics and Health
DNA	Deoxyribonucleic Acid
EIAR	Ethiopian Institute of Agricultural Research
ELISA	Enzyme-linked immunosorbent assay
EMDDI	Ethiopian Meat and Dairy Industry Development Institute
FAO	Food and Agricultural Organization of the United Nations
FTA	Flinders Technology Associates
GATK	Genome Analysis Toolkit
GCTA	Genome-wide complex trait analysis
gDNA	Genomic DNA
GEMMA	Genome-Wide Efficient Mixed Model Association
GGA	Gallus gallus (chicken) Autosome
GO	Gene Ontology
GTP	Growth and Transformation Plan
GWAS	Genome-Wide Association Studies
HB1	Hichner B1
HWE	Hardy-Weinberg Equilibrium
IACUC	Institutional Animal Care and Use Committee
IBD	Infectious Bursal Disease
IBS	Identity by Decent
IC	Ethiopian indigenous chicken
ILRI	International Livestock Research Institute
IPA	Ingenuity Pathway Analysis
Kb	Kilo-base (1000 bases)
LD	Linkage Disequilibrium

m	Meter
MAS	Marker Assisted Selection
MDV	Marek Disease Virus
MHC	Major Histocompatibility Complex
MoA	Ministry of Agriculture
ND	Newcastle disease
NDV	Newcastle Disease Virus
NGS	Next-Generation Sequencing
NPC	National Planning Commission
NVI	National Veterinary Institute
OD	Optical Density
PC	Principal Components
PCA	Principal Component Analysis
PPI	Protein-Protein Interaction
QC	Quality Control
QTL	Quantitative Trait Loci
SNP	Single Nucleotide Polymorphisms
UNISA	University of South Africa
USAID	United States Agency for International Development
VEP	Variant Effect Predictor
µl	Micro-litre

CHAPTER 1. INTRODUCTION

1.1. Background and Justification

Within the past decades, the human population has increased exponentially worldwide, and it has been projected that its growth will reach 9.73 billion by 2050 and 11.2 billion by 2100, which makes it more difficult for agriculture to supply the rising demand for food (FAO, 2017). Extreme poverty is one of the root causes of food insecurity and malnutrition, affecting more than a billion people worldwide. Food security has remained a significant issue worldwide, particularly in developing nations (Barret, 2010). Emerging countries food supply will need to increase twice by 2050, to meet population growth and dietary changes (IFAD, 2009; FAO, 2014). This will need to be achieved sustainably, maintaining the natural resources and environment globally (FAO, 2017). All the factors that determine food security: access, availability, stability, and utilization, are anticipated to be impacted by climate change. It will be necessary to provide help in bridging the protein gap with developing countries anticipated to make up 85% of the increased food consumption demand (FAO, 2007).

Chicken are the most common livestock species in the world (FAO, 2000). There were 25.8 billion chickens in 2021, up from 13.9 billion in 2000. In 2020, 40% of the world's meat production came from poultry (Statista, 2021). Over 98% of all poultry (ducks, chickens, and turkeys) maintained in Africa are chickens, which predominate flock make-up (Hassen *et al*., 2006). Africa produces only 5% of the world's chicken meat and eggs. Ethiopia is one of several developing nations where keeping chickens is common (Hassen *et al*., 2006), and where chicken meat contributes about 5% of the total national meat production (EMDDI, 2017). So, relative to other livestock species (e.g., cattle, sheep, and goats), chicken meat production remains small. Nevertheless, chickens represent a significant source of animal protein, even discounting egg production, for the Ethiopian smallholder household (Belay and Oljira., 2019). It is a livestock resource which requires little management.

In Ethiopia, there are over 57 million chickens in total, with indigenous, hybrid and exotic breeds accounting for 78.85%, 12.03%, and 9.11%, respectively (CSA 2020/2021). To advance egg and

meat production, a number of exotic chicken breeds have been introduced to Ethiopian farmers (Demeke, 2008). Mostly layer and dual-purpose chicken breeds have been distributed in the last two decades. Among the dual-purpose breeds of chicken, Sasso T451A is one of the chicken breeds that have been introduced to Ethiopia. Sasso T451A chickens are characterized by being a slow-growing, robust, and easy-to-manage chicken breed, which can be grown under different rearing systems from traditional to intensive production (SASSO, 2018). Compared to indigenous chickens at the same age, Sasso T451A chickens produce more eggs and they growth faster and larger (2.6–3.2 kg at 28 weeks age) (Osei-Amponsah *et al*., 2012).

Livestock production in Ethiopia is in a period of transition. The transition from backyard farming towards a more commercial form of farming is necessity to meet the needs of the rising population of the country (Ethiopian Growth and Transformation Plan (GTP I and II), https://www.agroberichtenbuitenland.nl/actueel/nieuws/2018/12/20/strategic-plan). In the poultry subsector, the traditional scavenging system is expected to be replaced with a semi-scavenging one at the smallholder level. It requires a coherent strategy and structure for poultry feed, breeding, health, disease prevention and control (NPC, 2018). Parallel to the transition of the poultry industry toward more intensification, the challenge of numerous diseases (caused by bacterial, parasitic and viral infections) will be on the rise. In developed countries, disease-related economic losses in the chicken industry can range from 10 - 20% of the total value of output, and they are probably even higher in developing nations (FAO, 2014). The combined effects of genetic improvement, optimal nutrition, vaccination strategies and impressive increases in disease protection have been accomplished by methodical efforts (Zhu *et al*., 2019). Selective breeding is an important additional method to be used to increase overall disease resistance for a stronger immune system (Cheng *et al*., 2013).

The prevalent disease, predation, nutrition, lack of management and acceptable breeds in the nation limit the amount of chicken that can be produced (Terfa *et al*., 2018). The most economically significant of these restrictions, which affect both chicken population and its production, are diseases and notably viral diseases. The mortality ("from egg to adult") has been estimated to be as high as 80% during disease epidemics (Zelalem *et al*., 2014). Numerous chicken diseases which include chronic respiratory disease, coccidiosis, Marek's disease, Newcastle disease (ND), and nutritional deficiencies have been identified in Ethiopia. These diseases represent a significant

source of economic losses (Mesfin and Bihonegn, 2018). Therefore, any progress in the production of free-range chickens will depend on the effective management of important poultry diseases, in particular the devastating ND (Tadesse *et al*., 2005).

Protection against NDV is through the use of vaccines generated with low virulent NDV strains. Immunity is derived from neutralizing antibodies formed against the viral hemagglutinin and fusion glycoproteins, which are responsible for attachment and spread of the virus. Given the objective of evaluating Ab response with respect to phenotypic and genetic variation, it would be important to outline related information, for example the basis of this Ab response. The disease specific response of the innate immune system may be a valuable indicator of the level of disease tolerance or resistance in the host. The innate immune response comprises factors that exist prior to the advent of infection and are capable of exclusion or rapid response to microbes. It has been demonstrated that immunological traits in poultry, such as Ab titers, are heritable (Lamont *et al*., 2003), raising the prospect of identifying loci (genes) linked to immune (disease resistance) traits. The genetic regulation of immunological traits has been mapped using microsatellite markers in previous ND studies (Yunis *et al*., 2002). Quantitative trait loci (QTLs) analysis has revealed association with immunological genes on chicken chromosomes 2-5, 9, 13, 16, 18-19, 22 and on the Z sex chromosome (Slawinska and Siwek, 2013).

It is challenging to measure traits like diseases resistance and response to infection (Psifidi *et al*., 2016). However, measuring antibody titer following infection or vaccination is promising avenue as a proxy of the innate system efficiency. Then genome-wide association studies (GWAS) may be conducted to find genetic markers associated with the disease resistance phenotype under study using DNA Beadchip technology, allowing genotyping thousands of genetic markers simultaneously. This approach is commonly used for human disease and economically important animal traits research. In chickens, many GWAS have been conducted using the Illumina 60K SNP Beadchip or the 600K SNP Affymetrix array. Using this method, major loci associated with disease (Raeesi *et al*., 2017), growth (Gu *et al*., 2011; Xie *et al*., 2012; Guo *et al*., 2017; Pertille *et al*., 2017), egg production (Liu *et al*., 2011; Wolc *et al*., 2012), carcass (Huang *et al*., 2018) and meat quantitative traits (Moreira *et al*., 2018), resistance to Marek's disease (Li *et al*., 2013), and immune responses to NDV (Luo *et al*., 2013) have been identified.

1.2. Research problem statement

Among the chicken infectious diseases, the highly contagious Newcastle viral disease is affecting both wild and domestic birds, making it one of the most contagious (Damena *et al*., 2016). In Ethiopia, ND outbreaks associated with the velogenic strain is known to cause serious economic losses and cause up to 80% mortality (Zelalem *et al*., 2014). Sasso T451A chicken flocks are now commonly raised in semi-scavenging village conditions in Ethiopia. However, the performance, genetic make-up, disease resistance, optimum management and adaptation to outdoor conditions have not been thoroughly assessed for this breed in the country. Accordingly, this study aims to undertake genomic and immune traits analyses of the Sasso T451A populations in Ethiopia. The research study will assess the association of SNP markers from genome-wide scans with Ab responses and production performance of Sasso T451A chickens raised in semi-scavenging village conditions at ILRI Addis Ababa poultry research facility.

1.3. Relevance of the research

The Next-Generation Sequencing (NGS) technology has accelerated the discovery of candidate genes associated to traits of economic importance. A study conducted by Benitez (2002) emphasized that accurate genetic data are dependent upon the availability of genetic variations. So, immune response is genetically variable, which gives rise to the possibility of enhancing disease resistance, either through traditional methods of animal breeding or by molecular genetic screening procedures. In this study, variation in immune response in Sasso T451A was assessed, correlated it with productivity traits and used genetic markers to contribute to our knowledge of the genetic control of such variation.

1.4. Aim and objectives of the study

The general aim of the study was to identify genes and genomic regions associated with the immune response of Sasso T451A chickens raised in Ethiopia in semi-scavenging system.

The specific objectives of this study are the following:

a) To evaluate the presence of phenotypic and genetic variability of Ab responses to Newcastle Disease Virus (NDV).
b) To identify and examine genomic regions associated with Ab response to NDV.

1.5. Research hypothesis

a) The Ab responses to a major infectious disease (ND) does not differ according to the genetic variation between individual chickens.
b) The immune response traits are not associated with the genomic regions.

1.6. Research question

a) Do genetic factors affect the immune response of individual chickens, and to what extent?
b) Is the immune response in chickens affected by the time of year of the challenge (batch effect)?

1.7. Dissertation layout

This study comprises of six chapters organised as follows:

Chapter 1: Introduction: Background of the study, which includes the research problem statement, relevance of the research, aims and objectives of the study, research hypothesis and question of our study.

Chapter 2: Literature review: It provides the summary of the literature (poultry industry, immune traits of chickens and use of genetic markers in chickens) and also provides a brief summary on the poultry production system, chicken breeds and an overview of Sasso T451A chicken in Ethiopia.

Chapter 3: Research methodology: It detailed information on methods such as sampling, genotyping including quality control, immune phenotypes, and genome-wide association analysis, genetic parameter estimation, SNP and candidate region

annotation, pathway, as well as functional enrichment analyses of the candidate regions.

Chapter 4: Results: This chapter provides all the findings of the current study.

Chapter 5: Discussion: Explanation and interpretation of the results in relation of previous studies.

Chapter 6: Conclusion and recommendations: Summary of the results and recommendations.

CHAPTER 2. LITERATURE REVIEW

2.1. Poultry industry

The poultry industry plays a vital role in the global economy, and it represents a major protein source for human consumption. The production of chickens around the world has changed significantly and increased during the past 50 years. Moreover, the consumption of chicken eggs and meat and animal-source foods has increased rapidly in the past decades (FAO, 2020). Indeed, poultry meat and eggs are among the main animal-supply ingredients extensively eaten across human societies, regardless of their cultures, traditions, and religions (FAO, 2008). The global industrial poultry sector includes three main segments: breeding of parental stocks, production of broiler and egg-layer, and processing of meat and eggs (FAO, 2014). It accounts for nearly 2/3 of chicken egg and meat production (Dolberg, 2007). In 2008, it was estimated that the commercial chicken (layer and broiler) markets produced more than 40 billion chickens yearly to satisfy the demands from consumers for meat (61 million metric tons) and eggs (> 55 million metric tons) throughout the world (FAO, 2008). In 2018 and 2020, the demand was 82.8 million metric tons of eggs and 137 million tons of poultry meat, respectively (FAO, 2020; FAO, 2021). The African continent contributes only 0.7% and 4.4% of the world chicken meat and egg production, respectively (FAO, 2014). In Ethiopia, indigenous, exotic and hybrid chickens contribute about 95.86%, 1.35% and 2.79% of the national poultry production, respectively. These three categories of breed contributed 2%, 0.2%, and 0.01% of poultry meat in 2016 and 11%, 1.7%, and 0.07% egg production East Africa, Africa and the world, respectively (FAOSTAT, 2018).

2.2. Poultry production systems in Ethiopia

Different management and production systems can be used to raise chicken. In Ethiopia, there is a distinct difference between the traditional, low-input approach and the modern, high-tech system (Yami, 1995). There are three main production systems for chicken in Ethiopia: small-scale, backyard or village farming and large-scale commercial farming. The different production systems are catagorized based on purpose of production, breed types, input and output levels, type of

producer, housing, length of broodiness, feeding, mortality rate, health care, growth rate, bio-security measures and number of chickens reared (Tadesse, 2015).

The backyard production system is practiced by nearly all rural families, excluding the nomadic population. This dominant type of poultry production system relies on indigenous (local) chickens. It is characterized by small flock size with little or no inputs, semi-scavenging or scavenging feeding and minimal level of bio-security with minimal health care and high mortality rate (Desalew *et al*., 2013). Therefore, the only significant financial input required is the base stock, some local grains, and perhaps some basic nightshades, with most of the time being spent at night in the family homes. The local chickens are reared in small flocks (4-10 hens), its market weight of < 1500 g at six months old and produce a maximum of 40-60 eggs/year/hen (Dessie *et al*., 2011). In such production system, local chickens are mostly kept, though a few hybrid and exotic breeds may also be present (Desalew *et al*., 2013). ND is the main source of economic loss because vaccination often only takes place in response to an epidemic.

Small-scale intensive production system is characterized by a medium level of supplementary feeding, minimal to low bio-security and small veterinary service inputs. Flock sizes in this production system typically vary from 50 to 500 exotic chickens. This production system is a recently developing system in peri-urban and urban areas, where exotic breeds of chicken are produced (meat and egg) along commercial lines (Alemu *et al*., 2009). The majority of Ethiopia's small-scale poultry farms are situated in and around Addis Ababa and Debre Zeit town in the Oromia region. Small-scale chicken farms could be kept as a primary source of revenue or as a secondary source of income. Here, the prevalence of diseases affecting poultry remain largely unknown. One of the rare examples is Kinunghi *et al*. (2004) study which reported that in such systems, chickens, with coccidiosis, experience mortality, slow weight gain and low eggs production.

In the large-scale commercial chicken farms, an average of $\geq$ 10,000 chickens are reared under indoor condition, with a medium to high level of biosecurity (Desalew *et al*., 2013). Here, we find exotic commercial lines which need expensive inputs like feed, housing, healthcare and general management. These commercial farms represent 2% of the Ethiopian poultry population. Here, the

chicken mortality rate up to adulthood only around 5% following the tight biosecurity measures in place (Bush, 2006). Large-scale commercial farms hens supply the foundation stock and feed for small-scale chicken farms (Nzietchueng, 2008).

2.3. Chicken breeds

Around the world, smallholder farmers raise a variety of poultry species. The most significant tropical species are pheasant, guinea fowl, turkey, goose, quail, ostrich, duck, and chicken. Around 1,600 various domestic chicken breeds are recognized internationally (FAO, 2020). In Ethiopia, chickens are the only domesticated poultry species. Breeds of chicken fall into three major divisions: local, exotic, and hybrid (CSA, 2020/2021). In the past, exotic chickens were only raised in intensively managed commercial farms. Currently, exotic and their crossbreeds may also be found in some backyard chicken production systems, where they require more input than indigenous village chickens (USAID, 2012).

2.3.1. Local chicken breeds

Local African chicken ecotypes are crucial to household livelihoods in both rural and urban areas: provide high-quality protein and are important resource for people particularly for women and kids. Local village chickens are kept an extensive chicken production system, which is best characterized as a low input-low output. Local chickens are hardy and more disease resistant than the high-producing strains and adapted to the harsh local environmental conditions (Manyelo *et al*., 2020). The attractive traits of local chickens are hard eggshells, high fertility, tasty eggs and meat, and hatchability (Melesse, 2000). They are phenotypically diverse in plumage shank colour, feather patterns, body size, comb types and colour. However, low production performance, late maturity and slow growth are there defining traits. Their eggs are small with a thick shell and deep yellow yolk color. On average, a local chicken produces 40 to 60 eggs annually, in comparison to the over 300 eggs produced by commercial layers (Moredaa and Mesekel, 2016). Low genetic potential, nutritional deficiencies, and seasonal influences are some of the factors affecting the local chicken's low egg output. Also, maternal genetic instinct in local chickens means that hens will go broody after they have laid a clutch of eggs into hatching and they will rear their chickens (Pym *et al*., 2006).

2.3.2. Exotic chicken breeds

Reports show that there are over 6.9 million hybrid and exotic chickens, representing 11.5% of Ethiopia's total poultry population (CSA 2020/2021). Exotic chickens were first imported into Ethiopia in the years 1953 were kept at Jimma Agricultural and Technical School (Wondmeneh *et al*., 2016). They were cockerels, pullets, and fertile eggs. Several exotic breeds of commercial chicken (Rhode Island Red, White and Brown Leghorns, Cornish, New Hampshire, Bovans, Australorp and Light Sussex) have been introduced over the past years. For these breeds to achieve their productivity, they require improved feed, vaccination, and therapeutic intervention (Tamir *et al*., 2015). The Ethiopian's Extension Department of the Ministry of Agriculture (MoA) has shown more interest and preference in a dual-purpose (egg, meat) chicken breed (Rhode Island Red) among the exotic breeds that were distributed to smallholder farmers. Moreover, one of the reasons for the importation of the Egyptian Fayoumi breed was the hope that it would eventually outperform other exotic breeds in Ethiopia's rural areas in terms of productivity, disease resistance, and adaptability (Wilson, 2010).

2.3.3. Hybrid chicken breeds

Hybrids or crossbreed chickens result from the crossing of two lines or strains of chickens to combine in the hybrid the desirable characteristics of both parental strains (e.g., productivity and adaptability). The Ethiopian Institute of Agricultural Research (EIAR) started a crossbreeding program to create a synthetic dual-purpose breed for local poultry production (Wondmeneh *et al*., 2016). The two exotic chicken breeds that are used are Rhode Island Red (R) and Fayoumi (F) as dam lines, and two local chicken breeds [local Netch (W) and Naked neck (N)], a white feathered chicken as sire lines. The local breeds were used as sire lines to produce the hybrids growth and egg production performance of the two crosses RW (R♀ X W♂) and (FN (F♀ X N♂), which are being compared with the exotic pure line and with each other' performance (Bekele *et al*., 2010).

2.4. Overview of Sasso T451A chicken in Ethiopia

To increase poultry productivity, different breeds of exotic chickens have been imported into Ethiopia. Among these, the Sasso T451A breed is increasingly becoming popular among smallholder farmers raising poultry under a traditional management system. In Ethiopia, Sasso T451A chicken are imported and distributed by EthioChicken, a private poultry farm company. The Sasso is a French, dual-purpose breed initially developed for France's 'Label Rouge' market to provide consumers with high-quality chicken meat and eggs. Today many different Sasso hybrid strains may be produced by crossing different parental Sasso lines of other production characteristics (SASSO, 2018). An F1 cross between the male line T44 and the female line SA51A, known as Sasso T451A, has been evaluated and found to be adaptable to a wide range of tropical settings (Yakubu and Ari, 2018). However, it shows different production characteristics depending on the agro-ecology. Also, its productivity on-farm at the smallholder level is generally lower compared to on-station performance (Aman *et al*., 2017).

2.5. The immune traits of chickens

While enhancing growth and reproductive traits are the primary production goals in poultry breeding, immune traits selection has received very little attention. With chicken diseases still quite serious, routine immunization remains one of the largest expenses in poultry industry and the majority of smallholder farmers are not reached or are unable to afford it. The host's defense immune response to a foreign (not recognized as self) molecule or pathogen typically defines the immune system (Geng, 2007). In chickens, immune response is affected by numerous environmental and genetic factors (Gavora, 2019). Given the characteristics of quantitative genetic variation, the genome must have numerous locations and multiple alleles that each influence a single trait (Dorshorst *et al*., 2011). An approach could be to choose individuals based on their genotype for a marker linked to an immune response QTL (for example, using marker-assisted selection (MAS). The finding of linkages between DNA markers and immune responses phenotypes is a prerequisite to this approach.

Numerous studies have demonstrated that selecting for improved immune responses can increase the genetic disease resistance of chickens (Bovenhuis *et al*., 2002; Dorshorst *et al*., 2011). In

addition to disease resistance, genetically enhancing the immune system can also improve vaccine effectiveness (Lamont *et al*., 2003). Increasing disease resistance genetically could lower the cost of immunization and other disease prevention measures, enhance the effectiveness of vaccines as well as decrease mortality and performance loss during illness outbreaks (Barbour *et al*., 2012).

Immune performance evaluation in chicken breeding commonly includes the following: sheep red blood cells, Ab response to influenza virus, heterophils to lymphocytes ratio, total serum concentrations of immunoglobulin Y and immune organ index (spleen, thymus), thymus weight at 100 days, spleen weight at 100 days (Zhang *et al*., 2015a) and ND virus. According to Nassir's observation, the velogenic strains of the ND virus are present all over Ethiopia (Nassir, 1998). Once outbreaks of ND occurred, the survivors have high Ab levels and are resistant for a while. Still, as the level of protection falls and with the emergence of new strains, the population becomes susceptible again, and the cycle is repeated.

The advent of genome-wide DNA arrays for poultry has enabled the study of the association of genomic markers and regions with important phenotypic traits. Results from such analyses are expected to facilitate selective breeding programs towards enhanced chicken productivity, health, and other traits (e.g., environmental adaptive traits). Previous work has demonstrated the utility of this approach for the genomic study of health and immune traits in African chickens (Psifidi *et al*., 2016; Banos *et al*., 2020). The variability in resistance or susceptibility to infections; and demonstrated the presence of relationship between LEI0258 marker polymorphisms and variations in chicken susceptibility to ND (Mpenda *et al*., 2020).

CHAPTER 3. MATERIAL AND METHODS

3.1. Study animals and sampling

This study used samples that were collected for a previous study and approved for this project by the ILRI Research Ethics Committee (ILRI-IACUC2020-10/ 2021/CAES_AREC/068). The samples were from a total of 2,573 Sasso T451A chickens, were raised in five batches, of which the last two batches were used in this study. A total of 1,022 Sasso T451A day old chickens were acquired from EthioChicken. The chickens were maintained at the International Livestock Research Institute (ILRI) poultry research facility, consisted of 507 batch four (194 males and 313 females) and 515 batch five (209 males, 306 females) chicken. They were maintained in a deep litter house during the brooder phase for 56 days from hatching. All chickens were vaccinated at the facility according to their vaccination protocol shown in Table 1, and they received therapeutic when needed. All chickens were tagged and sampled before being released into the paddock when they were approximately 56 days old.

At 56 days old, chickens were put together outside into a single large pen (400 m^2, 20 m x 20 m) at the ILRI poultry research facilities, where they were moving freely during daytime and kept indoor in poultry shed at night. The chickens occupied the pen for 4 weeks, after which they were moved to the adjacent pen for another 4 weeks shown in Table 2. Chickens were fed as per the recommended amount per chicken according to their age (Sasso protocol for T4451 hen) (SASSO, 2018) for the first 4 weeks. For the remaining 4 weeks, feed offered per chicken remained constant. As the chickens were scavenging within the pen during the daytime, their feed was supplemented with scavenging products (e.g., insects, plants, etc.). Chickens were naturally exposed to infectious diseases under these conditions. Throughout all phases of growth, the chickens had full access to water, and they were vaccinated with a commercial (National Veterinary Institute (NVI), Ethiopia) NDV live vaccine of the Hichner B1/LaSota strain, using the standard dose given by the manufacturer, by the ocular route at 7 and 21 days of age (NVI, 2016).

Each batch remained in the paddock and monitored for eight weeks. A total of 1.5 ml of blood from the wing vein was collected with a 2 ml syringe with a 23G x 1 (0.6mm x 25 mm) needle from each bird. Blood from 1022 chickens from day 56 and 762 chickens from day 112 was

allowed to coagulate overnight at room temperature prior to the removal of serum. After being separated, the serum was put into cryovial tubes, labeled, and kept at -20^{0}C until testing. Volume of 50 - 250 µl of whole blood was drawn with syringes and preserved in cryo-tubes filled with 1.5 ml absolute ethanol (100%) https://www.sheffield.ac.uk/nbaf-s/protocols_list. The blood was transported to ILRI Addis Ababa molecular laboratory, where it was transferred into QIAcard FTA Elute Micro cards, labeled and stored at room temperature until shipment for genome sequencing.

Table 1. Vaccination protocol at brooding facility

Age in Day	Disease vaccinated for	Name of the vaccine	Way of administration
0	MDV+ IBDV+NDV	Mareks * Gumboro + HB1	Sub-cutaneous + Spray
7	NDV	HB1	Ocular
14	Infectious Bursal Disease	Gumboro	Ocular
21	NDV	Lasota	Ocular
28	Fowl pox	Fowl pox	Wing-web
42	Fowl typhoid	Fowl typhoid	Sub-cutaneous

MDV= Marek's disease virus; **IBDV**= Infectious bursal disease virus; **NDV**= Newcastle disease virus; **HB1**= Hichner B1.

Table 2. Occupied plots by the subsequent batch

Batch of birds	Plot no. (first four weeks)	Plot no. (last four weeks)
Practice run	1	2
1st batch	2	3
2nd batch	3	4
3rd batch	4	1
4th batch	1	2
5th batch	2	3

3.2. Immune phenotypes

The measurement of NDV-specific antibodies was done in serum collected at 56 and 112 days of age using the IDEXX NDV ELISA kit for chickens (IDEXX Laboratories, Westbrook, ME, USA, Cat#99-09263) based on the principle of Indirect Enzyme Linked Immunosorbent Assay (ELISA). All collected samples (day 56, 1004 and day 112, 766) were tested (serum dilution 1:100) in duplicate with a negative and positive control being added to each plates. Optical density (OD) values of test samples and controls were read using an ELISA reader (BioTek®-ELx800™, USA) at 650 nm. In all cases, OD were converted into a ratio to the positive control (S:P ratio) using the following equation (Bettridge *et al*., 2014):

S:P ratio = (mean sample OD−negative control OD)/ (positive control OD−negative control OD)

By presenting the values on a scale, this data transformation made values ("1 = positive control and 0 = negative control") equal between plates. Plate to plate variations were accounted for by numbering the ELISA plates. Log-transformed Ab titers of NDV with significantly skewed distributions were normalized. To determine the statistical significance of variations in Ab response to NDV between batches four and five at 56 and 112 days of age, a Student's t-test in R was used, $P<0.05$ (Team, 2018).

3.3. Genotyping and quality control

DNA was extracted from the blood preserved on QIAcard FTA Elute Micro cards (QIAGEN, Cat. No. WB120410). The extracted DNA (n = 963, batch 4, 465 and batch 5, 498) was shipped to GeneSeek (Neogen Genomics, Lincoln, NE, United States). The genomic DNA was then genotyped by low-pass genome sequencing and with imputationed using the Gencove platform. The GATK software, using the default parameters, was used for SNPs calling. Indels were sequenced/imputed but excluded for the analysis (Mckenna *et al*., 2010). Finally, 14,123,057 SNPs remained for further analysis. Before statistical analysis, evaluation of the data quality and pre-processing of the data were performed to avoid introduction of bias into the analysis (Anderson *et al*., 2010). Raw SNP data were first subjected to quality control and loci with unknown chromosomal location were removed. PLINK v1.9 (Purcell *et al*., 2007) was used data quality control. All samples with high genotyping missing rate (> 10%) were excluded. SNPs were removed if they failed to pass the following criteria: minor allele frequency (MAF) > 0.01, call rate > 90%, *P*-value > 1×10^{-6} for Hardy-Weinberg equilibrium test. After a series of quality checks, 935 samples and 11,272,670 SNPs remained for the analysis.

3.4. Genome-wide association study

Population structure and relatedness are the main sources of confounding effects in genetic association studies (Astle and Balding, 2009). The linear mixed model method is efficient at preventing population stratification bias and it minimizes inflation from genetic effects (e.g., polygenic background). It is the commonest approach for a GWAS analysis with related individuals. Principal component analysis (PCA) was carried out and applied in PLINK analysis

toolset in order to evaluate population structure PCA, bearing in mind that clusters of SNPs in high linkage disequilibrium (LD) may bias the PCA results (Wang *et al*., 2009). First, using a 100 SNP sliding widows with a step of 10 SNPs, all autosomal SNPs were pruned for LD ($r^2 > 0.8$). The top three principal components (PC) that were used as covariates in the mixed model were then determined using these unlinked SNPs. Using the pruned SNPs, a pairwise kinship matrix was then built.

The EIGENSOFT package was used to evaluate genetic relatedness between individuals and population stratification within batches prior to the GWAS analysis (Price *et al*., 2006). The genome-wide efficient mixed model association (GEMMA) algorithm (Zhou and Stephens, 2014) was performed using the GWAS, and univariate linear mixed model were fitted separately for immune trait/batch/age as follows:

$$y = W\alpha + x\beta + u + \varepsilon,$$

where y indicates the vector of immune trait values for every individual; W is a matrix of covariates (fixed effects that contains the batch, age:56 or 112 days), calendar season [autumn (September to November, batch 4) and winter (December to February, batch 5)] growth traits, sex, the top three PCs and a column of 1s); α is a vector of the corresponding coefficients including the intercept; x represents a vector of genotypes of a marker; β is the effect size of the marker; u represents a vector of random effects with a covariance structure as $u \sim N(0, G\sigma^2 u)$, where G is the genetic relatedness matrix calculated from all SNP markers and σ^2 u is the polygenic additive variance; e is a vector of residual errors with $e \sim N(0, I\sigma^2 e)$, where I is the identity matrix and $\sigma^2 e$ is the residual variance. The significance threshold for the GWAS was defined using the Bonferroni correction method. These two thresholds were set for the data: $p < 1/N$ for suggestive significance and $p < 0.05/N$ for a 5% genome-wide significance level, where N is the number of the SNPs remaining after quality control. To analyze the findings from the GWAS, quantile-quantile plots and Manhattan were drawn, and genomic inflation factor (λ) was calculated with the median option by the qqman package in R for each trait (Turner, 2018).

3.5. Genetic parameter estimation

Genetic parameters were estimated for Ab response to NDV using a similar univariate linear mixed model than the one utilized for GWAS. With Genome-wide complex trait analysis (GCTA) (Yang *et al*., 2011), the estimates of the variance components were calculated using the restricted maximum likelihood analysis option. Using the same model, bivariate analyses were performed to estimate genetic and immune phenotypic correlations among the traits. We conducted these analyses separately for each chicken batch.

3.6. Annotation candidate region

The Galgal6 assembly was used to map all significant SNPs detected in the GWAS to the reference genome. For the annotation we used the Variable effect predictor (VEP) tool in Ensembl (http://www.ensembl.org/Tools/VEP). Additionally, using the Galgal6 assembly and the BioMart data mining tool (http://www.ensembl.org/biomart/martview/), we annotated genes positioned 100 kb downstream and upstream of the significant SNPs associated to Ab response to NDV.

3.7. Functional enrichment analyses of the candidate regions

STRING Genomics 11.0 (Szklarczyk *et al*., 2019) was used to classify the genes according to their biological pathways. It uses protein-protein interaction (PPI) networks and GO terms of the candidate genes. *Gallus gallus* was the background species and a PPI enrichment score > 0.15, the most stringent confidence interval, was used to construct the global network. The STRING specification calls for known PPI in research papers and curated databases. Additionally, it used protein homology, co-expression, gene neighbors, fusions, and co-occurrences to forecasts interacting genes.

CHAPTER 4. RESULTS

4.1. Antibody titers for Newcastle disease virus

Experimental chickens from two batches were used in this study. The Ab response to NDV, as assessed by an ELISA test, of individuals in both batches, four and five, was recorded at 56 and 112 days of age, to evaluate the effect of batch and sex on immune trait. The Ab response to NDV was highly variable across chickens, and a significant effect ($p < 0.05$) was observed at 56 days of age between batches four and five. Compared to batch four, batch five chickens had a weaker Ab response at 56 days and stronger Ab response at 112 days of age to viral vaccination. There was no effect of sex on any of the vaccine Ab responses tested, indicating no difference in the humoral immune response in male and female chickens. The adjusted mean ± standard error of Ab responses to NDV was 0.6 ± 0.02 and 0.16 ± 0.01 for batch four and 0.39 ± 0.02 and 0.97 ± 0.012 for batch five at day 56 and 112, respectively (Figure 1).

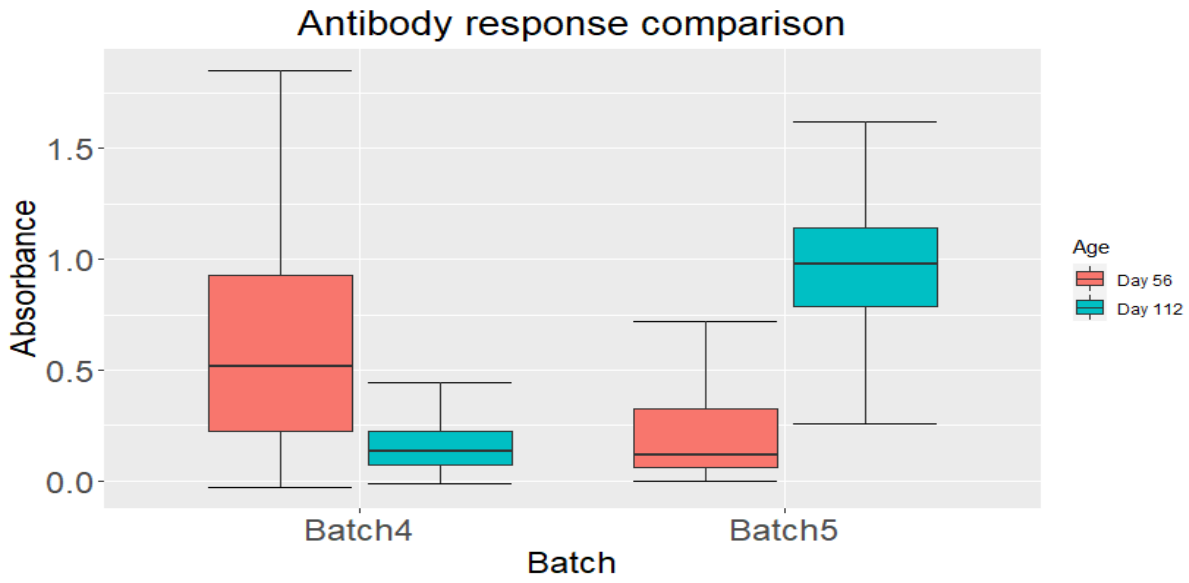

Figure 1. NDV-specific serum titers for each batch of chickens at day 56 and day 112.

4.2. Genetic Parameters

The ratio of additive genetic variance (Vg) to phenotypic variance (Vg + Ve) was used to compute heritability (h^2). The estimate of heritability for Ab response to NDV was performed using the GCTA. NDV Ab titer at day 56 and day112 had estimated heritability of 0.09 ± 0.14 and 0.13 ± 0.15, respectively (Table 3).

Table 3. Variance component estimates and heritability of antibody response to NDV

Age	Source	Vg	Ve	Vp	Vg /Vp	Pval
Day56	**Variance**	0.0106	0.1141	0.1247	0.09	0.27
	SE	0.0176	0.0178	0.0067	0.14	
Day112	**Variance**	0.0027	0.0187	0.0214	0.13	0.2
	SE	0.0032	0.0032	0.0012	0.15	

Vp =Phenotypic variance; **Vg** = Additive genetic variance; **SE** =Standard error; **Ve** =Environmental variance

4.3. Principal component analysis

The PCA analysis did not reveal any clear genetic separation between the two batches. The first PCA explained 10.0% and the second principal component explained 6.7% of the total variance (Figure 2). Consequently, to consider for differences in the population structure, the first two PCAs were handled as covariates and incorporated in the GWAS model as fixed effects.

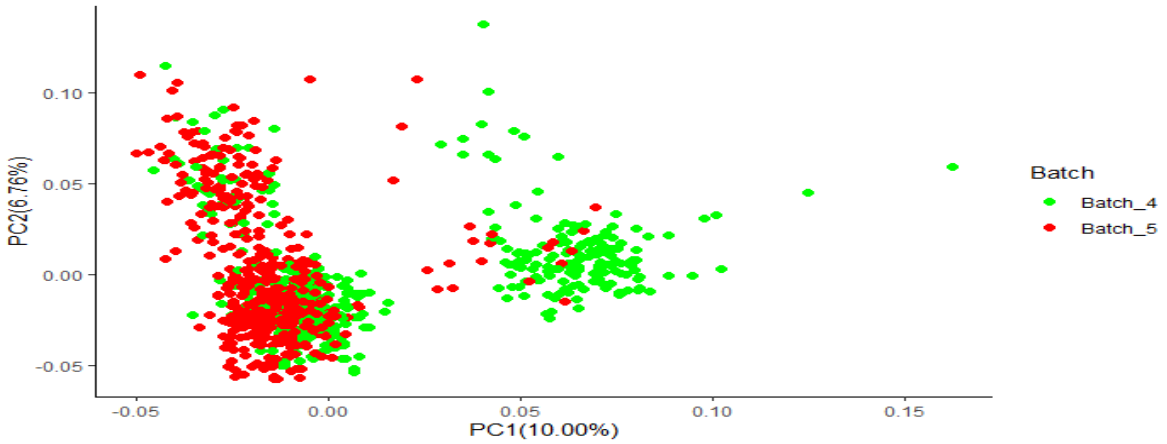

Figure 2. Principal component analysis of the two batches. Dots of different colors (red and green) represent individual bird of each batch (batch_4: green; batch_5: red)

4.4. Genome-wide association studies

The univariate GWAS was performed to detect genomic regions related to Ab response to NDV of Sasso T451A at 56 and 112 days of age. GWAS was performed for Ab response to NDV, and the findings are presented as Manhattan and QQ plots (Figure 3). For each analysis, genomic control inflation factors were greater (with a small margin) than 1 ($\lambda = 1.02$ and 1.00 respectively), which indicated low population stratification.

A total of five differentiated SNPs were found to be associated with antibody response to NDV, of which 2 of them are novel. These SNPs are located in the candidate genes rs733628728 ($p = 3.62 \times 10^{-7}$) at position at 15792460 and an unnamed SNPs ($p = 1.36 \times 10^{-7}$) at 258025 and at 198020 ($p = 3.92 \times 10^{-7}$) onchromosome 13, 33 and 30 at day 112 for batch four, and for SNPs rs316795557 ($p = 1.18 \times 10^{-7}$) at 26416538, and rs313761644 ($p = 2.32 \times 10^{-7}$) at 51943226 on chromosome 1 and 5 at day112 for batch five, respectively. No SNPs obtained the significant *p*-values for antibody response to NDV at day 56 for both batches (Table 3; Figure 3).

Table 4. Differentiated variants and potential candidate genes associated with Ab response to NDV

Traits	SNP ID	Chr	Position	MAF	Beta	p-value	Candidate gene
Batch4		33	258025	0.014	0.2584082	1.36×10^{-7}	
Day112	rs733628728	13	15792460	0.036	0.1622703	3.62×10^{-7}	
		30	198020	0.032	0.1741291	3.93×10^{-7}	
Batch5	rs316795557	1	26416538	0.243	0.1380258	1.18×10^{-7}	FOXP2
Day112	rs313761644	5	51943226	0.013	-0.4900926	2.32×10^{-7}	CEP170B

Chr= chromosome number; **MAF**= Minor allele frequency

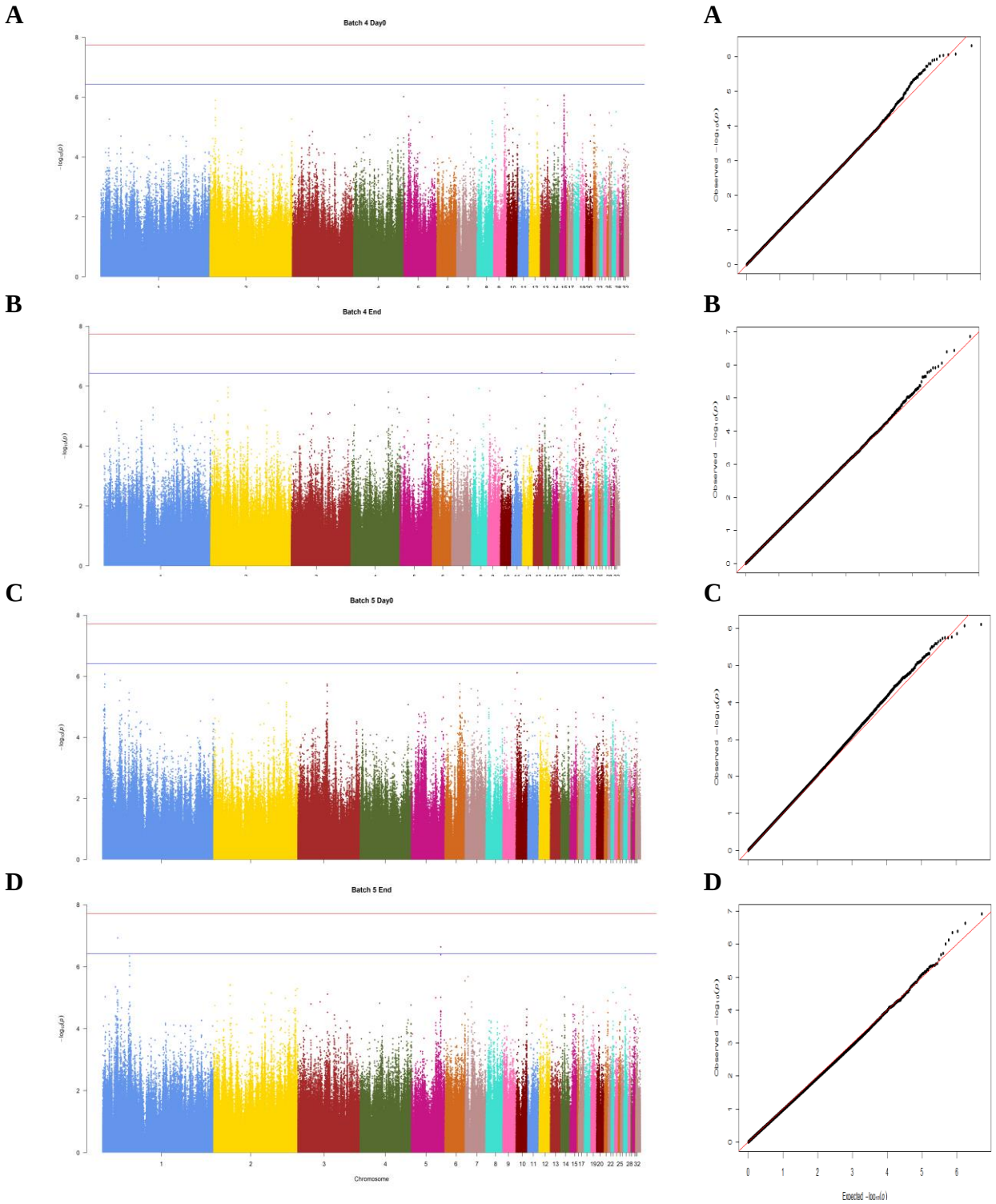

Figure 3. Quantile–quantile (Q–Q) (right) plots and Manhattan (left) of SNP-based GWAS for batch 4 day56 (**A**), batch 4 day112 (**B**), batch 5 day56 (**C**), and batch 5 day112 (**D**). The Q–Q plots revealed the expected −log10(p), while the Manhattan plots revealed −log10 (observed p) for SNPs (y-axis) against their corresponding positions on each chromosome (x-axis). The horizontal dashed and solid lines in Manhattan plots represent the genome-wide suggestive (−log10(p) = 3.74) and significant (−log10(p) =1.86) thresholds, respectively.

4.5. Annotation of SNPs and candidate regions

The annotation and location of all significant SNPs (n=21) detected by using GWAS analyses are represented in Table 4 (both batches and age group at 112 days). These SNPs were located in intron, downstream and upstream of a gene intergenic region. The genomic intervals 100 kb downstream and upstream of the GWAS identified significant SNPs were referred to as candidate regions, and annotated genes within those specific regions were identified. The list of candidate genes for Ab response to ND at 112 days for both batches are summarized in Table 4. The list includes nine genes protein phosphatase 1 regulatory subunit 3A *(PPP1R3A)*, transient receptor potential cation channel subfamily C member 7 *(TRPC7)*, dynamin-2-like *(DNM2)*, cell division cycle 37 *(CDC37)*, tyrosine kinase 2 *(TYK2)*, trafficking protein particle complex 5 *(TRAPPC5)*, B-cell receptor-associated protein 31 *(BCAP31)*, WD repeat domain 13 *(WDR13)*, and phospholipase D family member 4 *(PLD4)* (Table 4).

Table 5. Gene ID and name, chromosome and size of the candidate regions including 100 kb upstream and downstream the significant SNPs

Gene stable ID	Gene name	Chromosome	Region
ENSGALG00000009435	*PPP1R3A*	1	26.5-26.53 Mb
ENSGALG00000006297	TRPC7	13	15.7-15.76 Mb
ENSGALG00000051104		13	15.796-15.799 Mb
ENSGALG00000040741	DNM2	30	90-110 kb
ENSGALG00000041323		30	137.1-137.8 kb
ENSGALG00000048930	CDC37	30	170-179 kb
ENSGALG00000030599	TYK2	30	180-196 kb
ENSGALG00000050608		30	200-214 kb
ENSGALG00000039088	TRAPPC5	30	242-247 kb
ENSGALG00000035236	BCAP31	30	269-271 kb
ENSGALG00000044536	WDR13	30	273.5-276.5 kb
ENSGALG00000050708		33	167.5-168.4 kb
ENSGALG00000047992		33	180-250 kb
ENSGALG00000048833		33	215.2-217.6 kb
ENSGALG00000049509		33	313.8-314.6 kb
ENSGALG00000034369		33	347.1-347.9 kb
ENSGALG00000051385		5	51.888-51.890 Mb
ENSGALG00000041565		5	51.928-51.942 Mb
ENSGALG00000011639		5	51.945-51.975 Mb
ENSGALG00000011646	PLD4	5	51.985-51.996 Mb
ENSGALG00000047641		5	52.04-52.042 Mb

4.6. Gene annotation and functional enrichment analysis

All mapped genes for Ab response to ND showed the presence of enriched gene clusters related to regulation of transcription, endoplasmic reticulum to Golgi vesicle-mediated transport, DNA-templated, cytokines and interferons signaling, a variety of cellular processes, neural mechanisms, inflammatory response, metal-ions binding and transport, and ATP binding. The GO analysis showed no statistically significant results except panther GO-slim molecular function, which are olfactory receptor activity (GO:0004984) and odorant binding (GO:0005549), fold enrichment (FC >45) and FDR (P<0.001).

Using STRING analysis, the network proteins that are encoded by the 11 candidate genes revealed more interactions among themselves compared to those expected for a random set of proteins of the same size drawn from the genome (PPI enrichment p-value $P < 0.305$; expected number of edges = 4; number of nodes = 10; avg. local clustering coefficient = 0.4; average node degree =1; and observed number of edges = 5) (Figure 4). This indicates that the current group of proteins appear to be a random assortment of proteins that are not very well related; these proteins have not received a lot of research attention and STRING may not yet be aware of their relationships (Szklarczyk *et al*., 2019).

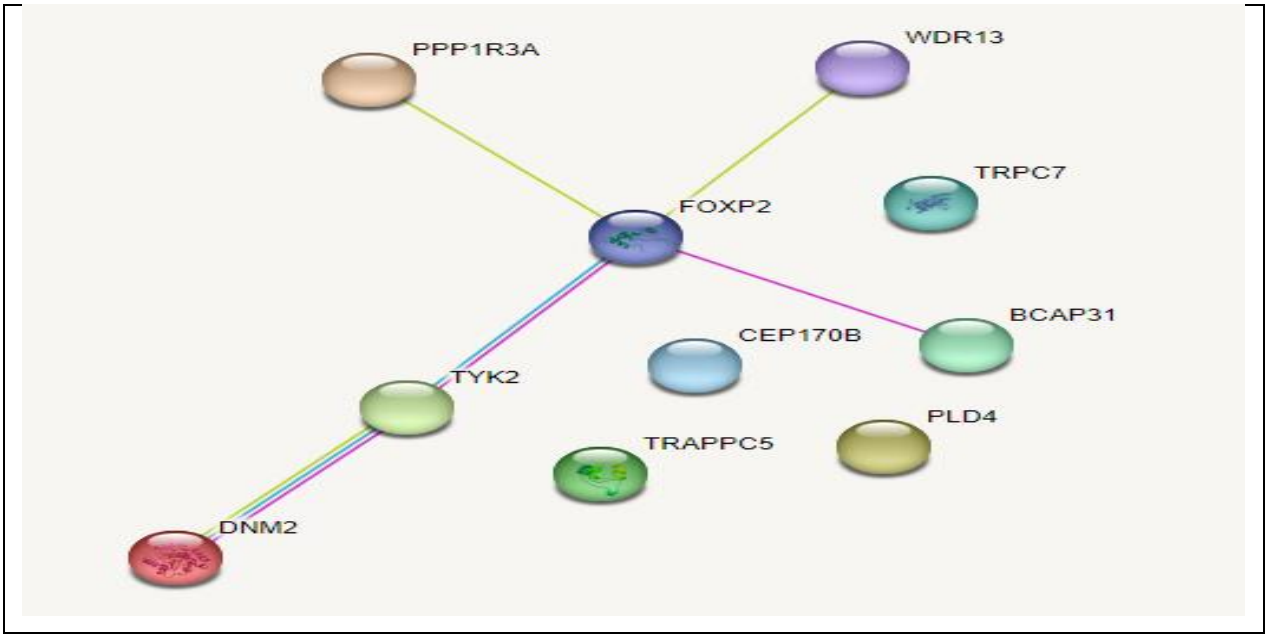

Figure 4. Functionally significantly enriched genes protein-protein interactions following STRING analysis.

CHAPTER 5. DISCUSSION

Genome-wide association study is a research method utilized to identify genomic variants statistically associated to recorded phenotypes including economically important traits (disease) in domestic animals such as disease resistance or tolerance (Raeesi *et al*., 2017). Here, we investigated the genome of Sasso T451A chickens to identify genetic variation linked to the immune response of NDV. According to Fischer *et al*. (2013), the host immune response to viruses is a complex process with a previous study suggesting a polygenic control for the immune response to ND (Saelao *et al*., 2019).

Overall, the heritability estimates were lower (0.09 - 0.13) than previous reports on the same phenotypic trait (NDV-specific Ab responses) (Lwelamira *et al*., 2009; Luo *et al*., 2013). Nevertheless, much of this kind of studies estimated heritability under no environmental disturbances. Heritability can vary over time because the variation as a result of environmental factors, where genes engaged in specific traits may manifest in a different way (Charmantier and Garant., 2005). To our knowledge, heritability estimates for the Ab response to NDV in Sasso T451A chicken are being reported for the first time in this work.

The PCA results (Figure 2) could partly be attributed to the fact that the two batches were of the same breed (Rothschild *et al*., 2018). Furthermore, PCA results are compatible with a three-way crossing origin for the studied population. Compared to 2-way crossbred chickens, three-way crossbred chickens exhibit better egg characteristics and have lower mortality (Khawaja *et al*., 2013). The improvement of traits like annual egg production, feed conversion, age at sexual maturity, and intake is also here better addressed.

It is essential to highlight that the region identified in the current study to be significantly ($P < 3.92E-7$) associated with the antibody response to NDV differs from those identified in previous analyses. In broiler chickens, the QTLs for the Ab response to ND were found on chromosomes 2 and 18 (Yonash *et al*., 2001); whereas Biscarini *et al*. (2010) found a total of 13 QTLs on chromosomes 3, 4, 5, 9, 13, 16, 22, and Z. In addition, Wang *et al*. (2015) identified 6 QTLs on chromosomes 2, 4, and Z. The major histocompatibility complex (MHC) which is linked to

chicken resistance (Zhang *et al*., 2015b) was found on chromosome 16. These different findings could have taken place for a number of reasons, including genetic composition of the experimental populations, the number of SNPs or marker density, the type of the targeted Ab reaction, time post-vaccination and the dose of NDV applied, choice of statistical models and limited power of most QTL mapping studies (Saelao *et al*., 2019). The significant thresholds are different from the traditional limits commonly used to detect candidate regions for functional associations between Ab response to ND and SNPs. In the current investigation used the Bonferroni correction method.

Other prior studies used layer and broiler chickens (Yonash *et al*., 2001; Biscarini *et al*., 2010), but the current study utilised Sasso T451A (dual-purpose) chicken. The majority of earlier investigations used microsatellite markers, while SNP-based GWAS was employed in the current investigation. The focus of this study was on the secondary instead of the primary Ab response to ND virus. In the secondary and primary Ab response, the dominant class of Ab produced is immunoglobulin Y (IgY) and IgM, respectively; thus, chicken's ranking on Ab response to the first immunization may be different from that to the second, resulting in the identification of distinct QTLs (Biscarini *et al*., 2010). This study was found suggestive QTL for Ab response to NDV at day 112 on chromosomes 13, 30, and 33 and 1 and 5 at day 112 for batch four and five, respectively. Nevertheless, besides the previously reported chromosomes 1, 5, and 13 (Luo *et al*., 2013, Wang *et al*., 2015, Saelao *et al*., 2019), there were no previous investigation on the QTLs on chromosomes 30 and 33 that controlling ND antibody response.

MicroRNA, antibodies, and additional molecules produced by disease-resistant genes help the host in fending off pathogen-caused harm (Dar *et al*., 2018). By the advent of many molecular technologies and assays in chickens, many anti-disease genes (e.g., SEMA5A, IFN, MH, MX, NRAMP1, ZYXIN, ANTI-ALV, CD1CB, TGFBR2TVB, CD1B, MHC, CHMP2B, ROBO1and ROBO2) have so far been discovered (Deist *et al*., 2017; Lillie *et al*., 2017).

This study revealed 11 putative genes (PPP1R3A, TRPC7, DNM2, CDC37, TYK2, TRAPPC5, BCAP31, WDR13, FOXP2, CEP170B and PLD4) detected SNPs suggestively associated with Ab response to ND in Sasso T451A at day 112. The gene ontology (GO) annotation shows that each of these genes is responsible for the regulation of binding, transport, cytokine activity, immune

responses (innate and adaptive immunity), transcription, and development. Among these genes, six were found close to the significant SNPs (*PPP1R3A, WDR13, FOXP2, BCAP31, TYK2, DNM2*). These genes should be investigated further to ascertain their associations with Ab response to ND in Sasso T451A chicken in Ethiopia. Until now, none of these potential Ab response to ND genes have been directly linked to the immunological response in chickens (Adhikari and Davie, 2018; Elbeltagy *et al*., 2019).

CHAPTER 6. CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

To our knowledge, this is the first study aimed to identify genes and genomic regions associated with Ab response to NDV in Sasso T451A chickens raised in Ethiopia. The outcomes stipulate valuable understandings on the SNPs and candidate genes that are elaborate in the genetical architecture of Ab response to NDV in two age groups and batches chickens. The SNPs (regions) that are associated with Ab response to NDV can be used as information in MAS. In this study, five genomic regions that are significantly associated with Ab response to NDV were identified (rs733628728, rs316795557 (*FOXP2*), rs313761644 (*CEP170B*) and two are novel genes. The genetic basis of ND will facilitate the discovery of strategies to assist in genetic selection of chickens for NDV resistance and better vaccine response. The enhancement of genetic resistance to ND will furthermore help in protecting the Ethiopia poultry industry and improve global food security. Moreover, the genes and variants uncovered in this study demand more investigation in order to comprehend the underlying molecular pathways for actual use.

6.2. Recommendations

Further studies to unravel what constitutes an Ab response to NDV are needed to help in facilitating future breeding programmes. Producing chickens that are able to perform optimally in difficult and challenging condition will eventually increase the supply of good quality protein for human consumption. This information could contribute to breeding decisions for chicken production in NDV endemic areas.

6.3. Future Studies

To comprehend the underlying molecular mechanisms of action, it is important to do additional research on the variations found in this study. Similarly, further studies are needed to determine the functional role of the QTLs in the antibody response to NDV.

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