The epidemiology and ecology of infectious diseases in Ethiopian village chickens and the role of co-infection in infection risk

Thesis submitted in accordance with the requirements of the University of Liverpool

for the degree of Doctor in Philosophy by

Judy Madelaine Bettridge.

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Declaration

I declare that the work in this thesis is original, and has been carried out by myself or with due acknowledgement. It has not been submitted in another form for any other degree or professional qualification.

Judy Bettridge

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List of Abbreviations

CKCL	chicken kidney cell lysate
CSA	Central Statistics Agency of Ethiopia
CV	coefficient of variation
DA	development agent
EIAR	Ethiopian Institute for Agricultural Research
ELISA	enzyme-linked immunosorbent assay
ETB	Ethiopian Birr
FAO	Food and Agriculture Organization of the United Nations
FGD	focus group discussion
FTA	fast technology for analysis of nucleic acids
GAM	generalised additive model
GPS	global positioning system
HA	haemagglutinin
HAI	haemagglutination inhibition
HAU	haemagglutinating unit
HPAI	highly pathogenic avian influenza
IAEA	International Atomic Energy Agency
IBD	infectious bursal disease
IBDV	infectious bursal disease virus
ICC	intraclass correlation coefficient
LPAI	low pathogenicity avian influenza
MDV	Marek's disease virus
MPI	multidimensional poverty index
NA	neuraminidase
NaOH	sodium hydroxide
ND	Newcastle disease
NDV	Newcastle disease virus
NGO	non-Governmental Organisation
OD	optical density
OIE	Office International des Epizooties (World Organisation for Animal Health)
PBS	phosphate buffered saline
PBST	phosphate buffered saline + Tween-20
PCA	principal component analysis
PRA	participatory rural appraisal
RBCs	red blood cells
RDA	redundancy analysis
RRA	rapid rural appraisal
SNP	Single nucleotide polymorphism
s:p ratio	ratio of sample to positive control
UN	United Nations
vvIBD	very virulent Infectious bursal disease

Abstract

Bettridge, J.M. The epidemiology and ecology of infectious diseases in Ethiopian village chickens, and the role of co-infection in infection risk.

The scavenging village chicken is important to millions of smallholders in Ethiopia, as in other less-economically developed countries, for its contribution to the economic, nutritional and social well-being of farmers, especially women and children. Infectious diseases are frequently cited as the greatest constraint to village chicken production, and in Ethiopia, most mortality is attributed to seasonal outbreaks of Newcastle disease (ND). This study conducted four cross-sectional surveys over an 18-month period in two geographically distinct regions of Ethiopia, to examine a range of bacterial, viral and parasitic infections in randomly-selected village chickens, and to look at their 6-month survival rate. The two chicken populations of the different regions were found to be different in terms of their population dynamics and phenotypic characteristics, and these may be driven by farmer demands, which are dictated by the local economic and cultural value placed on specific qualities in the chickens. Over the course of the study, no large outbreaks were observed in the eight villages which took part in the study, and only 9 out of 1280 birds (0.7%) were found to be serologically positive for ND. However, even in the absence of large outbreaks, around 20% of the birds in the study were reported to have died of disease within the 6-month follow-up period, and a further 13% lost to predation. Both location and seasonal variation influenced a bird's fate, as did farmer decisions, such as choosing birds with specific characteristics to sell or eat. Rather than large outbreaks, the rainy season appeared to be associated with increased small-scale losses, and a variety of signs were described, suggesting several pathogens may be involved. No single infection measured at the time of sampling was a good predictor of subsequent death from disease; instead different pathogens appeared to be important in each region, and reduced the probability of survival through a variety of mechanisms. Positive correlations between *Pasteurella* and *Salmonella,* and between Marek's disease and parasitic diseases were identified, but fewer birds than expected were identified with pathogens from both these groups, perhaps suggesting a decreased chance of survival for co-infected birds. Strong seasonal variation in prevalence was not observed for any of the infections in the study, suggesting that seasonal rises in disease mortality are unlikely to be attributable to a single infection, but other factors may play a role, including an increased probability of co-infection. This makes it difficult to prioritise control strategies for individual diseases; instead development programmes may find broad-based strategies, such as improving hygiene and chick management may be more beneficial to minimise the small-scale losses. Programmes also need to be tailored to local needs rather than assuming a blanket strategy will work equally well for all farmers or regions. Any development strategies to control single diseases should consider potential impacts on non-target infections, due to the existence of multiple interactions between pathogens in this system.

Chapter 1

General introduction and thesis objectives

There is widespread recognition of the importance of poultry keeping to poor rural households in developing countries throughout the world (Copland and Alders, 2005; Mack et al., 2005). A substantial amount of research over the past 25 years has focused on ways to improve village chicken production in order to benefit the poorest and most marginalised members of society. The Food and Agriculture Organization of the United Nations (FAO) together with the International Atomic Energy Agency (IAEA) conducted a Coordinated Research Project in 13 African countries over a five-year period beginning in 1998, and concluded that vaccination against Newcastle disease virus (NDV) was the most critical intervention in all cases, and that other interventions such as improving feed, housing or controlling parasites were only effective if used in conjunction with vaccination. All 13 country projects demonstrated an economic return on the investment in NDV vaccines (Dwinger and Unger, 2006). Ethiopia was not one of the countries involved in this programme.

Although there is recognition within Ethiopia of the potential benefits to the development of village poultry, both by researchers and at government levels, the emphasis there has been different to that put forward by the FAO/IAEA study. In Ethiopia, research has focused on attempting to improve productivity by incorporating high-performing commercial lines of chickens into village production systems. Disease control appears to have received much less attention until relatively recently, and as such, Ethiopia may be lagging behind other countries in the region with respect to knowledge of poultry diseases in the village chicken population. There is no reason to suspect that Ethiopia is substantially different from other surrounding countries with regard to the methods of chicken production or constraints experienced by village chicken producers, or that the methods employed by other countries would not substantially benefit Ethiopian producers. In addition, it is highly likely that the existing genetic improvement programmes would benefit from incorporating some knowledge of infectious disease epidemiology in the village situation. This review aims to

summarize some of the existing literature on infectious diseases in village chickens, with particular reference to the current situation in Ethiopia.

1.1 The role of village poultry in human development in Ethiopia

The FAO splits the poultry industry into four broadly defined production sectors (FAO, 2004). Sectors 1 and 2 deal with intensively reared, industrialised poultry produced for commercial purposes. Sector 3 describes a small to medium commercial enterprise with low or minimal biosecurity and birds usually entering live bird markets. The key features of Sector 4 production are an extensive management system with minimal inputs or biosecurity and birds often consumed locally. This sector is variously referred to as smallholder, family, rural, indigenous, traditional, scavenging, village or backyard poultry. Although the terms are frequently used interchangeably, Ahlers et al. (2009) differentiates village poultry, which are usually unconfined and scavenge around the home and village, with or without small amounts of supplementary feed from kitchen waste or surplus grains; from backyard poultry, which are more typically found in urban or peri-urban areas and raised in an enclosure. These authors use family poultry as an umbrella term to encompass both these types of production system.

These small poultry flocks can also be categorised according to their function. McLeod et al. (2009) described the “safety net” flock; kept for their contribution to poor families’ livelihoods and social dynamics. Birds are typically indigenous breeds which require few inputs and have low outputs of both eggs and meat. They usually contribute little in terms of economic income, but may be sold or given away as required, typically for small expenses, such as school fees or medicines. The “asset-builder” flock is seen as a potential route out of poverty, where the flock may represent a significant proportion of the owner’s assets and income. In order to be a success, good market connections are required, and it

can be quite a high-risk enterprise, as owners invest money, time and food resources into the flock with no guarantee of good returns.

There are numerous advantages to developing village poultry production systems. Poultry are easily accessible, even to the poorest households or ones with a lack of able-bodied workers, as they require minimal land, labour or financial inputs. They can scavenge for food, and do not compete for food resources with humans, and they are normally in close proximity to the household, under minimal supervision, enabling them to be managed by women and children while placing few additional burdens on these groups. Their role in managing this household asset gives women greater control over the income from sale of poultry and eggs, and this can be used to support child education. As such, the improvement programs for family poultry have the potential to contribute to several of the UN's Millennium Development Goals (Alders and Pym, 2009).

The Federal Democratic Republic of Ethiopia, like many other developing countries, has recognised both the need for and the benefits of poultry development schemes. Since 2010, the United Nations (UN) has assessed poverty levels in member countries using the Multidimensional Poverty Index (MPI), which identifies multiple deprivations in the same households in education, health and standard of living. The most recent survey data available for estimating MPI figures for Ethiopia were collected in 2011. In Ethiopia 87.3% of the population lived in multidimensional poverty and 71.1% in severe poverty, ranking the country 173rd out of 186 in the UN Human Development Index (UNDP, 2013). Ethiopia is multiethnic, multilingual and multicultural, with 9 regional states and around 83 individual languages (http://www.mfa.gov.et/). Although it supports a wide range of livelihood activities, through traditional pastoralism to urban lifestyles, agriculture is the predominant activity, accounting for around 47% of GDP and around 85% of employment (CIA, 2013). Oromiya is the largest regional state in Ethiopia, both in terms of land area and

population. At the time of the last Ethiopian census, approximately 88% of the population of the Oromiya region of Ethiopia were living in rural households (CSA, 2007). The latest published estimates by the Central Statistics Agency of Ethiopia put the total poultry population at approximately 49.3 million (CSA, 2010/11), of which around 18.8 million (38%) are in Oromiya. Although it is described as "poultry", the data presented only refers to chickens, as the numbers of other farmed poultry species in Ethiopia are negligible. The report estimates that over 97% of Ethiopian chickens are indigenous breeds.

With both a large rural (human) and a large poultry population, several Ethiopian researchers have identified the potential for development of village chickens as an economic and nutritional resource (Yami, 1995; Nasser et al., 2000; Dessie and Ogle, 2001; Duguma, 2009; Moges et al., 2010b). There are several published descriptions of the village production systems throughout Ethiopia and identification of numerous constraints faced by village poultry keepers, including disease, predation, lack of feed, water and shelter; and poor access to veterinary services and to markets (Dessie and Ogle, 2001; Halima et al., 2007; Dinka et al., 2010). Poor productivity is also described; hens show high levels of broodiness which limits egg production, and high losses among chicks limit the number of birds which can be sold for meat. In addition, high losses among the adult birds mean that a considerable proportion of eggs must be hatched and reared in order to obtain replacement stock, further limiting the availability of both eggs and birds for sale or consumption (Dessie and Ogle, 2001)

1.2 Poultry development programmes

Kitalyi (1998) recommended a step-wise program for the development of backyard poultry into a semi-commercial enterprise. Starting with the basic indigenous stock, initial developments should include good hygiene, shelter, preferential treatment of chicks and

control of the most devastating diseases, such as Newcastle disease (ND). Later improvements in the management include developing feeding and other disease control programmes, followed by improving the stock by introducing high-yielding traits and developing marketing strategies.

Exotic lines of chickens were distributed to small-scale farmers in Ethiopia by research institutes, governmental and non-governmental organisations (NGOs) from the 1950s, as live birds, fertile eggs and later as a cockerel-exchange programme, where farmers exchanged indigenous cocks for those from exotic, commercial lines (Teklewold et al., 2006). In 1996, the Ethiopian Ministry of Agriculture developed a poultry extension package for rural farmers which involved training a member of the household in various aspects of poultry management, and providing a nucleus flock of Rhode Island Red chickens. These birds were vaccinated against NDV prior to distribution by the poultry multiplication centres throughout the country (Dessie and Jobre, 2004), although it is unclear whether vaccination schedules were able to be maintained after distribution. These programmes were not a great success, as the exotic birds showed a poor tolerance to the harsh conditions, and *f*armers have complained that this distribution of exotic cocks, pullets and fertile eggs has negatively impacted on the local poultry's brooding ability and adaptation to low-input feeding systems (Dinka et al., 2010). Bell (2009) argues that it is not genetic make-up which is limiting the productivity of backyard chickens, and one of their key advantages is that they are able to find their own feed, thus making them far more profitable per unit of money invested than a commercial bird, for which feed has to be purchased. In Ethiopia, the adoption of exotic birds was found to be greater amongst farmers who offered supplementary feed to their poultry (Teklewold et al., 2006), and farmers who had adopted Rhode Island Red birds rated their scavenging behaviour as inferior to that of indigenous birds (Dana et al., 2010). The same author reported that "adaptation", consisting of disease and stress tolerance, flightiness, ability to escape

predators and scavenging vigour, was rated as the most important attribute of village chickens by farmers, although they would like to see improvements in egg production and growth, as bodyweights play a substantial role in dictating the market price. Farmers also rated the taste of eggs and meat from indigenous birds more highly than their exotic counterparts

New strategies in Ethiopia are now being developed which focus on utilising the indigenous birds, and several recommendations have already been made as to what needs to be done in terms of improving chicken production (Moges et al., 2010b). These broadly agree with those made by Kitalyi in 1998, but prioritise genetic improvement of the indigenous breeds, and shifting to market-oriented production systems. Rather than placing the emphasis on disease control, basic hygiene and nutrition, which in Kitalyi's work were the foundation on which further improvements could be built, Moges's report mentions these aspects only briefly, and provides no specific recommendations as to how they may be implemented. It would appear that for several years after the death of Dr. Mohammed Nasser, who contributed much to Newcastle Disease research in Ethiopia (Dessie and Jobre, 2004), there was only scant research devoted to poultry disease, and the development agenda concentrated heavily on ways that the existing chicken distribution programmes may be improved. In order to utilise the genetic merits of the indigenous breeds, a programme has been in development at the Ethiopian Institute for Agricultural Research (EIAR) for several years. The premise is to use selective and cross-breeding to develop a dual-purpose chicken which meets the requirements of farmers for adaptation to a scavenging system, whilst having higher outputs of eggs and meat than the existing ecotypes (Dana et al., 2011).

Although this approach may appear promising, life-history theory (based on the idea that organisms have finite resources which are competitively allocated to growth, maintenance and reproduction) suggests that adaptive changes must come with costs, often described

as “trade-offs”, and that the immune response can be evaluated as a life-history trait, and be affected by such trade-offs (Zuk and Stoehr, 2002). Therefore artificial selection for growth and high egg production might be predicted to compromise other traits, including immunity to infectious diseases. Indeed, there is evidence from selected chicken lines that selection for growth frequently compromises specific humoral or cellular responses to a novel antigen (van der Most et al., 2011), whilst numerous studies in other avian species have observed increasing parasite burdens in birds which mature early, lay more eggs and rear more clutches, leading to speculation that there are “costs” associated with resisting infections which are borne by individuals which invest more into reproduction (Møller, 1997; Zuk and Stoehr, 2002). Individuals achieve greater reproductive success when not constrained by limited nutritional resources, but there is also some evidence that immune responses are also decreased when resources are limited (Lochmiller et al., 1993).

Maintaining a functional immune system is vital for animals in an environment where they can be exposed to a range of diseases. In commercial settings, exposure to infections can be better controlled and feed provision matched to animals’ energy requirements, making fast-growing animals more profitable. The design of the current selective breeding programme in Ethiopia means that the advantages seen in the environment of an intensive farm, where vaccinations and biosecurity measures have been put in place to control disease, and all feed is provided, may not be transferred to the village situation, where limited feed resources are a frequent constraint. This may exert additional pressure on immune responses in individuals selected for their ability to divert more resources into growth and reproduction. In addition, the risk of losing some important disease resistance genes from the “improved” population, which has, of necessity, been developed from a small foundation flock, may make them more vulnerable than the source population from which they were derived. Therefore some knowledge of the diseases present in the field situation may assist the success of any poultry development programme put in place.

1.3 Disease as a constraint to village poultry production

Disease is consistently highlighted by village poultry keepers as one of the biggest constraints to production (Guèye, 1998; Mack et al., 2005), but the key to an effective intervention programme requires specific knowledge of which infections are present in the population. Numerous serological surveys have been reported in the literature for a wide range of infectious diseases in village chickens in Ethiopia and throughout the world. Serological surveys have the advantage of being relatively cheap and easy to perform, and clearly demonstrate that village chickens have been exposed to many potential pathogens. The main problem with serological surveys is that they can demonstrate exposure to the disease without providing much information about its impact on the population. A high seroprevalence indicates that a large number of animals have been exposed, but does not necessarily correlate with a high impact on that population – for example, infectious bursal disease in a flock of adult chickens may pass unnoticed, as birds will not show clinical signs (Eterradossi and Saif, 2008). Conversely a low seroprevalence may be found both in cases where the exposure to a pathogen is low, but also where a highly virulent pathogen, such as velogenic NDV, occurs in a classic epizootic outbreak, with case fatality rates of close to 100% (Martin, 1991).

Estimating the relative importance of various diseases in a population is therefore complex. In Ethiopia, the lack of knowledge of poultry disease by local veterinary extension services combined with the lack of an effective reporting network, which makes it very difficult for those who do have specialist knowledge in this area to detect outbreaks, means that most poultry disease goes unreported and uninvestigated (Halima et al., 2007). Alamargot (1985) performed a series of post-mortem examinations in order to determine which diseases were causing the most losses in Ethiopian poultry. However 193 out of the 198 necropsies were birds from commercial farms, and as such his findings cannot necessarily be

extrapolated to village poultry. Reports of outbreak investigations in the Ethiopian literature on village poultry have been confined to areas where there had been distribution of exotic birds (Mazengia et al., 2009), and as such may not represent what is occurring in indigenous poultry; either because of disease introduction with the exotic birds, which are reared intensively before distribution, or because exotic birds are likely to be more susceptible to endemic diseases, due to their poor adaptation to the village situation.

Diseases in village chickens which have to date been reported in Ethiopia include Newcastle disease (Tadesse et al., 2005; Mazengia et al., 2010; Chaka et al., 2012); infectious bursal disease (Degefu et al., 2010; Mazengia et al., 2010; Chaka et al., 2012; Jenbreie et al., 2012; Kassa and Molla, 2012; Zeryehun and Fekadu, 2012); salmonellosis (Berhe et al., 2012; Berihun et al., 2012; Alebachew and Mekonnen, 2013);, pasteurellosis (Chaka et al., 2012), and mycoplasma (Chaka et al., 2012). Marek's disease has also been identified in village chickens kept under intensive management (Duguma et al., 2005). Parasitic diseases, including coccidiosis (Ashenafi et al., 2004b; Luu et al., 2013), helminths (Tolossa et al., 2009; Molla et al., 2012) and ectoparasites (Belihu et al., 2009; Tolossa et al., 2009) have also been demonstrated to be highly prevalent in the country. An overview of each is given here, together with a brief review of the literature which may be relevant to the diseases' epidemiology in the village situation in Ethiopia.

1.4 Newcastle Disease Virus

1.4.1 Biology and Pathology

(Summarised from Alexander and Senne (2008)

Newcastle disease virus (NDV) is a Type I avian paramyxovirus. It is a single stranded, negative sense RNA virus with haemagglutination and neuraminidase activity. There is wide variation between different strain types, but they can be grouped according to five main

categories: **Viscerotropic velogenic** and **neurotropic velogenic** NDV both cause acute, lethal infections in all ages of chickens, with mortality up to 100% in housed flocks. The two strains respectively cause haemorrhagic lesions of digestive tract and a syndrome comprising respiratory and neurological signs. **Mesogenic** strains cause respiratory infections and mortality in young birds and a drop in egg production in laying hens, whilst **lentogenic** viruses cause mild or inapparent respiratory infections. The fifth virus type is an **asymptomatic enteric** virus, which cause gut infection without obvious disease. Virus pathotype is not usually clear-cut, and signs of milder strains may be exacerbated by concurrent disease or adverse conditions. Immunity to one strain is cross-protective, and milder strains are often used in vaccine production.

NDV may infect avian species other than chickens; ducks, geese, and many wild species can be infected and transmit virus, but show no clinical signs; turkeys and game birds can be clinically infected. It is also occasionally reported to cause zoonotic infections, causing conjunctivitis, sometimes accompanied by fever and flu-like symptoms (Swayne and King, 2003). In chickens, disease may present with a variety of signs, including sudden death, lethargy, fluffed feathers, respiratory signs, green diarrhoea, torticollis, paralysis, oedema of head and neck, or a decrease in egg production (Alexander and Senne, 2008).

1.4.2 Epidemiology

NDV is shed both from the respiratory tract and in faeces, and infection can be through inhalation or ingestion, with an incubation period of between 2 and 15 days. Although the virus is heat-labile, it may persist for up to 6 months in faeces. However, under village conditions, it is unlikely to persist for more than 1 month in the environment (Alders and Spradbrow, 2001). Spread can also occur through contact with contaminated people, animals and equipment, feed, water, vaccines and poultry products, including viscera from dead birds fed to chickens, or to cats and dogs, which can shed virus for a short period in

faeces. Although respiratory spread is important in housed flocks, under village conditions, where there is less direct contact, faeco-oral spread is probably more important, and spread throughout a village may take weeks to months (Martin, 1991). Epizootic outbreaks usually occur when an infected bird is introduced into a fully susceptible flock, with up to 100% mortality, whilst endemic disease can occur under village conditions, where birds of varying age and infection status can freely mix. Where breeding birds have survived an outbreak, lower-level mortalities will then occur in susceptible (primarily young) birds, until a large population of susceptible birds has built up, when epizootic outbreaks may occur. These outbreaks are often seasonal, associated with climatic stress, changes in food availability, movement of birds through markets, or periods of increased hatching and rearing which increase the susceptible population (Awan et al., 1994).

The circulation of several different strains of virus may complicate the picture under village conditions. Lentogenic or avirulent strains can be maintained in circulation for prolonged periods, and may give partial immunity to velogenic strains. Infected birds may then carry and shed virus, but without the associated clinical signs (Martin, 1991; Awan et al., 1994). Recent studies in Ethiopia have confirmed the presence of both virulent and lentogenic strains circulating within rural household flocks (Chaka et al., 2013a; Fentie et al., 2013). Concurrent parasitic disease appears important in determining susceptibility (Martin, 1991) but although ectoparasites can be infected, they do not transmit disease (Alexander and Senne, 2008). Experimental studies have shown that helminth infections can reduce the antibody response to vaccination/ challenge with NDV, although the cellular immune response still protects birds from clinical disease (Horning et al., 2003). Other risk factors, such as exposure to wild birds, and restocking practices, have been examined in Uganda, but no significant associations were found (Otim et al., 2007).

Worldwide, the significance of NDV as a major constraint to village chicken production is well recognised, and the benefits of vaccination, as a cost-effective intervention, have been demonstrated in 13 different African countries (Dwinger and Unger, 2006). In Tanzania and Mozambique, effective NDV control programmes have resulted in increased chicken numbers and subsequent increases in households', and particularly women's, purchasing and decision-making powers (Alders and Pym, 2009).

In Ethiopia, comparatively little attention has been paid to controlling Newcastle disease in village poultry to date. A review of the disease in Ethiopia published in 2004 (Dessie) reported only two serological studies, from a DVM and an MSc thesis, and complained of a lack of monitoring capacity in the state Veterinary Services department. In the 10 years since this review was published, more work has been done on NDV in Ethiopia, but the literature is confusing. Several studies of the effects of disease on village poultry production have been in the form of participatory rural appraisal (PRA) and/or questionnaire-based studies. Although Ethiopian farmers can recognise and describe a variety of diseases (Sambo, 2012), they tend to designate any acute disease with high mortality as "*fengele"* (Chaka et al., 2012), which was described by Dessie and Ogle (2001) as a syndrome comprising of inappetance, watery, yellowish droppings, paralysis and eventually death. The term "*fengele*" has, in subsequent studies, become synonymous with Newcastle disease, even though this syndromic description could potentially include a number of other disease conditions.

Studies based on interviews or PRA work report outbreaks of "*fengele*" to be seasonal and to occur annually during the rainy season (Dessie and Ogle 2001, Halima 2007, Dinka 2010), although since villagisation in the 1980s, losses occur throughout the year (Dessie and Ogle, 2001). This is not inconsistent with ND occurring in both epidemic and endemic patterns in different areas of Ethiopia; which would be borne out by the wide range of reported levels

of seropositivity (Zeleke, 2005b; Tadesse et al., 2005; Regasa et al., 2007; Mazengia et al., 2010; Chaka et al., 2012), which range from 6 – 64%. However, it is difficult to make any comparisons between these studies, due to the differences in sampling techniques and test procedures used. Although there is little doubt that NDV is important in Ethiopia, the literature which discusses disease in more general terms as a constraint may be confusing the issue, because there appears to have been some spurious assumptions made which have since been cited several times without sufficient evidence. This has taken the form of:

- *Mortality is higher in the wet season*
- *NDV is a disease which causes widespread mortality*
- *Therefore NDV outbreaks are occurring in the wet season*

Although many other diseases have, in very recent years, begun to receive more attention from those in the veterinary field, those without specialist knowledge of poultry disease often appear to attribute all disease problems to NDV, without appreciation that other diseases may be involved in the seasonal *"fengele"* occurrence, and thus may over-emphasise its importance. A potential concern is that, should any vaccination programmes for NDV be implemented in future, these may not meet local people's expectations for controlling *"fengele"*, unless this disparity is carefully explained. Komba (2012) reported continued disease outbreaks in chicks and growers in Tanzania, despite achieving good vaccine coverage with a thermostable NDV vaccine, suggesting other diseases also needed to be controlled for.

Vaccination of village chickens in Ethiopia is still in its infancy. Although the exotic birds distributed in the government programmes were vaccinated prior to distribution, there is almost no literature describing vaccination of indigenous birds. A recent paper by Nega et al. (2012) assessing I2 thermostable NDV vaccine in village chickens in Ethiopia reported a

reduction in mortality of 82%; however this appears to have been assessed by asking owners to estimate mortality rates before and after vaccination. Nonetheless, it would appear that NDV vaccination would be a promising intervention for Ethiopian farmers to adopt, as it has been in many other countries in the region.

1.5 Avian Influenza

1.5.1 Biology and Pathology

(Summarised from Swayne and Halvorson (2008))

Avian influenza is a type A influenza virus; a negative sense single-stranded RNA virus with segmented genome of 8 segments of the orthomyxovirus family. Strain typing is based on serological type of haemagglutinin (HA) and neuraminidase (NA) proteins. 16 HA and 9 NA types are recognised. In addition, viruses are classified according to pathotype – i.e. their ability to cause pathogenic effects in the chicken host. They are either highly pathogenic (HPAI) or low pathogenicity (LPAI). This classification is made either on the intravenous pathogenicity index in 6-week-old chicks, or by using molecular methods. A variety of pathogenic effects may be seen in the field, regardless of the laboratory definition of pathotype, as host species, age and immune status play important roles in determining the outcome of infection. HPAI infections usually have close to 100% morbidity and mortality, with clinical signs of pathologies in multiple organs – including cardiovascular and neurological signs. Sudden death, and severe depression and anorexia with loss of production are usually seen. LPAI infections may show no signs, or common clinical presentations will include respiratory signs, loss of production, occasionally diarrhoea and generalised signs of lethargy and decreased appetite. Mortality is usually less than 5%.

1.5.2 Epidemiology

HPAI viruses can infect man, and have done so sporadically, usually after direct contact with birds in markets or villages infected with the H5N1 strain. Circulation of the infecting virus among humans is usually very limited, but can cause severe flu-like illness and mortalities in affected individuals. Because of their devastating impact on the poultry industry and their zoonotic potential, all HPAI viruses and any LPAI viruses of the H5 or H7 subtype are notifiable. This is due to the ability of LPAI H5 and H7 viruses to mutate to HPAI during circulation in poultry (Swayne and Halvorson, 2008). Because of the clinical similarities of HPAI to NDV, its presence in developing countries is thought to be under-reported, as people may regard mass mortality of chickens as normal, and see no value in reporting it (Cardona et al., 2010). In addition, because HPAI is notifiable and a stamping-out policy is usually applied, losses in a village situation occur from culling, as well as disease, but are not usually compensated. In Indonesia and Cambodia, where outbreaks have occurred, the impact on rural families has been severe (Rushton et al., 2005). HPAI has never been reported in Ethiopia, where general and targeted surveillance measures are in place. Notifiable LPAI was last reported in Ethiopia in 2006. (O.I.E., 2013)

1.6 Infectious Bursal Disease Virus (IBD, Gumboro)

1.6.1 Biology and Pathology

(Summarised from Eterradossi and Saif (2008)).

Infectious bursal disease virus is an avibirnavirus, a non-enveloped RNA virus, which can infect chickens, turkeys, ducks, guinea fowl and ostriches. Serotype 1 causes clinical disease in chickens under 10 weeks, while Serotype 2 is not associated with clinical disease. Chickens over the age of 10 weeks are not clinically affected; nor are any other avian species. The virus targets developing B-lymphocytes in the bursa of Fabricius, hence its age-

specific presentation. It causes severe, acute disease in birds of 3-6 weeks, with watery diarrhoea, ruffled feathers, anorexia, and prostration. Morbidity is very high, and mortality is usually from 5-40%; although a very virulent subtype (vvIBD), first isolated in the Netherlands in the late 1980s, can cause up to 100% mortality. There are distinctive post-mortem lesions with peri-bursal oedema, swelling and haemorrhages; echymotic haemorrhages in muscle; and swelling and discolouration of the kidneys. Birds under 3 weeks can have a mild or subclinical form of disease, which may cause depletion of lymphoid tissue in the bursa in birds less than 2 weeks of age. This results in a markedly decreased humoral response and increases susceptibility to several other infections or may cause vaccine failure, although there is still a normal antibody response to IBD virus. Birds over 10 weeks usually do not develop clinical signs.

1.6.2 Epidemiology

IBD virus particles are highly resistant to heat and disinfection and can persist in the environment for long periods (at least 4 months). Spread is possible through water, feed and fomites. Its epidemiology is principally known through infections in large commercial flocks, and may be somewhat different in a village situation. The incubation period is only 2-3 days, and in a housed fully susceptible flock, morbidity can be 100%, receding after 5-7 days. There is no carrier status: repeat outbreaks are caused by survival of the pathogen in the environment. Recurring outbreaks may be silent, as birds are often infected at under 3 weeks old (Eterradossi and Saif, 2008). Studies in village poultry have detected high proportions of positive samples, even where clinical disease is rarely observed (Abdu et al., 1986). Studies in Mauritania and Tanzania reported proportions of positive samples up to 46 and 42% respectively (Bell et al., 1990; Permin and Bisgaard, 1999).

IBD was reported in Ethiopia for the first time in 2002 – before that, the country was thought to be free of the disease. The report focused on an outbreak in commercial

broilers in Debre Zeit (Zeleke et al., 2005a). A report for the same area in 2004-2005 identified lesions suggestive of IBD on one farm (Chanie et al., 2009). The first study on the incidence of IBD in Ethiopian village poultry was in two areas in the Amhara region which had received “improved” chicks from a commercial farm (Mazengia et al., 2009), and it has been suggested that this was the cause of the introduction of the disease to village poultry. Serological studies have since demonstrated infection in indigenous birds in several areas of the country (Chaka et al., 2012; Jenbreie et al., 2012; Kassa and Molla, 2012; Zeryehun and Fekadu, 2012), with the proportion of positive samples ranging from 75 to 96%. However, at least two of these studies have purposely selected areas where there is regular distribution of chicks from the government multiplication centres to village producers. Two papers on the molecular characterisation of virus isolates have identified very virulent IBD strains from commercial and indigenous birds (Negash et al., 2012; Jenberie et al., 2013).

Control of IBD through vaccination, even in commercial farms, remains difficult. Determining the timing of vaccination for chicks is highly dependent on the level of maternal antibody. A wide variety of vaccine strains are commercially produced, mostly derived from classical virus strains, which do not all successfully protect against the vvIBD strains. “Hot” vaccines, which break through maternal antibody, are now in frequent use in countries which have vvIBD circulating, although their use risks causing bursal lesions, and as such may affect the response to other vaccinations or cause immunosuppression (Müller et al., 2012). Very little appears to have been published on the use of IBD vaccines in the village situation. One report on the use of IBD vaccines in day-old chicks distributed to farmers in Egypt concluded it reduced mortality (Azzam et al., 2004). There is also a report of a joint project between the Australian Centre for International Agricultural Research and Indonesia, to develop a vaccine for use in village poultry, although it is unclear if this has since made it into commercial production (Ignjatovic and Parede, 2009). Failure of commercial vaccines to protect industrialised flocks in Ethiopia has prompted efforts to

attenuate the local vvIBD strain to produce vaccines suitable for use within the country (Jenbreie, personal communication), although these will, again, be primarily intended for use on commercial units.

1.7 Marek's Disease Virus

1.7.1 Biology and Pathology

(Summarised from Schat and Nair (2008) and Payne and Venugopal (2000))

Marek's disease virus is an oncogenic herpesvirus of the genus Mardivirus. Serotype 1 viruses are now classified as the species Gallid herpesvirus 2. This species is further divided into pathotypes, known as mild, virulent, very virulent and very virulent plus. Serotypes 2 and 3 are non-oncogenic. Hosts other than chickens include quail, turkeys and pheasants.

The pathogenesis is complex and split into stages. Infection through the respiratory route is followed 3-6 days post-infection by an early cytolytic phase in B cells and activated T cells, causing marked immunosuppression. The virus spreads throughout the body, including to the feather follicles, from which it is shed into the environment. The second stage involves latent infection in CD4+ cells of varying duration; and the third and fourth stages involve late cytolytic infection with immunosuppression, infiltration of nerves and neoplastic transformation of the infected T-cells, resulting in lymphomatous lesions in various organs.

Different forms of disease are seen depending on the age of the chicken, the strain of the virus and the resistance of the host. Classical forms, which mainly exhibit neural involvement, are mainly seen in chickens between 2 and 12 months of age. Young chickens infected with virulent strains may develop early mortality syndrome 8-14 days post-infection. Paralysis usually develops 8-12 days post-infection, and some birds which recover from this may go on to succumb to lymphomas within a few weeks. Infection with the very virulent strains may result in acute mortality syndrome, where birds die during the cytolytic

phase of infection, well before the development of lymphomas. There is also an uncommon manifestation of disease where transient paralysis of 24-48 hrs develops in birds between 5 and 18 weeks of age, which involves encephalitic infections. Morbidity in housed flocks is usually between 10 and 30%, though may be as high as 70% with acute forms of disease. Mortalities may increase rapidly, then cease, or continue over several months.

1.7.2 Epidemiology

Transmission is direct or indirect, as the virus particles are shed in feather dander which can contaminate the environment and be spread by the airborne route, on fomites or on people. Spread through a housed flock is rapid, especially in young chicks. Virus particles in contaminated houses can survive for over a year (Schat and Nair, 2008). The epidemiology of disease in village poultry is largely unknown, although it is thought that clinical disease is rarely seen, as it is most likely to manifest under conditions where there are high concentrations of poultry and associated high levels of early challenge (Wajid et al., 2013).

Reports of Marek's disease in Ethiopia are confined to exotic chickens reared on commercial farms (Lobago and Woldemeskel 2004) or in village ecotypes kept experimentally in confined conditions (Duguma et al., 2005). Marek's disease is considered to be of importance in village birds, but there are few published studies looking at the disease under village conditions. One study in Mexico found around 8% of commercially-reared birds placed in village conditions at 3 weeks of age developed signs of MDV infection. Disease developed in both vaccinated and unvaccinated birds (Rodriguez et al., 1997). A seroprevalence study in the Galapagos looked at four farms keeping backyard poultry, each with more than 50 birds. Seroprevalence ranged from 20-50% between farms (Soos et al., 2008). A study in Kenya comparing seropositivity between domestic and wild poultry and game bird species only looked at eleven free-range village chickens, but seven of them had MDV antibodies (Morgan, 1971). A study in Iraq isolated MDV virus from just

under 50% of sampled birds, and found little difference in the proportion of positive samples between commercial and village birds (Wajid et al., 2013). All viruses isolated in this last study showed genetic similarities to well-characterised very virulent isolates.

Susceptibility to MDV has a strong genetic component, and it has been suggested that village birds, in which the virus may have been circulating for long periods, may have been naturally selected for resistance (Wajid et al., 2013). However, one Ethiopian paper suggested that the local ecotypes were highly susceptible to MDV when reared under confined management and there was variation between the ecotypes. Unfortunately in this study the pathotype of the infecting virus was unknown (Duguma et al., 2005).

1.8 *Pasteurella multocida* (Fowl Cholera)

1.8.1 Biology and Pathology

(Summarised from Glisson et al. (2008))

Pasteurella multocida is a gram-negative, non-motile, non-sporulating rod-shaped bacterium. Different strains have been grouped by serotype and sequencing to give three subspecies: *P. multocida* subspecies *multocida, P. multocida* subspecies *septica* and *P. multocida* subspecies *gallicida.* Pathogen virulence depends on both the strain and host species. A wide range of avian hosts are susceptible and it has also been isolated from a variety of mammalian hosts, although only swine isolates have been shown to be pathogenic for poultry. Infections can be acute or chronic, and healthy birds can carry the organism for prolonged periods in the pharynx or cloaca.

Most infections occur in older birds over 16 weeks of age, with acutely infected birds showing signs of fever, lethargy, mucoid secretions from the mouth, watery or greenish diarrhoea, and sudden death. Pathological lesions are those associated with septicaemia. Chronic infections are usually localised and may be suppurative, with sinusitis, arthritis,

torticollis, conjunctivitis or pharyngitis most commonly observed. In infections in housed flocks, around 20% mortality is usually seen, although up to 45% has been reported.

1.8.2 Epidemiology

Infections are most likely introduced by chronically infected birds. Although it has been isolated from insects including acarid parasites of chickens, they probably do not transmit the disease (Glisson et al., 2008). Clinical signs and duration of infection have been shown to be worse in concurrent infections with *Ascaridia galli* under experimental conditions (Dahl et al., 2002).

The proportion of positive samples in village poultry is in Ethiopia has been reported at 63% (Chaka et al., 2012), and at levels of 52% in Zimbabwe (Kelly et al., 1994). Mortality rates in village poultry attributed to *P. multocida* infections appear to be low; Biswas et al. (2005) in a longitudinal study in Bangladesh attributed less than 7% of deaths to pasteurellosis, and it may be that most strains in village poultry are of low pathogenicity, hence the high exposure rates reported. Healthy birds may become chronic carriers, even in the absence of visible fowl cholera outbreaks. The proportion of village birds infected has been reported at rates of 0.7% in Tanzania by isolation of the pathogen from tracheal swabs of scavenging birds (Muhairwa et al., 2001) and rates of 1.1% were found in family flocks in Kenya (Mbuthia et al., 2008). In this last study, bacterial carriage from birds sampled in markets has been reported to be significantly higher than that on farms.

1.9 *Salmonella enterica*

1.9.1 Biology and Pathology

(Summarised from Shivaprasad and Barrow (2008) and Chappell et al. (2009))

Salmonella enterica are gram-negative, slender rod-shaped, facultatively anaerobic bacteria. Several serovars are capable of infecting chickens, including the motile

paratyphoid serovars, *S.* Enteritidis and *S.* Typhimurium, which infect a wide range of species and are both major public health issues. On the other hand, *S.* enterica serovar Gallinarum and serovar Pullorum infections are predominantly restricted to chickens, although there are some reports of outbreaks in turkeys, game birds and parrots. *S.* Gallinarum is the causal agent of Fowl Typhoid, which affects birds of all ages, while *S.* Pullorum is the causal agent of Pullorum disease, which causes substantial mortality in chicks up to 2-3 weeks old. While the paratyphoid serovars of *Salmonella* are flagellated, and cause intestinal inflammation early in infection, the non-flagellate serovars Gallinarum and Pullorum cause little inflammation, are taken up by macrophages and dendritic cells, and may result in severe systemic disease. Disease severity is highly variable, depending on factors such as the age and strain of bird, management factors and route and dose of exposure. Mortality from systemic salmonellosis can be up to 100% in young birds.

Infection takes place in three stages; intestinal invasion, development of systemic infection and eventually immune clearance, death or development of a subclinical carrier state, where the bacteria persist within macrophages. Clinical signs for both Fowl Typhoid and Pullorum disease include early death in birds hatched from infected eggs, weakness, depression, poor appetite, ruffled feathers, pale combs, poor growth/ decreased egg production, diarrhoea and dehydration. Post-mortem lesions can be seen in the liver, spleen, heart, pericardium, lungs, yolk sac and joints in young birds. In adults, lesions are most often found in the ova and pericardium. Paratyphoid infections may cause similar signs in very young chicks, but may be asymptomatic in adults.

1.9.2 Epidemiology

Most *Salmonella* transmission is only horizontal, via the faeco-oral route, and persistently infected birds are probably an important route of spread. In a housed flock, spread can occur through cannibalism of infected birds and eggs, or through faeces, contaminated

litter, feed or water, and mechanical spread by personnel, rodents or flies may also occur (Shivaprasad and Barrow, 2008). *S.* Pullorum can also be vertically transmitted, as it frequently persists in a carrier state following infection, and can recrudesce at point of lay in females (Chappell et al., 2009). *Salmonella* does not survive well in the environment, possibly up to a week in faeces, but it can be killed by direct sunlight. The route of spread in village flocks is not studied but may be more likely to be direct transmission.

Both *S.* Pullorum and *S.* Gallinarum possess 'O' antigens 9 and 12, and the OIE Terrestrial Manual states that the same antigen is used for serological detection of both (O.I.E., 2012). In addition, cross reactions to *S.* Enteritidis or *S.* Typhimurium are likely. Two recently published studies in Ethiopia which tested sera from backyard flocks using rapid slide agglutination tests report the proportion of *S.* Pullorum-positive birds as 39% (Berhe et al., 2012) and *S.* Gallinarum-positives as 45% (Berihun et al., 2012), although neither describes the antigen used, so it is unclear whether they can specifically attribute results to the reported serovar. Serological studies using an *S.* Gallinarum antigen in rural poultry in other African countries report positivity of 6.3% in Tanzania using an agglutination test (Mdegela et al., 2000) and up to 58% in Morocco using an ELISA test (Bouzoubaa et al., 1992). The latter study also reported *S.* Pullorum rates in Morocco to be around 6% using an agglutination test. Isolation of *Salmonella* from village chicken faeces in Ethiopia has been reported in one study, with 41% of samples yielding a positive culture, although no typing was performed (Alebachew and Mekonnen, 2013). *S.* Gallinarum and Pullorum have been isolated from intensive poultry farms in Ethiopia (Aragaw et al., 2010).

1.10 Coccidiosis

1.10.1 Biology and Pathology

(Summarised from McDougald and Fitz-Coy (2008))

Coccidiosis is caused by a protozoal infection by intracellular apicomplexan parasites of the *Eimeria* genus. All species are host-specific, and there are nine known to infect chickens. The parasites have a direct life cycle: Oocysts are shed in faeces of infected hosts, which then sporulate, and sporozoites are released after ingestion. These then undergo two to four rounds of asexual reproduction (schizogony –formation of schizonts) in epithelial cells in the gut, before producing gametocytes which combine to form an oocyst. The cycle takes 4-6 days. Pathologies are caused by the rupturing of schizonts to release merozoites, causing damage to cells, and the host inflammatory reactions to gametocytes. Gross lesions appear as plaques in the intestines, haemorrhage, mucoid exudates and enteritis. Different *Eimeria* species are found in different parts of the intestinal tract, and have different pathogenic effects. This is used to help in identification of species, although molecular methods are becoming more widely applied. *Eimeria brunetti, necatrix* and *tenella* are considered to be more pathogenic, whilst *E. praecox* and *mitis* are less so, although the extent of lesions in all infections will also depend on the age of the bird and infective dose, which is likely to vary across production systems. Marek's disease and IBD may interfere with immunity to coccidiosis, resulting in more severe disease, and secondary enteric infections with *Clostridium perfringens* or *S.* Typhimurium can occur due to tissue damage.

Most clinical infections are seen between 3-6 weeks of age, as the majority of young chicks have protection from maternal antibodies and older birds usually develop a strong immune response after infection. There is no cross-protective immunity between different species, so some birds can develop subsequent infections. *E. necatrix* infections tend to occur later, between 8-18 weeks of age, and *E. brunetti* infections may occur early or late.

1.10.2 Epidemiology

Ingestion of viable sporulated oocysts is the only method of infection. These can survive for several weeks in soil, but are quickly killed by exposure to heat or drying, so risks are higher in cool, damp weather. They may be spread mechanically (McDougald and Fitz-Coy, 2008).

The proportion of birds positive for coccidiosis under village conditions in Ethiopia has been reported to be between 13% and 61% in local breeds (Ashenafi et al., 2004b; Gari et al., 2008). Clinical coccidiosis in village birds from the same studies, defined either as live birds showing depression and haemorrhagic diarrhoea or as birds with intestinal lesions at post mortem, was reported at 12-15%. Ashenafi et al. (2004b) reported higher levels of infection in highland areas in Ethiopia compared to lowland areas, although only one market was selected in each area for comparison. A study in Kenya found no significant difference in the proportion of positives across different climatic regions (Kaingu et al., 2010). Species reported in Ethiopia are *E. necatrix, E. acervulina, E. maxima, E. tenella, E. brunetti* and *E. mitis* (Lobago et al., 2005; Gari et al., 2008).

1.11 Nematodes

A number of parasitic nematodes are found in poultry. A brief description of some of the more prevalent African species is given below, summarised from Permin and Hansen (1998) and Yazwinski and Tucker (2008).

1.11.1 *Ascaridia galli*

These are large, (Males 50-76mm, Females 60-116mm) thick, yellowish-white worms, primarily found in the small intestine, but occasionally reported from crop, gizzard, body cavity, oviduct and egg. This species has a direct life cycle; eggs become infective after 7-28 days and can survive low environmental temperatures, with larvae maturing after 28-30 days. Pathogenic effects include retarded growth, anaemia, hypoglycaemia, increased

mortality and infection can increase the effects of coccidiosis. Birds develop an age-related resistance, and arrested larval development often occurs in birds over 3 months. Birds' nutritional status can also affect resistance, particularly levels of Vitamins A and B, calcium and lysine.

1.11.2 Heterakis gallinarum

A small (Males 7-13mm, Females 10-15mm) white worm found in the caeca of chickens, turkeys, game birds and waterfowl. It has a direct life cycle, although eggs ingested by earthworms are still infective. The eggs are indistinguishable from those of *A. galli*. Larvae live in the walls of the caeca for 12 days then become free in the lumen. The pathogenic effects include caecal inflammation, and nodule formation and secondary infections, with hepatic granulomata occasionally reported. *H. gallinarum* is a vector for the protozoan *Histomonas meleagridis* (Blackhead), which is a particular problem for turkeys.

1.11.4 Subulura (Allodapa) brumpti

These are small (Males 7-10mm, Females 9-14mm) worms with curved tails, found in the lumen of the caecum. Their intermediate hosts are beetles or cockroaches. There are no known pathogenic effects.

1.11.3 Capillaria spp.

There are many species of this nematode which infect the chicken. The size varies depending on species, with most being 10-25mm but some species reaching 60mm long. Species can be divided into those found in the crop or oesophagus (*C. annulata, C. contorta*); those found in the small intestines (*C. caudinflata, C. obsignata, C. bursata*) and those found in the caecum (*C. anatis*). Life cycles are either direct or involving an earthworm intermediate host, depending on species. All species are pathogenic, causing inflammation and ulceration of tissues. All ages of bird may be affected. Weight loss,

weakness and poor growth are the predominant signs, but heavy burdens, especially of the crop worms, may cause significant mortality in young birds.

1.11.5 Cheilospirura (Acuaria) hamulosa

These are medium (Males 9-19mm, Females 16-25mm) worms found in the gizzard lining, where they cause small lesions and nodules in the gizzard endothelium and musculature. However, they have little pathogenic effect except in heavy infestations. Intermediate hosts include grasshoppers, weevils and beetles.

1.11.6 Dyspharynx spiralis

Small (Males 7-8mm, Females 9-10mm) worm rolled in a spiral, found in the proventriculus with their heads buried in the mucosa. Intermediate hosts are isopod spp. such as the woodlouse. Pathologies caused by these worms involve ulceration, inflammation and pronounced thickening of the proventriculus, with signs of weight loss and anaemia, and potentially heavy losses of young birds.

1.11.7 Epidemiology

The proportion of birds under village conditions positive for nematode infections in previous studies in Ethiopia has been reported to be around 75%. The most common species reported are *A. galli* (32-55%), *H. gallinarum* (17-48%) and *S. Brumpti* (17-27%). Other species have been reported at rates of less than 5% (Eshetu et al., 2001; Ashenafi and Eshetu, 2004a; Tolossa et al., 2009; Heyradin et al., 2012; Molla et al., 2012). The majority of infected birds are infected with more than one species. The altitude and climatic conditions have been suggested to affect infection prevalence, with highland areas being linked to lower proportions of infected birds (Eshetu et al., 2001). However, neither Tolossa et al. (2009) nor Molla et al. (2012) found any such differences between different climactic regions. Although the proportion of positives may be high, parasites tend to be over-distributed, with around 10% of birds carrying large burdens which may have clinical

impact (Magwisha et al., 2002). Little work on the extent of the clinical impact of helminth infections in rural poultry appears to have been carried out. Weight loss or retarded growth, anaemia, weakness and mortality are known to be problems in experimental infections.

1.12 Cestodes

A brief description of some of the more important species of cestodes in Africa is given below, summarised from Permin and Hansen (1998) and McDougald (2008).

1.12.1 *Davainea proglottina*

A small worm - less than 4mm, with fewer than 9 proglottids – found in the duodenal mucosa. High burdens of over 3000 worms have been found in individual birds. The intermediate hosts are slugs and snails. This worm is pathogenic in young birds, causing reduced growth, emaciation and weakness.

1.12.2 *Raillietina tetragona and Raillietina echinobothrida*

These large worms (25-34 cm) are found in the small intestine and cause a hyperplastic nodular enteritis. Clinical signs include weight loss and decreased egg production. The intermediate host is the ant.

1.12.3 *Raillietina cesticillus*

This 15cm worm is found in the duodenum or jejunum, but is not thought to be pathogenic. Intermediate hosts are several beetle species.

1.12.4 *Amoebotaenia sphenoides (Amoebotaenia cuneata)*

This very small worm – less than 4mm – is found between the duodenal villi. The intermediate host is the earthworm. The exact pathogenic effects unclear, but infections are usually associated with haemorrhagic enteritis, or chronic wasting.

1.12.5 *Choanotaenia infundibulum*

This is a distinctive white worm, up to 23cm in length, found in the proximal intestines. It has several intermediate hosts including *Musca* (housefly) spp. and several beetles. It has been described as “moderately pathogenic” although there are no reported controlled experiments.

1.12.6 *Hymenolopis cantainana*

This is a very slender, thread-like worm, 2cm in length, found in the small intestines. Intermediate hosts are scarab (dung) beetles. It is not believed to be pathogenic.

1.12.7 *Epidemiology*

The level of cestode infections in village birds has been reported in studies in Ethiopia at values between 55 -86% (Ashenafi and Eshetu, 2004a; Tolossa et al., 2009; Heyradin et al., 2012; Molla et al., 2012). Variation between different regions with different altitude and climate conditions was reported, with three studies reporting lower proportions of infected birds in highland areas (Eshetu et al., 2001; Tolossa et al., 2009; Molla et al., 2012). Infection with multiple species was commonly reported. The more prevalent species are reported as *R. tetragona, R. echinobothrida, A. sphenoides* and *H. cantainana*. The more pathogenic *D. proglottina* has been reported at much lower rates of between 1% and 8%.

1.13 Ectoparasites

A number of ectoparasites of poultry have been described. Many have a worldwide distribution, whilst others are restricted to the tropics. A brief description of those poultry parasites previously described in the Ethiopian literature is listed below, summarised from Lapage (1956), Permin and Hansen (1998) and Wall and Shearer (2001).

Ticks

1.13.1 Argas persicus

This is a soft bodied red-brown tick, all life stages of which feed on poultry. Adults are active at night, but hide during the day in cracks in housing. The life cycle can be completed in 1 month, or stages can survive up to a year in the environment. This tick has significance as a vector for two haemoparasites of poultry; *Aegyptianella pullorum* and *Borrelia anserina* (causing spirochaetosis).

Mites

1.13.2 Dermanyssus gallinae

Commonly known as the chicken mite or red mite, this parasite spends most of the time off the host, living in crevices in housing, and feeding on hosts at night. Its life cycle can be completed in 7-9 days. Large infestations can result in decreased weight gain and egg production.

1.13.3 Ornithonyssus bursa

The tropical fowl mite is a mite whose entire life cycle takes place on the host. They are found on the fluff of the feathers especially around the vent, and certain life stages feed on blood, which may result in anaemia, weight loss, decreases in egg production and even mortality. They can transmit Fowlpox, NDV and pasteurellosis. Short-lived zoonotic infections have been reported, but the mite cannot survive away from the bird host.

1.13.4 *Cnemidocoptes mutans*

The scaly leg mite, as its name suggests, burrows under scales on legs and feet, causing cornification and hypertrophy of tissues, which can lead to distortion and crippling in severe cases. This mite is also found on a range of wild birds.

1.13.5 *Cytoditus nudus*

The air sac mite is found in respiratory passages and air sacs and is transmitted in respiratory mucus. Also has occasionally been found in body cavities on the serosal surface of organs. It is moderately pathogenic, causing dyspnoea, cough, secondary respiratory infections and peritonitis.

1.13.6 *Epidermoptes bilobatus*

Known as the skin mite, this is found all over the body and is a cause of dermatitis, pruritus and loss of production.

Fleas

1.13.7 *Echidnophaga gallinacea*

The common name, sticktight flea, describes the behaviour of this parasite, the adult of which lives permanently attached to face and wattles, where they can survive for many months. They can cause anaemia and a decrease in egg production in adult birds and reduced growth and mortality in chicks.

1.13.8 *Ctenocephalides felis*

The cat flea has been reported on one bird in one Ethiopian study. The pathogenic significance of this flea in chickens is unknown, but unlikely to be significant.

Lice

1.13.9 *Menacanthus stramineus*

Although commonly called the chicken body louse, its hosts include many other wild bird species. Its entire life cycle occurs on the host, with eggs glued to host feathers. This is a chewing louse, feeding on skin and feathers, and may cause skin lesions and a decrease in egg production. In severe infestations it may cause anaemia, restlessness, poor digestion

and occasional mortality in young birds. It is thought to limit *Ornithonyssus* mite infestation.

1.13.10 Menopon gallinae

Alternative names include the shaft louse and feather louse. This is a small (2mm) pale yellow, chewing louse, which feeds on blood from young quill feathers. Like *Menacanthus,* it causes skin lesions and decrease in egg production.

1.13.11 Gonoides gigas

This large species has the common name of tropical brown chicken louse. It usually occurs in too small numbers to be pathogenic.

1.13.12 Goniocotes gallinae

This is commonly called the fluff louse, as it is predominantly found in down feathers. It is usually only present in small numbers and not pathogenic.

1.13.13 Cuclotogaster hetrographus

Named the head louse, as its predilection sites are the head and neck. It can cause pathology in young birds, namely anaemia, weakness and poor growth.

1.13.14 Epidemiology

Two studies on ectoparasites in Ethiopian village chickens found 92 and 84% of birds infected with at least one species of ectoparasite (Belihu et al., 2009; Tolossa et al., 2009). Other studies do not give an overall finding for ectoparasites, but report separately for individual species. Lice were the most frequent ectoparasite identified in all studies, particularly *Menacanthus stramineus,* with the proportion of infected chickens ranging from 26 – 65%; and *Menopon gallinae,* reported at a rate ranging from 20 -60% (Abebe et al., 1997; Belihu et al., 2009; Tolossa et al., 2009; Mekuria and Gezahegn, 2010). Mites and ticks were found at much lower frequency. The flea species *Echidnophaga gallinacea* was

found in three of the studies, identified in 1.3, 12.8 and 51% of birds (Belihu et al., 2009; Tolossa et al., 2009; Mekuria and Gezahegn, 2010), although there was no clear reason for this difference. None of the studies found differences between different climatic regions, although two studies reported levels of infestation to be higher in males than females. Co-infection with multiple species would appear to be common, although none of the studies specifically examined this aspect.

1.14 Co-infections in village chickens

Interactions between pairs of infections have long been studied under experimental conditions in poultry. Pre-infection with Marek's disease has been observed to alter the clearance of certain *Eimeria* species (Biggs et al., 1968), while both *Pasteurella multocida* and *Salmonella enterica* serovar Enteritidis infections have been shown to be more severe in the presence of pre-infection with the nematode *Ascaridia galli* (Dahl et al., 2002; Eigaard et al., 2006). *Ascaridia galli* has also been shown to reduce the antibody response to vaccination with NDV (Horning, 2003). The explanation for these interactions is thought to lie with the response of the chicken immune system. In the case of pre-infection with the cytolytic MDV, which impairs the adaptive immune response to several pathogens, this effect of enhancing *Eimeria* infections is perhaps unsurprising. The effect of *Ascaridia galli* infections on responses to three different pathogens is thought to be mediated through the altered differentiation of T-cells. Protection to extracellular infections, such as macroparasites usually requires antibody and primes the immune system towards a Th2-type response. However, protective cell-mediated responses to intracellular microbial pathogens, such as NDV and *Salmonella,* are primarily driven by T helper 1 (Th1) cells, and the priming of the immune system towards a Th2 response by Ascarid infection reduces its subsequent ability to produce an adequate Th1 cell response (Degen et al., 2005).

Despite the wide range of infections reported in village chickens, the vast majority of observational studies focus on single infections. Even those studies which investigate more than one species of pathogen, for example Tolossa et al. (2009), who reports on several species of both endo- and ecto-parasite, neglect to report even the most basic figures of birds infected with more than one species. A serological study in Zimbabwe (Kelly et al., 1994) only stated that there was no relationship between seropositivity for any of the infections studied, which included infectious bronchitis virus, reticuloendotheliosis, IBD, pasteurellosis, *Mycoplasma*, NDV, avian encephalomyelitis, avian leucosis and avian reovirus infections. However, a longitudinal study by Biswas et al. (2005) in Bangladesh suggested that around 10% of mortalities were caused by mixed infections, of which the most common combination was *Salmonella* and *Pasteurella multocida*. Only 2.3% of cases involving multiple infections did not involve at least one of either NDV or *Pasteurella*.

One recent example of a study investigating multiple infectious diseases is the work by Chaka et al. (2012) in Ethiopia, which reported that 86% of birds had antibodies to more than one of the four diseases under investigation, and that over 75% of these co-infections involved IBD. However, over 90% of birds had IBD antibodies in the study at each time-point measured; therefore this is perhaps not surprising. There was no comment on whether the involvement of IBD in so many co-infections was more or less than expected under an assumption of independence. The study also reported that birds were seropositive to a greater number of diseases in the wet, compared to the dry season. However, the only disease which increased in seroprevalence from the dry to the wet season was *Mycoplasma gallisepticum*, which is known to affect birds longer and more severely during cold weather. This organism is frequently reported to occur in housed flocks in conjunction with other pathogens such as NDV or infectious bronchitis virus, which may predispose to *M. gallisepticum* infection, or with *Escherischia coli*, which may complicate it (Ley, 2008).

It would therefore appear that despite increasing knowledge of how pathogens may interact in experimental and intensively farmed poultry, both the frequency and the significance of multiple infections in village poultry are still only poorly understood. There is evidence from studies in wild mammal populations that existing infections may pose greater risks for further infections than environmental variables (Telfer et al., 2010), and that host survival may be severely affected by multiple, coincident infections (Jolles et al., 2008). A recent study in zebu cattle in Kenya also found that co-infection with *Trypanosoma* species or strongyle nematodes increased the risk of death in *Theileria parva* infections, clearly demonstrating the importance of co-infections in livestock under field conditions (Thumbi et al., 2014). As it might be anticipated that chickens in a village situation are likely to be regularly exposed to multiple pathogens, this is clearly an area which needs addressing.

1.15 Aims of this thesis

There is currently a need for the Ethiopian development programmes for village chickens to incorporate an aspect of infectious disease control. Although NDV is highly likely to be important, we hypothesise that several other pathogens may be contributing towards the seasonally described "*fengele*" outbreaks, which occur in the wet season. The survival and transmission of many infectious agents is better under cool, damp conditions, and as such there may be seasonal differences in exposure to many important pathogens. There may also be interactions between infections, such that singly infected birds may survive, but co-infected birds may succumb, or certain infections may predispose to further infection with an additional pathogen. In addition, other non-infectious causes may play a role in the reported seasonal patterns observed. Examples of such factors would be cold or nutritional stress; seasonal differences in bird movements or management practices; and seasonal breeding, which affects the size of the susceptible population. To inform the Ethiopian

development programmes, especially the breeding programme, which aims to start incorporating disease resistance traits into its future selection criteria, there is a need to better describe and quantify not just the presence but the impact of a range of infections, and to better understand their potential interactions with each other and with their hosts.

The aims of this thesis are:

1) To better understand the Ethiopian village production system with a specific focus on practices that may alter the risk of infectious diseases.
2) To quantify the impact of disease on Ethiopian village poultry production and describe any temporal and spatial variation
3) To investigate the epidemiology of a range of pathogen types, including micro- and macro-parasites in Ethiopian village chickens and measure their temporal and spatial variation
4) To investigate potential associations between observed pathogens which may alter the infection risk

As this thesis is part of a wider study, the “Chicken Health for Development” project, which also examines the population genetics of Ethiopian indigenous chickens and the socio-economic aspects of chicken keeping, a further aim is to inform and work alongside these lines of research, such that a detailed observation of the system of village chicken keeping in Ethiopia in its broadest sense may be obtained.

Chapter 2

Materials and methods

2.1 Acknowledgements

This work has been undertaken as part of the Chicken Health for Development project, a multidisciplinary collaboration between institutions in the UK and Ethiopia, investigating the health, genetics and socio-economic aspects of Ethiopian village chicken production. A large number of people have been involved with the study design and data generation, and their contributions and affiliations are listed here. During the production of this thesis, I have occasionally had recourse to use some of the data from other parts of the project, primarily collected to answer different research questions, in order to contextualise and explain some of the findings from my own analyses. I would like to thank all those who have generously allowed me to use their data in this thesis. Any errors are entirely my own.

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2.2 Introduction

This chapter covers the design of the epidemiological study, the collection of the data, the laboratory methods and the major statistical methods which have been used in this thesis. Subsequent chapters deal with different aspects of the results, but as all data were collected as part of the same observational study, the methods of data collection are identical throughout. Therefore to save repetition, the materials and methods are described in detail here, whilst subsequent chapters will only highlight the most relevant aspects and any additional statistical methods specific to a particular analysis.

2.3 Study design

The multidisciplinary nature of the study meant that there were certain requirements which needed to be fulfilled in designing the sampling strategy. In the original design, it was planned to compare one lowland and one highland region. The two regions needed to be geographically distant, with a low probability that chickens from the two areas would be genetically related, i.e. there should be no direct trade of chickens between them. Two geographically separate woredas (administrative districts) in the Oromia region of Ethiopia were purposely selected, both with chicken populations which had been previously well-characterised: Horro, in the western highlands, and Jarso in the east. Woredas are divided into smaller administrative districts, known as kebeles, which may comprise several villages or sub-areas. Two market sheds (a group of villages linked by trading networks, as described by Tadelle (2003)), were purposely selected within each region, and two kebeles were selected within each of the four market sheds. This nested design was to allow an examination of the population structure and any differences in exposure to infections, as there is theoretically little movement of chickens between market sheds.

Kebeles were selected in consultation with local representatives of the Department of Agriculture and the communities. Selection was made on the basis that the community was willing to participate, that the area was believed locally to be representative of the woreda, and on knowing there was no recent improvement programme in these areas, since introduction of exotic breeds or vaccination programmes would interfere with the study of either the genetics or the serological detection of infections, respectively. In the event, it was decided to abandon the original plan of selecting villages in the lowland areas of Jarso, due to potential risks of kidnapping and bandit activity in the lowland areas, which are close to the border with the Ethiopian Somali region. Therefore two highland market sheds in the Jarso region were selected. A map of the study areas is shown in Figure 2.1.

The sampling was designed to run over two years, and to sample both before and after the main rainy season (when most disease outbreaks reportedly occur), in order to capture seasonal changes in seroprevalence, and to assess whether these were consistent between years. Sample size calculation, accounting for the expected degree of clustering, was performed at the design stage, and it was planned to sample 1600 birds across the entire study. Due to the later decision to incorporate follow-up visits to farms, it was later decided to reduce the number of sampled households in the second year of the study for logistical and financial considerations.

2.4 Data Collection

2.4.1 Ethics statement

Ethical approval for the study was obtained from the University of Liverpool research ethics committee (reference RETH000410). A later amendment was made after the study design was adjusted to collect follow-up data on the birds, to allow participants' names to be recorded. Due to low literacy levels among participants, a statement explaining the study

objectives and outlining sampling methods was read to the farmers before they elected to participate. They were also informed that they could withdraw from the study at any time, and asked to contact their local development agent (DA) in the event of any subsequent problems. DAs are employees of the agricultural extension service provided by the Ethiopian Government whose role is to provide farmers with access to training, research and technologies (http://www.moa.gov.et/policies-and-strategies). Oral permission was obtained for data collection and bird sampling. All data were anonymised, and only very limited personal data was collected from the study participants. Local ethical approval for animal sampling was not required.

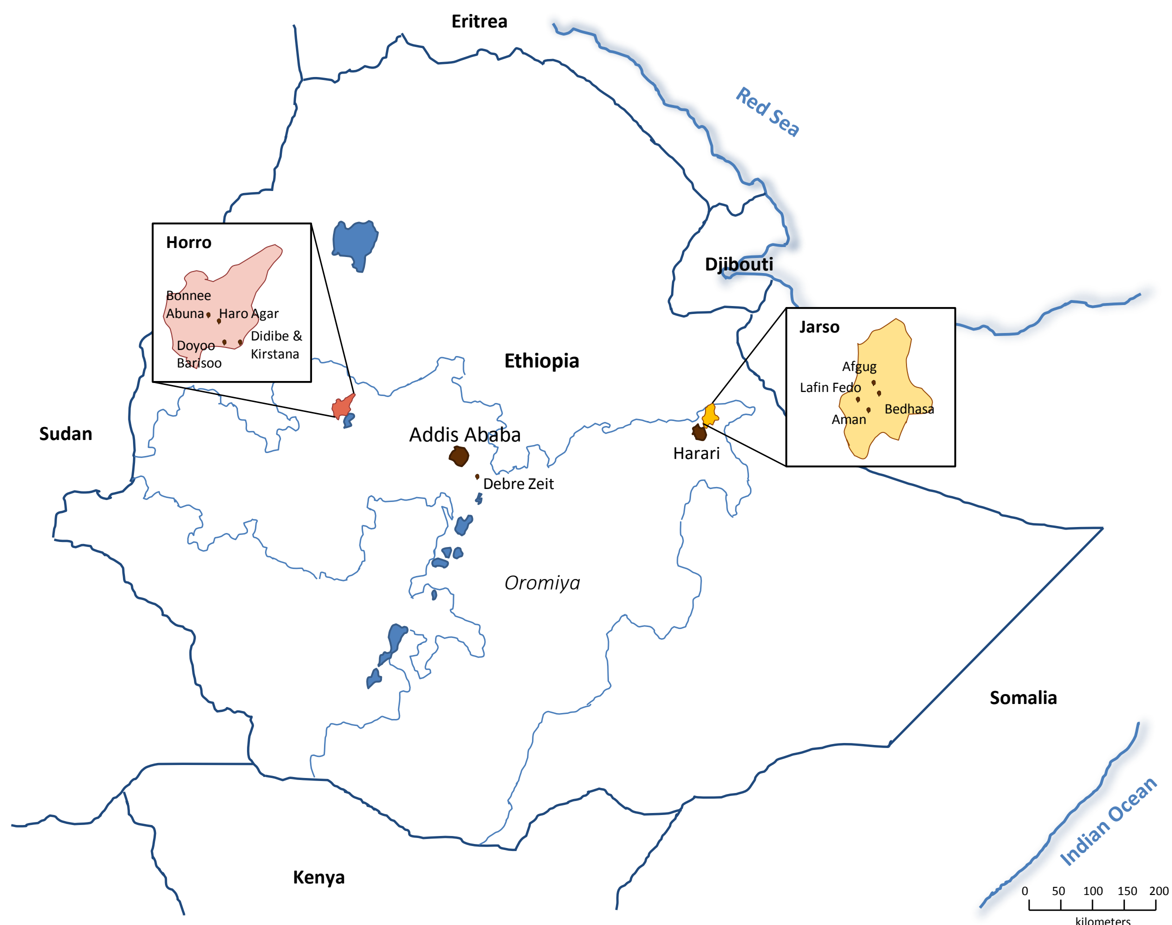

Figure 2.1 Map of Ethiopia, showing sampling areas.

2.4.2 Rapid rural appraisal (RRA)

In January 2011, a rapid rural appraisal (RRA) was conducted in four of the study kebeles; one from each of four market sheds. These were carried out by some of the Ethiopian members of the study team, accompanied by district veterinarians or officials from the local agriculture office. Focus group discussions (FGD) comprised of up to 50 farmers, of which between 20 and 50% were female. Group discussions typically took two hours. Following the FGD, field visits were conducted in each of the kebeles to directly observe village poultry production. Discussions with key informants, such as district administrators and animal extension workers, were used to collect additional information and triangulate the FGD. The results of these were fed back to the rest of the study team and informed questionnaire development and fieldwork planning.

2.4.3 Sample selection

A pilot study was performed in one kebele in the Horro region in April 2011, where questionnaires and sampling techniques were trialled in the field. Sera collected during the pilot study were used in the development of laboratory tests. Questionnaires and data collection techniques were further refined before the study commenced.

Each kebele was visited on four occasions; in May/June and October/November in each year of the study (2011-2012). These visits were timed for before and after the main rainy season, which occurs between June and September. Each visit to the kebele took place over 2-4 days. Lists of farmers in each kebele, grouped by village or area, were compiled by the local DAs. Within each kebele, a number of contiguous sub-areas were selected in order to produce a list of approximately 400-700 households. Systematic random sampling was used to select 104 potential participants in each kebele by selecting every *n*th name from each list with a starting place chosen at random. The selected names were split into four groups, one for each sampling season, so that different households were selected to

sample on each visit to the kebele. Approximately 30% more names than were required were selected to allow for exclusions or non-participation.

Ethiopian staff were recruited to collect the field samples, and conduct the questionnaires in the local language. Training was provided beforehand, and where necessary in the field if problems were identified. Selected households were visited by the study team, and farmers were interviewed to confirm that they had no exotic birds or vaccinations used in their flock. They also needed to own two indigenous-type birds of at least six months of age in order to be included in the study.

Two birds were selected from each household to be sampled, and where possible one cock and one hen were chosen; otherwise two hens were sampled. If the household owned more than one cock over six months of age, each bird was allocated a number then, using random number tables, one was selected for sampling. Hens were sampled using the same method, but if possible, birds related in the first degree to the cock were excluded, based on the farmer's knowledge of their birds. This was to minimise the relatedness of birds where possible for the genetics study. Once the team had made the selection from all available birds, they were caught, usually by members of the household, and placed securely in baskets.

2.4.4 Questionnaires

A questionnaire interview was carried out with a member of the household, in order to collect data on the management practices and the disease history of the flock. Interviews were conducted in the local language either with the person with main responsibility for caring for the birds or with the head of the household, and answers recorded in English. A copy of the questionnaires used is in General Appendix A. Basic questions for this study remained the same throughout, but some additional questions were asked for one or more seasons, as complimentary projects by other students were carried out simultaneously.

Household latitude and longitude were recorded using handheld Global Positioning System (GPS) units. Altitude was not recorded, due to poor agreement between individual units, but was subsequently found from the location data using Google Earth software.

2.4.5 Clinical examination

A form was provided with a checklist of the information which needed to be collected for each bird (General Appendix A) for fieldworkers to complete. This form was labelled with a unique code to identify the household and bird, and all samples collected from that bird were labelled with the same code, in the form of pre-printed stickers. Bird codes were also linked to the household questionnaire.

Information about the clinical history of the two sampled birds was obtained by interviewing a member of the household. A series of photographs were taken of each bird, comprising a lateral, dorsal and frontal view, and close-up shots of the head, feet and skin. These were identified by taking a photograph of the label with the bird's unique identifying code before commencing the photo series. Photos were also imprinted with the date and time, to help with identification. Photographs were primarily to help with characterisation of the bird phenotypes, but also served a useful role in identifying individuals.

The birds were each then subjected to a clinical examination, and scoring for ectoparasites. The checklist provided comprised a series of tick boxes for fieldworkers to complete, to ensure that birds were examined thoroughly and consistently. Information was collected on various clinical signs exhibited, and also on certain morphological traits, such as feathering, for the genetics study. Birds were weighed in the baskets, and ascribed a body condition score on a 0 – 3 grading system (Gregory and Robins 1998).

A scoring system was also devised for selected ectoparasites. Ticks, fleas or mites were scored by counting the number found on an examination of the most likely places to find them – the naked areas on the face, under the wings or around the vent. Lice were scored

by performing a timed count of 3 areas of the body – one side of the keel, the back and the rump - and a total count of lice under the tail feathers and at the base of the flight feathers of one wing, as described by Clayton and Drown (2001). Birds were also scored for scaly leg mite, by grading the amount of hyperkeratosis; none, mild or severe. If ectoparasites were observed or suspected, a sample of the parasite or a skin scraping from the leg was collected and stored in 70% ethanol.

A 1.5ml blood sample was taken from the wing vein of each bird. This was collected into a syringe which had been flushed with sodium citrate. The sample was gently mixed by inverting the syringe prior to being transferred to eppendorf tubes for storage. In addition, some blood was placed on FTA cards and, for birds sampled in the first two sampling periods, two blood smears were made at the time of sampling and fixed in methanol for 30 seconds. Before releasing sampled birds, they were fitted with a leg ring, the colour and number of which was recorded on the checklist.

Wherever possible, a faecal sample was collected from the basket where the bird had been confined, or where the bird was observed to defecate after release. If it could not be collected from the bird, a sample was taken from the household environment. Fieldworkers were required to record in the checklist whether or not the faecal sample was known to come directly from the sampled bird. Blood and faecal samples were stored and transported in a refrigerated container to the laboratory in Debre Zeit, where plasma was separated from the cell pellets and stored at -20°C. The faecal samples were kept at 4°C until processed. Ectoparasite samples were stored in ethanol at room temperature.

2.4.6 Follow-up data collection

A follow-up visit was carried out for households interviewed during the first three rounds. This visit occurred around 6 months later, at the time of the next visit to the kebele. Farmers took part in a short interview questionnaire (General Appendix A) to ascertain

what further disease events they had experienced in the intervening period, and the fate of the two previously sampled birds was determined. Surviving birds were re-examined, including body condition scoring and re-weighing, and a second photograph series was taken. If a bird had not survived, farmers were asked to report what had happened to it, and to provide some brief description of signs if it had died from disease.

2.5 Laboratory testing – serological methods

A laboratory was established at the Ethiopian Institute of Agricultural Research (EIAR) in Debre Zeit, which lies in the central highlands of Ethiopia, approximately 45km southeast of the capital, Addis Ababa. All serological and coprological examinations were carried out in this laboratory. Methanol-fixed blood smears and ectoparasites in 70% ethanol were exported under license to the University of Liverpool for microscopic examinations, and FTA cards were exported under license to the University of Nottingham, where they formed the basis of the genetics studies. The laboratory manuals used during the project which give full details of the Enzyme-linked immunosorbent assay (ELISA) and haemagglutination inhibition (HAI) test procedures, including preparation of reagents, are shown in General Appendix B.

2.5.1 Infectious Bursal Disease Virus (IBDV) ELISA

Serum samples were screened for antibodies to IBDV using a Flockscreen antibody ELISA kit (x-OvO, Dunfermline, UK). Samples were diluted 1 in 500 with the sample diluents provided, and added in triplicate to the antigen-coated wells. The positive and negative controls were provided with the kit and added in the last two positions on each plate. After a 30-minute incubation, the plates were manually washed with the wash buffer, and an enzyme conjugate was added. The plates were incubated for a further 30 minutes, then washed a second time before adding the enzyme substrate reagent. These were incubated

for 15 minutes and a stop solution was added. For the first two rounds of testing, the stop solution had to be replaced with 0.5M sodium hydroxide (NaOH), due to difficulties in transporting the kit's stop solution from the UK on a commercial airline. Plates were read after 5 minutes on the spectrophotometer at an absorbance of 562, as the recommended 550 wavelength was not available. Where the test was not valid after 5 minutes, plates were read again at intervals, as the colour continued to develop. All plates gave a valid reading within 15 minutes.

2.5.2 Pasteurella multocida, Salmonella O9 serotype and Marek's Disease virus ELISAs

2.5.2(a) Preparation of Antigen

Antigens for the bacterial serology tests were prepared according to the method described by Beal (2004). A nalidixic acid-resistant isolate of *Salmonella enterica* serovar Gallinarum was grown in Luria Bertani broth and an isolate of *Pasteurella multocida* grown in brain-heart infusion broth. Both were incubated overnight at 37° C in an orbital incubator (150 rpm). Bacterial cells were pelleted by centrifugation at 4100 g for 40 min and washed twice with phosphate buffered saline (PBS). Cultures were re-suspended in 40ml PBS, then heat inactivated at 65° C for 4 hours. They then underwent three freeze–thaw cycles at -70° C before sonication (10 x 20 second bursts) on ice. The suspensions were clarified by centrifugation at 4100 g for 40 min followed by centrifugation at 30,000 g for 20 min to remove insoluble fractions. Protein concentrations of the soluble antigen preparations were measured using the Fluka protein quantification kit rapid (Sigma-Aldrich, Gillingham, UK) and aliquots stored at -20 °C.

Antigen for the Marek's Disease virus (MDV) ELISAs was kindly provided by the Institute of Animal Health (Compton, UK), and consisted of a suspension of chicken kidney cells infected with a CV1988 strain of MDV, which had been lysed with 4 freeze-thaw cycles. In addition, the Institute provided non-infected chicken kidney cell lysate (CKCL) as a control.

2.5.2(b) Antibody detection

Flat-bottomed polystyrene ELISA plates (Greiner Bio-one, Stonehouse, UK) were coated with antigen in 100 µl/well of carbonate buffer (pH 9.6) overnight at 4 °C. Dilutions of the different antigens are given in Table 2.1. Following removal of the antigen, plates were blocked with PBS Tween-20 (0.05%) (PBS-T) supplemented with 3% skimmed milk powder (diluent) for 1 hour at 37 °C, and washed once with PBS-T. Serum samples were diluted 1:100 in the diluent and plates were incubated at 37 °C for 1 hour and washed three times with PBS-T. Bound antibodies were detected by incubating with 100 µl rabbit anti-chicken IgY (Sigma-Aldrich, Gillingham, UK) diluted 1:1000 in diluent for 1 hour at 37°C. After three washes in PBS-T, bound antibodies were detected following the addition of 4-Nitrophenyl phosphate disodium salt hexahydrate tablets (Sigma-Aldrich, Gillingham, UK) dissolved in glycine buffer (pH 10.4) with 1mM zinc chloride and 1mM magnesium chloride. Plates were incubated at 37 °C in the dark for 15 min, after which the reaction was stopped with 3M sodium hydroxide and absorbance read at 405 nm using an ELx808 Absorbance microplate reader (BioTek, Potton, UK).

In order to test for antibodies to MDV, samples were required to be tested against both infected and uninfected chicken kidney cells, to rule out cross-reactivity of the sera under evaluation to cellular antigens. To try and minimise the influence of external conditions such as atmospheric temperature, or timing differences on the test results, sera were tested against infected and uninfected cells on two plates run simultaneously.

Table 2.1 Dilution of antigen used in the laboratory-developed ELISA tests

Antigen	Dilution in carbonate buffer
S. gallinarum antigen (6.4µg/ml)	1/80
P. multocida antigen (11.1 µg/ml)	1/80
MDV-infected chicken kidney cell lysate	1/20
Non-infected chicken kidney cell lysate	1/20

2.5.3 Controls and ELISA validation

The commercial kit for the IBD ELISA included positive and negative control sera. For the laboratory-developed assays, negative controls were sera collected from specific-pathogen free birds in the UK. Positive controls for each of the laboratory tests were produced as described below. The performance of the controls for the four assays is shown in the Appendix to Chapter 2.

2.5.3(a) *Pasteurella*

Positive controls were generated by vaccinating four adult white Leghorn birds at the research station in EIAR with an ovine *Pasteurella multocida* vaccine. Birds were given two doses of 300µl subcutaneously 19 days apart, and sera were collected 3 weeks after the second vaccination.

In order to validate the laboratory-developed *Pasteurella* ELISA, it was tested against a commercial kit (Flockchek, Idexx Laboratories, The Netherlands). Twenty field samples at a 1:50 dilution and both the laboratory and the kit positive and negative controls were tested in triplicate on both the commercial and laboratory ELISAs, run simultaneously.

Both the kit and the laboratory negative controls tested negative on both plates. The laboratory positives generated by vaccination were detected as only weakly positive or negative by the commercial kit. Nineteen of the twenty field samples were detected as positive, and the last one gave an equivocal result. All of the positive controls and all twenty field samples gave positive results on the laboratory ELISA (i.e. they had an optical density (OD_{405}) at least three times greater than the negative control). However, the kit positive was detected as being only weakly positive on the laboratory ELISA.

Apart from one equivocal test, both ELISAs showed good agreement in detecting antibody-positive sera. The laboratory test was likely to be less specific than the commercial kit, although this was not possible to demonstrate, as there was a lack of negative field

samples available for testing. However, the laboratory test gave higher optical density readings for samples at the same dilution, and was likely to be more sensitive than the kit, which is particularly developed to evaluate vaccine efficacy. The most positive of the sera from the vaccinated birds was chosen to use as a control for testing the field samples (Positive 1); in addition a second positive control for the laboratory test was made by pooling sera from 4 positive field samples (Positive 2).

2.5.3(b) Salmonella O9 serotypes

An initial positive control was made from pooled sera collected from specific pathogen free chickens infected experimentally with S. Enteritidis in the UK. Pilot samples showed that many field samples had much higher titres than this; thus a new positive serum was selected from among the pilot samples for the testing of the study samples (Positive 1). As this was used up, a second positive was made by pooling positive field sera, and tested alongside the original control sera on 7 plates before being used alone.

2.5.3(c) Marek's Disease Virus

An initial positive control was provided by the Institute of Animal Health, consisting of antiserum against the HPR516 strain of MDV. A positive serum from among the pilot samples was identified and validated as the positive control for the testing of field samples (Positive 1.) Pooled positive field samples were used to make up a replacement positive as the first one ran low (Positive 2) and was run in parallel with Positive 1 on 14 plates.

2.5.4 Plate and sample exclusion

The commercial IBD kit provided guidelines to assess the validity of the plates. These required that:

a) Mean Negative control absorbance must be <0.2
b) Mean Positive control absorbance must be at least 2 times the optical density of the negative control absorbance with a minimum difference of 0.2 OD.

Two IBDV plates were excluded because no positive control was added, and samples were tested again on different plates.

Out of 39 plates accepted, all satisfied the criterion that the positive should be at least 2 times the optical density of the negative, and all satisfied at least one of the other two criteria. One plate had a negative control value which was too high (Plate B9: negative control OD_{562} = 0.3025). Eighteen plates failed to meet the criterion of a minimum difference of 0.2 OD_{562}. This has occurred across all seasons of testing, so appears to be unrelated to substituting NaOH for the stop solution in the first two seasons. There appeared to be a marked difference in colour development between different bottles of substrate, noticeable when two different bottles of substrate solution were used on the same plate. Therefore it may be an issue related to the transport / storage of the substrate reagent. Since it was not possible to retest all these plates due to lack of materials, we have accepted these plates, even though they do not meet the recommended criteria. However, we have adjusted for differences between plates in the analysis, and lowered the manufacturer's recommended cut-off slightly, to account for the relatively low OD_{562} values.

No prior criteria were set for acceptance of the laboratory-developed ELISA tests, with the exception of the MDV ELISA, where positive controls were required not only to have a high OD_{405} against the infected cell lysate, but also to have a low OD_{405} against the uninfected CKCL (Zelnik et al. 2004). We set a minimum difference of 0.1 for the positive control against infected and uninfected cells. Otherwise, for all assays, plates were evaluated against all previous plates as the assays were carried out, and a subjective assessment made as to whether they were valid, based on the normal range of values for their controls (Figure 2.2).

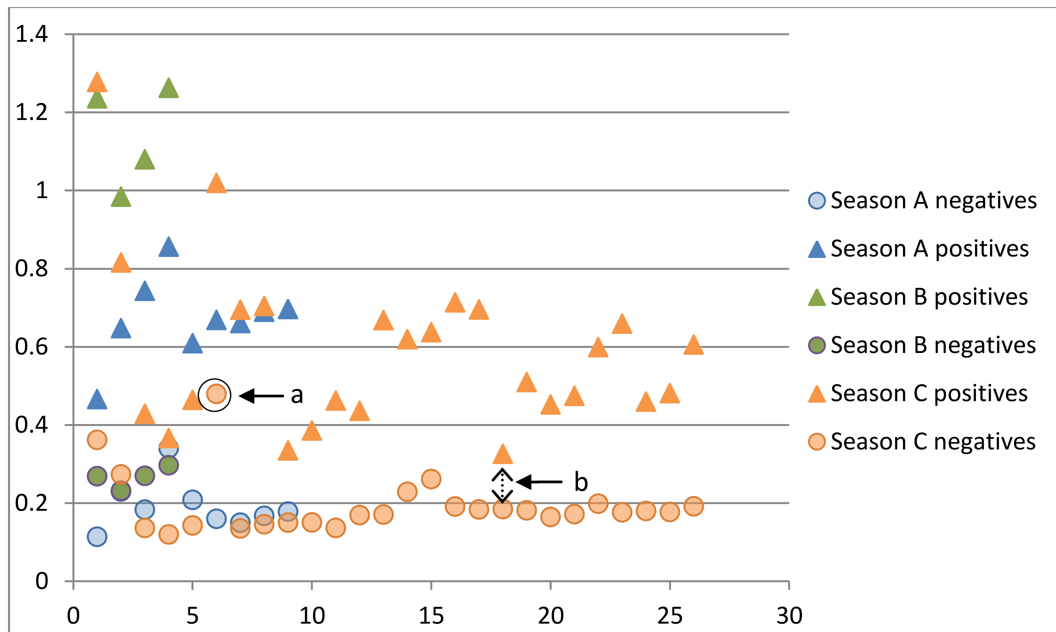

Figure 2.2 Example of the daily log of positive and negative control values for the Salmonella ELISA plates over different batches of testing. The log was used to help determine invalid plates by comparing the control OD values to their normal range. Labels indicate plates which were rejected, because of (a) a high negative control OD and (b) the positive control OD was less than twice that of the negative control.

Four *Pasteurella* ELISA plates were rejected, because the mean positive control absorbance was less than twice that of the mean negative control absorbance, either because of unusually high negative or unusually low positive readings. Two *Salmonella* plates were rejected; one because the negative control OD_{405} was too high, and one because the positive control was less than twice the negative control absorbance. One MDV test plate was rejected, as the OD_{405} of the positive and negative controls was too high against the uninfected cells.

Samples were tested in triplicate where possible, or in duplicate when reagents began to run low. This affected all ELISA tests except *Pasteurella,* and samples from all regions and seasons of collection, with the general exception of those collected in the third season. The co-efficient of variation (CV) was calculated for all samples and all those with a CV >25%

were retested. Where samples were tested in triplicate, one replicate may be excluded if it had an absorbance noticeably different from the other two readings, if this would bring the CV below 20%.

The test for MDV antibodies required that samples be tested against lysates of both virus-infected and uninfected chicken kidney cells; the OD_{405} against the uninfected cell lysate was then subtracted from the OD_{405} against the infected lysate to give a net value which was used in further calculations. Samples with a net value greater than 3x the standard deviation of the net values (after accounting for the effect of the different test plates) were deemed to be cross-reacting to the cells in which the virus was grown and were discarded from further analyses.

2.5.5 Newcastle Disease Haemagglutination Inhibition (HAI) assays

2.5.5(a) Test procedure

HAI assays were carried out according to the procedure described in the OIE Terrestrial manual (2009). Briefly, 250µl of PBS was dispensed into each well of a V-bottomed microtitre plate, 250 µl of test sera were dispensed into the first well of each row, and two-fold dilutions were made across the plate. Dilutions were made up to 1/64, in order to be able to test more samples per plate. Four haemagglutinating units (HAU) of virus in 250µl of PBS were then added to each well, and plates were incubated for 30 minutes at room temperature. A volume of 250µl of a 1% solution of chicken red blood cells (RBCs) was then added to each well, gently mixed and allowed to settle for 45 minutes at room temperature.

Agglutination was read by gently tilting the plates, and the haemagglutination (HI) titre was the highest dilution of serum which caused complete inhibition of 4 HAU of virus. A line of 6 red blood cell control wells, containing 250µl of a 1% chicken RBC solution and 500µl of

PBS, were included on each plate, and only those wells in which red blood cells streamed at the same rate as the control wells were considered to show inhibition.

A virus control was also included on each plate. This consisted of twofold dilutions of the virus suspension in volumes of 250µl, plus 250µl of PBS and 250µl of chicken RBCs. This was included to confirm the virus solution had been correctly titrated and was able to agglutinate chicken RBCs. Positive and negative control sera were also included on each plate. Plates were rejected if the negative control gave a titre > 1/4, if the positive control was not within one dilution of the known titre, if the virus was not within one dilution of the expected titre, or if the red blood cell control did not stream. Samples were considered as positive if they inhibited agglutination at a dilution of 1/16 or above.

2.5.5(b) Validation

Inter-reader variation was assessed for the two main readers who carried out the HAI assays. This consisted of both readers being asked to read four plates independently of each other and provide the titre for each of the 12 samples on each plate. I made a comparison of the two scores, and where the two scores differed, I and one other observer also read the plates and allocated a third score.

The four readings of the positive control agreed either exactly or to within one dilution. However, Reader 1 scored the negative control two- to fourfold higher than Reader 2, and a similar pattern was seen for the readings of the test samples. Where Reader 1 had classed a sample as negative, there was good agreement between the two scores. However, of the 10 samples which Reader 1 classed as positive, none were scored as positive by Reader 2, or by the third arbitrator. Where there was disagreement between the two readers, the third score invariably agreed with Reader 2’s assessment of the titre.

In addition, we were able to test 17 samples with an NDV ELISA test (Biocheck, Hounslow, UK). This confirmed as negative 12 samples which both readers had scored as negative.

One sample which both readers scored as positive was not classed as positive by the ELISA. One sample which Reader 2 had classed as positive, and Reader 1 had classed as equivocal was classed as positive by the ELISA, and one sample which Reader 1 had classed as positive and Reader 2 as negative was classed as negative by ELISA. Two samples tested by ELISA were read by only one reader, of which the ELISA agreed with the negative reading by Reader 2, but disagreed with the positive reading by Reader 1.

From this, it was evident that there was considerable subjectivity in the reading of the test, and Reader 1's assessment appeared to be much less specific than Reader 2's interpretation. In order to try and establish a consistent measurement of samples, it was therefore decided to accept only the samples scored as negative by Reader 1, and for Reader 2 to retest all samples which Reader 1 had given a titre 4 or greater, providing there was sufficient serum left to do so.

2.6 Laboratory testing - Faecal Examination

Faecal samples were examined for *Eimeria* oocysts and nematode and cestode eggs using a modified version of the concentration McMaster technique (Permin and Hansen 1998). Due to the small volumes of faeces for individual birds and the necessity, in some cases, of keeping part of the samples for later DNA extractions, it was not possible to use the recommended 4g of faeces. Counts were therefore done on 1g of faecal material from each bird.

1g of faeces was suspended in 14ml of water. Where necessary, this was soaked for a few minutes to break up hard clumps: The mixture was then stirred, and put through a strainer, and the whole of the resulting suspension was centrifuged at 1200 RPM for 7 minutes. The supernatant was removed, and the sediment re-suspended in 6ml saturated salt solution shortly before counting. Two chambers of a McMasters slide were filled with the faecal

suspension and each species counted separately. The total number in two chambers for each species was multiplied by 20 to give the resulting eggs per gram.

Initially, eggs were tentatively identified to genus or species level, by using published keys (Lapage 1956, Soulsby 1982, Permin and Hansen 1998) and a selection of photographs were sent to an expert in the field, to confirm identification as far as possible. Because of the difficulty in identifying a number of eggs, it was decided to concentrate only on nematode eggs which could be attributed either to *Ascaridia galli* or *Heterakis gallinarum,* although it was not possible to confidently differentiate the two species. Although other types of nematode eggs were seen in low numbers, there were no other frequently occurring species. Eggs identified as being of the class *Cestoda* were also grouped as a single entity, as were oocysts of the *Eimeria* genus.

2.7 Ectoparasite Identification

Ectoparasite samples were of two types: scrapings from birds observed to have hyperkeratosis of the legs and/or feet, and arthropod specimens directly observed during examination. Skin scrapings were cleared by placing them on a slide with a drop of 10% potassium hydroxide for 10 minutes. They were then examined microscopically for the presence of *Cnemidocoptes* mites. Other arthropod specimens were examined using light microscopy to identify the species using published keys. (Soulsby, 1982; Walker, 1994; Price and Graham, 1996; Taylor, et al., 2007; Price et al*.,* 2003; Emerson, 1956; Matsudaira and Kaneko, 1969; Marshall, 2003; Ledger, 1980; Osborn, 1896; Tuff, 1977; Bowman, 1999; Wall and Shearer, 2001; Permin et al*.,* 2002). A stage micrometer was used to measure individual specimens to assist in identification. A photographic record of specimens was made using a digital camera (Digital Sight DS-L1, Nikon Instruments Inc., USA) mounted onto the compound microscope or a low magnification USB digital microscope (Traveler

USB Microscope, Sertonics Ltd., UK) for viewing at 40-200x magnification. A subset of samples was sent to an expert in livestock parasitology at the University of Edinburgh for confirmation of the putative classification assigned by the student undertaking the project.

2.8 Data handling and statistical methods

2.8.1 Database management

Data for the various components of the project (kebele statistics, socio-economic data, household locations, flock management, disease experience, reproductive traits, phenotypic traits, bird examinations, laboratory data, genetic single nucleotide polymorphism (SNP) data, ectoparasite identifications and follow-up data at household and bird level) were managed independently by the people who had a primary interest in or responsibility for each area (Table 2.2). Microsoft Excel spreadsheets were used to input data by all parties, and each was responsible for checking data quality. Coded labels linking laboratory and questionnaire data were used throughout, in order to subsequently link data collected from each household with its bird occupants. However, for convenience, the socio-economic and genetic researchers used their own codes in parallel with the linking codes for identification.

For data collected during the fieldwork, a random selection of 60 bird questionnaires and 32 household questionnaires were checked for accuracy of data entry. Each variable was also examined to detect patterns in missing data, or any outlying values, which were checked against the original questionnaires. Descriptive variables, such as veterinary and ethnoveterinary treatments or peculiar clinical findings were grouped thematically, to examine whether new variables should be created to describe these characteristics, and to ensure consistency among the descriptive terms used.

After checking and cleaning, data from the spreadsheets was imported into a database (Microsoft Access, 2010) where different spreadsheets were linked. Database queries were then set up to retrieve specified information for different research questions, which were exported and stored as Excel spreadsheets (Figure 2.3).

Data exploration, tabulation and graphical summaries, statistical tests, regression modelling, ordination analyses, mapping and spatial tests were all performed using R software (R Development Core Team, 2008).

Table 2.2 People contributing to different data types

Data type	Primary responsibility	Data input
Kebele statistics	Takele Taye	Takele Taye
Socio-economic data	Zelalem Terfa	Zelalem Terfa
Household locations	Judy Bettridge	Judy Bettridge
Flock management Disease experience Bird examinations Follow-up bird data Follow-up household data	Judy Bettridge	Judy Bettridge Eskindar Aklilu Stacey Lynch Ian Tuerena Lisa Liu Becca McKown Sally Hutton Alex Harvey Emmanuel Sambo
Laboratory data	Judy Bettridge	Stacey Lynch Judy Bettridge Marisol Collins Camilla Brena Kasech Melese
Reproductive data	Eskindar Aklilu	Eskindar Aklilu Judy Bettridge
Phenotypic data	Takele Taye	Takele Taye
Genetic SNP data	Takele Taye	Takele Taye
Ectoparasite identification	Marisol Collins	Marisol Collins

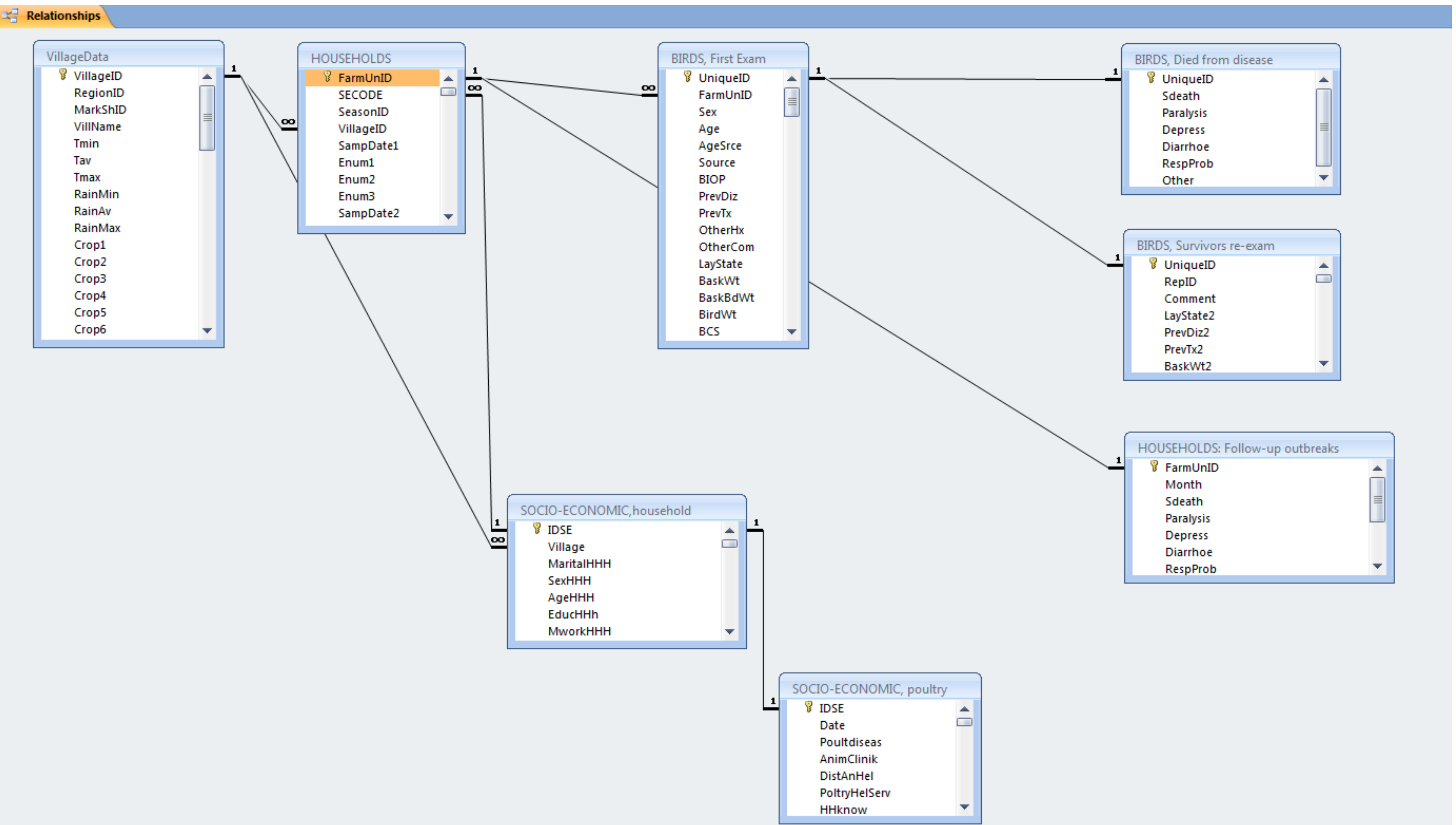

Figure 2.3   Access database layout, showing different data links

2.8.2 ELISA data transformations

Optical densities for all of the ELISA samples are converted into a ratio to the positive control (s:p ratio) using Equation 2.1.

$$\text{s:p ratio} = \frac{\text{Mean sample OD} - \text{Negative control OD}}{\text{Positive control OD} - \text{Negative control OD}} \tag{2.1}$$

This is designed to make values comparable between plates by allowing all values to be expressed on a scale where 0 is equal to the negative control and 1 is equal to the positive control, and normally a cut-off value will be designated in order to discriminate positive and negative samples. Figure 2.4 shows the distribution of s:p ratios for the commercial IBD ELISA, with the manufacturer's recommended cut-off value of 0.306. There is a clear "shoulder" in the distribution, with the negative and positive controls either side of this, allowing a cut-off to be easily determined.

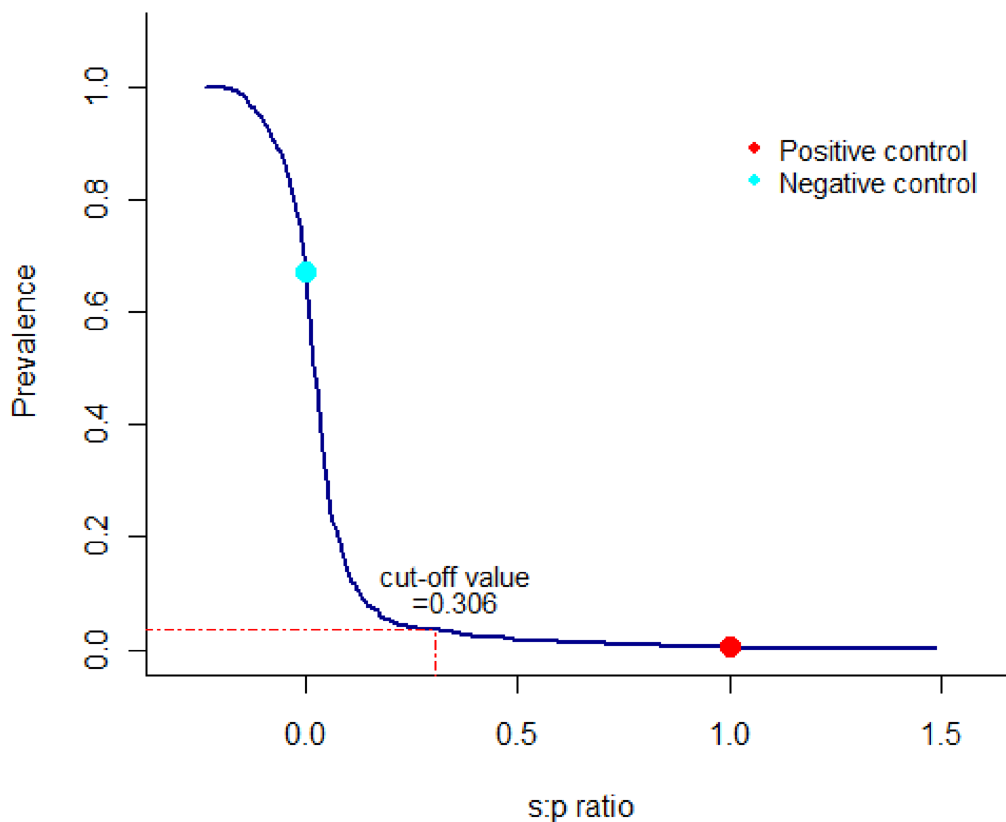

Figure 2.4 Distribution of IBD s:p ratios, showing the placement of the cut-off, and the negative and positive controls.

For the ELISAs that were developed within the laboratory, determination of a cut-off value was much more difficult, as neither a panel of negative sera were available with which to validate the tests, nor a "gold-standard" test with which to compare our results. Plotting the distribution of the s:p ratios did not demonstrate a clear "shoulder" in the region between the negative and positive controls, but showed a smooth, continuous distribution of the range of values. Thus even a small change in the cut-off value chosen resulted in a very different estimation of prevalence for the *Salmonella* and *Pasteurella* in particular (Figure 2.5), both of which showed a long tail of samples with optical densities that were many times higher than the positive controls.

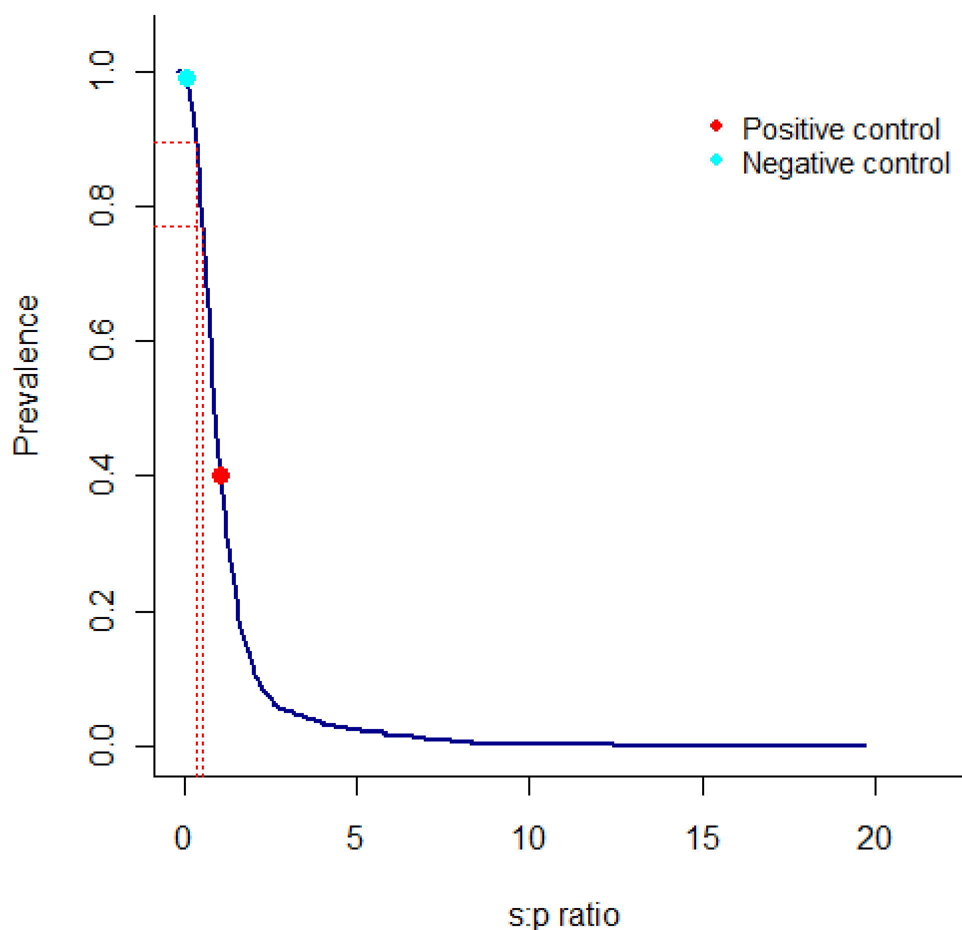

Figure 2.5 The change in prevalence with the alteration of the cut-off value for the Salmonella data

For the *Salmonella,* the explanation for these very high samples may be to do with the different subspecies of *Salmonella* which would induce antibodies which would be detected by the assay. The O9 serotype includes both *S.* Enteritidis and *S.* Pullorum, which induce quite different antibody profiles (Chappell et al. 2009). The positive control used was from an *S.* Enteritidis-infected bird, but *S.* Pullorum infection may induce much greater antibody responses (Chappell et al. 2009). The latter may also be maintained in a carrier state, so high antibody titres may be sustained over a longer period. *Pasteurella* can also induce a carrier state, although in poultry the immune responses to this infection are largely unstudied (Wigley 2013).

Due to both the difficulty in determining cut-off values for the in-house ELISAs and the loss of potentially valuable information from dichotomising the continuous data, we elected to work with the s:p ratios when trying to make comparisons in exposure between birds, areas or seasons. However, there were two issues with the raw data. Firstly, as new positive controls had to be introduced for later plates, it was necessary to estimate a value for the original control on a small number of plates (1 *Salmonella* and 8 MDV plates), in order to have a consistent benchmark scale with which to compare samples. These estimates were made using predicted values from a regression model which used the negative and second positive controls to predict the value of the first positive from all the plates where the two controls had been run in parallel.

Secondly, due to the high variation in control readings between plates, (see Appendix 1) we found that the s:p ratio conversion (Equation 2.1) was inadequate by itself to allow us to compare readings made on different plates, as the sample values were strongly influenced by the control values of individual plates. If the positive or both control readings were unusually high, samples tended to have lower s:p ratios, whereas if the difference between the controls was small, then s:p ratios would be correspondingly greater (Figure 2.6).

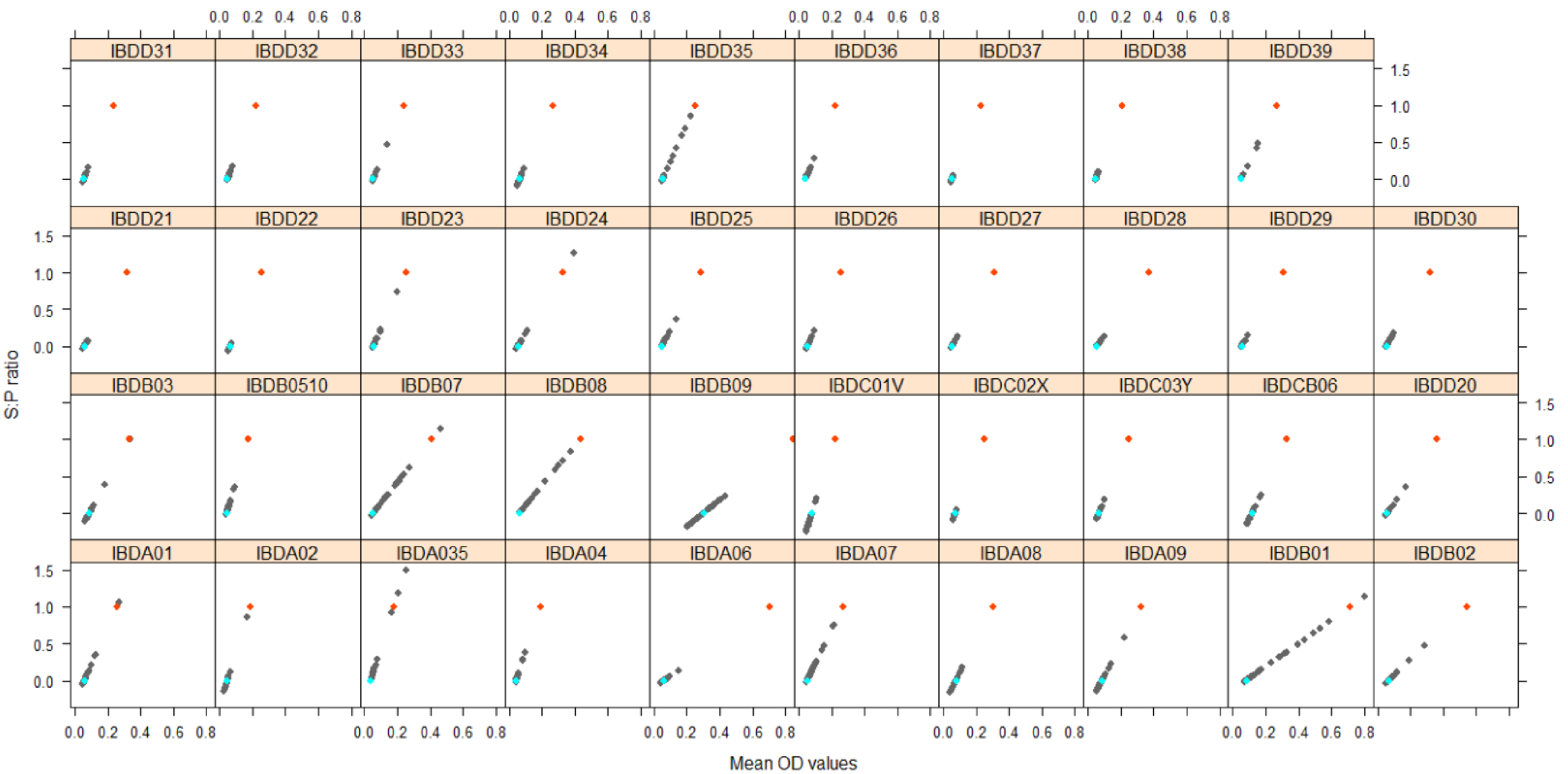

Figure 2.6 Sample OD values and s:p ratios for each plate for the IBD ELISA

Where some samples were tested on more than one plate to assess continuity, it was found that the greater the optical density of the sample, the more variation there was between s:p ratios for the same sample calculated from different plates. In order to adjust for between-plate variation, plate was included as a factor in analyses of the ELISA data.

2.8.3 Multilevel random-effect models

Multilevel models are used when data has a complex, often nested or hierarchical structure. Individuals drawn from the same group might be expected to be more alike than those from different groups, therefore this clustering needs to be taken into account when analysing the data. Random effects models account for this clustering by estimating the variance between groups in the population. The groups themselves are assumed to be a random selection from a larger population. The simplest multilevel model with a single explanatory variable is

$$y_{ij} = \beta_0 + \beta_1 x_{ij} + u_j + e_{ij} \tag{2.2}$$

where y_{ij} is the value of y for the i th individual in the j th group. This model assumes an overall linear relationship between the explanatory variable x and the response variable y. This relationship is represented by a line with intercept β_0 and a slope β_1. However, the residual u_j represents the difference between the group mean for the individuals in group j from the overall mean, and so the intercept for group j is given by $\beta_0 + u_j$. The group residuals are assumed to come from a normal distribution with mean zero and variance σ_u^2 (Summarised from Steele (2008)).

Our data have two main structures; samples within plates and chickens within households (which are further nested within kebeles, market sheds and regions). I have used multilevel modelling throughout this thesis to explore various types of data and investigate potential relationships. An outline of the processes I have used, and how the models have been adapted for various data types follows.

2.8.3(a) Adjusting for variation in the ELISA data

Early explorations suggested that the plate on which a sample was tested was a source of important variation, for the reasons described above. To account for this, in all models where ELISA s:p ratio is a response variable, plate is included as a random effect. However, where s:p ratios are used as explanatory variables for another outcome (such as bird survival, or a different infection), a different approach was required, as the large number of plates precluded this being fitted as an interaction term.

Before fitting s:p ratios as explanatory model variables, they were first adjusted using a random-effects intercept-only regression model with plate as a random effect.

$$\text{s:p ratio}_{ij} = \beta_0 + u_{\text{plate } j} + e_{ij} \qquad (2.3)$$

The bird-level residual e_{ij} was then taken as an estimation of the individual's antibody response. This has the effect of decreasing the variation estimated to be attributable to the plate, and also re-centres the distribution around 0 (Figure 2.7). This residual value for each ELISA I have called the adjusted s:p value.

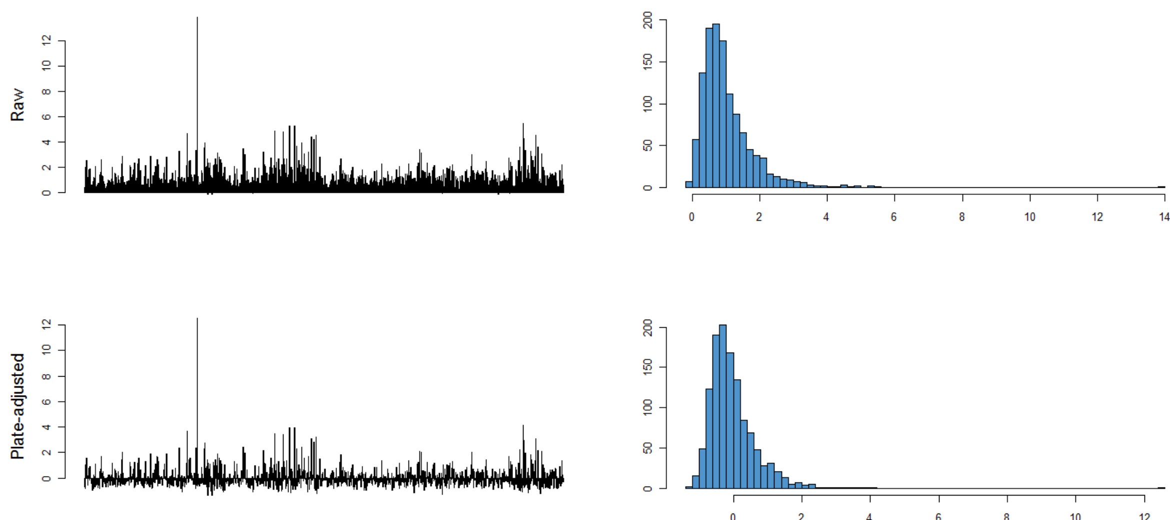

Figure 2.7 Values for the Pasteurella s:p ratios before (top) and after (bottom) random-effect modelling to adjust for between-plate variation.

2.8.3(b) Adapting models to different data types: Binomial and multinomial outcomes

(References summarised from Steele (2008) and Faraway (2006)).

For response data which is not continuously distributed but can only take one of a fixed number of outcomes, the linear model is not appropriate. If we have a binary outcome, the response variable Y_i for $i = 1,...,n_i$, where n is the number of independent trials, can be modelled using the binomial distribution

$$p(Y_i = y_i) = \binom{n}{y_i} p_i^{y_i}(1 - p_i)^{n_i - y_i} \tag{2.4}$$

This distribution has a mean np and a variance np(1-p), which is not constant, but varies with the mean. Because of this, and the fact that probabilities are strictly bounded between 0 and 1, the linear regression model, which requires constant variance and predicts probabilities outside these boundaries cannot be used to predict the response variable directly. However, if the individual trials which comprise the response Y are subject to the same q predictors, then a linear predictor can be modelled

$$\eta = \beta_0 + \beta_1 x_{i1} + ... + \beta_q x_{iq} \tag{2.5}$$

The linear predictor is transformed to give p by means of a link function. We have used the logit link function

$$\eta = ln\left(\frac{p}{1-p}\right) \tag{2.6}$$

as this is most easily interpretable in terms of an odds ratio. Odds give the probability of an event happening compared to the probability of it not happening; therefore η gives the log odds of an event. Using equation 2.5, it can be seen that a unit increase in x_{i1}, if all other variables are held constant, will increase the odds of an outcome by exp β_1.

Multinomial outcomes, where response data are nominal rather than ordered, are similar, but have the requirement that the probabilities for each individual outcome must add up

to 1. However, individual components of the multinomial response may also be considered as Poisson responses if the total number of trials is sufficiently large. The Poisson distribution usually describes count data, when the number of times something happened is known, but the number of times it did not happen is unknown. This is useful for modelling rates of an outcome during a fixed time period and responses take the form of positive integers. Multinomial responses can be treated as individual Poisson responses if a **response factor** is introduced, which has an observation for each level of multinomial outcome. This acts like a dummy variable, such that for each set of observations for an individual, one response will be one and the rest zero. The explanatory variables are replicated for the individual for as many response categories as required.

Using our data on the outcomes for chickens at the follow-up visit as an example, a chicken could be assigned to one of the categories, “Survival”, “Died of disease”, “Used for gain” or “Lost to non-disease causes”. These categories were mutually exclusive (i.e. a bird could not be assigned to more than one) and non-ordered. Table 2.3 shows how the data is reorganised from a multinomial response to allow it to be analysed using a Poisson log-linear model.

As with the binomial models, the regression gives a linear predictor, which is transformed to give an estimate of the mean μ of the Poisson-distributed outcome, using the log link function

$$\beta_0 + \beta_1 x_{i1} + \ldots + \beta_n x_{in} = \eta_i = ln(\mu_i) \quad (2.7)$$

In other words, the log outcome rate can be expressed as a linear function of the set of explanatory variables. The equation can be rearranged to give

$$\mu_i = (e^{\beta_0})(e^{\beta_1 x_1}) \ldots (e^{\beta_n x_n}) \quad (2.8)$$

Table 2.3 Data reorganisation from a multinomial response to allow Poisson log-linear modelling.

Unique ID	Region	Sex	Survival	Disease	Gain	Loss
HA1A01A	H	M	0	0	0	1
HA1A02A	H	M	1	0	0	0
HA1A02B	H	F	1	0	0	0
HA1A04A	H	M	0	1	0	0
...	...	...	...	...	...	...

Unique ID	Region	Sex	Response category	Y
HA1A01A	H	M	Survival	0
HA1A01A	H	M	Disease	0
HA1A01A	H	M	Gain	0
HA1A01A	H	M	Loss	1
HA1A02A	H	M	Survival	1
HA1A02A	H	M	Disease	0
HA1A02A	H	M	Gain	0
HA1A02A	H	M	Loss	0
HA1A02B	H	F	Survival	1
HA1A02B	H	F	Disease	0
...	...	...	...	...

This equation can be used to estimate the relative risks for the explanatory variables. If, for example we wished to estimate the relative risk of having 30 lice compared to 5 lice, this could be written as

$$e^{\beta x30} \div e^{\beta x5} = e^{\beta x25} \qquad (2.9)$$

For the multinomial responses, each outcome is modelled as a function of the interactions between the explanatory variables with the response category for that variable. Thus, for our example here, the rate of survival of Males in Horro would be given by the equation

$$Y_{Survival} = \left(e^{\beta_{Survival}}\right)\left(e^{\beta_{Male,\ Survival}}\right)\left(e^{\beta_{Horro,\ Survival}}\right) \qquad (2.10)$$

As both the explanatory variables here are categorical, the exponential of the coefficients gives the relative rate compared to the reference category for that variable. Where the variables are continuous, the exponential of the coefficient shows the relative rate change for every unit increase in that variable.

2.8.3(c) Selection of model variables

When considering variables to include in the models, I started by considering all possible variables which I might expect to have a potential influence on the outcome of interest, and how I might expect them to influence each other. Explanatory variables were checked for potential correlations with each other using tabular and/or graphical summaries, correlation coefficients and chi-squared tests. Univariable regression models were constructed to check for relationships between the explanatory variables and the outcome and, for continuous variables, Generalised Additive Models (GAM) were used to examine whether the relationship could be adequately modelled with a straight line, or whether variables needed to be re-defined in order to better describe the relationship.

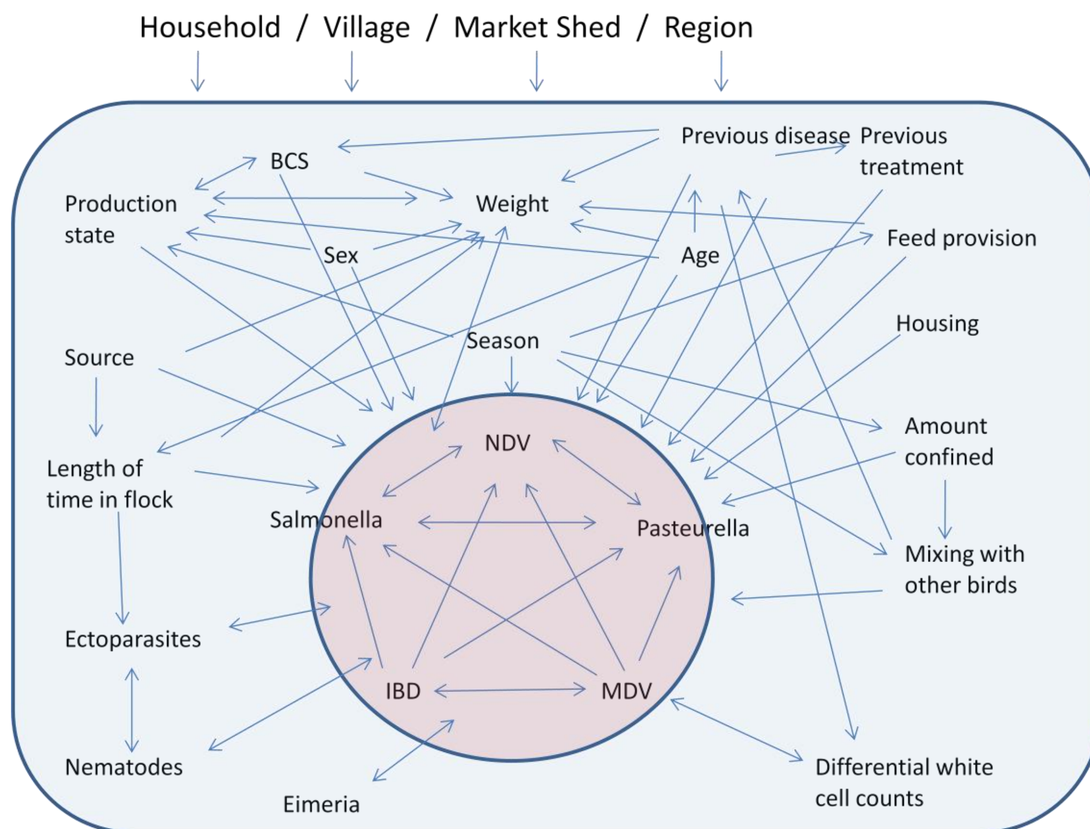

Figure 2.8 Drawing of potential relationships between variables

Before constructing multivariable models, I sketched diagrams for each outcome such as the one shown in Figure 2.8, to help visualise potential networks of variables. This assisted in controlling for potential confounding variables during the model selection process. Models were constructed using a stepwise process, in which individual variables were added to the null model one by one, based on knowledge of what might be expected to be of greatest biological significance. Interactions were tested with other variables. Non-significant variables were removed, including variables which later became non-significant when additional variables were added to the model. Likelihood ratio tests and the Akaike Information Criterion were also used to judge whether the addition of more variables significantly improved the fit of the model.

2.9 Mapping and spatial correlations

Mapping of household points was carried out using the RGoogleMaps package in R (Loecher 2011), which allowed a satellite map of the terrain in a grey scale to be downloaded and points plotted according to their geographical coordinates. The package adjusted for map projections, and so gives a more precise location than using latitude and longitude values on a two-dimensional scatterplot.

After performing some of the random-effects regression models, particularly those looking at an infection outcome, the household-level residuals were plotted on maps. This allowed a visual examination of where those households with unusually high or low infection values might be situated. A spatial test statistic (Eagle, unpublished) was used to assess whether the variation in households over a range of distances was less than might be expected compared to a random distribution of the data points, which might suggest spatial clustering of the data.

2.10 Principal component analysis and redundancy analysis

Analyses were performed using R software (R Development Core Team, 2008) using the vegan package (Oksanen et al. 2011), and according to the methods described by Borcard (2011).

Principal component analysis (PCA) is a technique for reducing a data set of p random variables such that the majority of information – the variances of each of the p variables and the correlations or co-variances between the variables – is preserved in a smaller number of derived variables. It is a purely descriptive method, used to represent the main trends of the data, which can then be visualised. The first PCA axis explains the largest variance or, when fitted as a straight line to the data, gives the least total residual sums of squares but, unlike explanatory variables in multiple regression, it is not derived from measured data but a theoretical variable estimated from the outcomes alone. If a single variable does not adequately capture all the information in the data, a second axis is fitted, uncorrelated with the first axis, which also best captures the information. Further axes may be added, subject to the same constraint, that they are uncorrelated with all higher axes, up to the number of variables in the dataset. However, in practice, higher axes that explain only small amounts of variation are generally ignored (Zuur, Ieno and Smith 2007). The Kaiser-Guttman criterion, which compares each axis to the mean of all eigenvalues, suggests that only those axes greater than the mean should be interpreted and others are essentially random variation (Borcard 2011).

Redundancy analysis (RDA) combines regression with principal component analysis (Borcard 2011). It regresses a matrix of centred response data on a second matrix of explanatory variables and the fitted values are combined in a matrix. A PCA of the fitted values produces canonical eigenvalues and a matrix of canonical eigenvectors, which can be used to calculate the coordinates of the sites, either in the space of the original

response variables, or in the space of the explanatory variables (i.e. on the fitted response variables). The residual values from the multiple regressions can also be submitted to a PCA to obtain an unconstrained ordination of the residuals.

The adjusted R^2 value was used to test whether the inclusion of explanatory variables was a significantly better fit than the null model, and a forward selection process was used to select those significant variables which explained the greatest proportion of the variance in the response data (Borcard, 2011). Permutation tests were used to test how many RDA axes explained a significant proportion of the variation. The Kaiser-Guttman criterion was applied to unconstrained axes, to determine those which explained variation of interest. Biplots and triplots were produced, which were scaled according either to the distances between observations (scaling 1 in the vegan package) or the correlations between variables (scaling 2).

2.11 Discussion

The main laboratory and statistical methods have been described here in detail, and only the most relevant aspects will be highlighted where they recur in subsequent chapters of this thesis. Other methods, where used, will be described in the appropriate chapters. The aim is to prevent unnecessary repetition, but also to highlight some of the difficulties encountered with some of the laboratory data, and explain the rationale for some of the decisions taken in the best way to handle the serology results in the statistical analysis, as this is highly relevant to some of the findings in later chapters.

The major issues were generally related to high variability in the serology results. The likely causes of these include degradation of reagents, arising from transport and storage issues where a cold chain could not be maintained; variation in water quality, as the laboratory in

Ethiopia had filtered, but not purified water; high variation in ambient temperatures in the laboratory and operator variation. The decision to carry out the laboratory testing in Ethiopia was made partly to avoid the difficulty of importing samples which could potentially include unknown pathogens into the UK, but mainly to provide some diagnostic capacity within the agricultural research centre. Thus problems with setting up and running a laboratory in a less economically developed country, such as acquiring reagents, and maintaining a regular water supply were largely unavoidable. One of the functions of the laboratory was to provide training for students and technicians in the performance of diagnostic tests; therefore the large number of operators running the tests was also inevitable.

The decision to use serology, rather than identification of infectious agents, was also a practical decision, as almost all reagents are available within country, tests are cheap to run, and the issue with collecting and transporting samples from remote field sites is far less of a problem for sera, which are relatively stable. In ideal circumstances, the ELISA tests would have been validated by testing them against a panel of known negative and positive sera. Unfortunately, it proved impossible to find birds or sera in Ethiopia which were known to be negative, and only a very limited amount could be brought from the UK. This is clearly an issue for other researchers and diagnostic facilities in Ethiopia, and may limit the development of local diagnostic capacities until this issue is addressed.

With regard to our data, the inability to validate the tests was a major factor in the decision to use the continuous data, rather than dichotomising it. However, it is also true that the continuous data may provide some additional valuable information, for example in the case of the *Salmonella* ELISAs, where high values potentially represent infection with a different serovar. I have tried as far as possible to control for some of the variability introduced by the test itself, by fitting the test plate as a random effect in regression analyses, although as

there was often spatial and temporal correlation between the samples tested on the same plate, this method may potentially underestimate the effects of kebele and sampling season, attributing them instead to between-plate variation.

Appendix 2.1 Summary of ELISA testing and performance of control sera

IBD		Negative control	Positive control
• 1310 tests		All plates	All plates
• 39 plates	Mean	0.066	0.319
• 21 days over an	Standard deviation	0.042	0.149
18-month period	Range	0.039 – 0.303	0.172 – 0.849

Pasteurella		Negative control	Positive 1	Positive 2
• 1319 tests		All plates	All plates	40 / 46 plates
• 46 plates	Mean	0.226	1.036	1.105
• 33 days over a	Standard deviation	0.071	0.349	0.258
9-month period	Range	0.136 – 0.424	0.319 – 2.094	0.556 – 1.498

Salmonella		Negative control	Positive 1	Positive 2
• 1244 tests		All plates	37 / 38 plates	8 / 38 plates
• 38 plates	Mean	0.195	0.664	0.75
• 17 days over an	Standard deviation	0.058	0.242	0.140
18-month period	Range	0.11 – 0.36	0.34 – 1.28	0.50 – 0.86

MDV		Negative control	Positive 1	Positive 2
• 1282 tests	Infected cells	All plates	24 / 32 plates	22 / 32 plates
• 32 pairs of plates	Mean	0.189	0.577	0.896
• 17 days over a	Standard deviation	0.055	0.146	0.270
2-month period	Range	0.130 – 0.360	0.340 – 0.930	0.520 – 1.420
	Uninfected cells			
	Mean	0.197	0.260	0.354
	Standard deviation	0.076	0.068	0.094
	Range	0.100 – 0.480	0.180 – 0.400	0.240 – 0.570

Chapter 3

Chicken keeping in two highland regions of Ethiopia:

Farmer practices and bird characteristics

3.1 Introduction

The Ethiopian Ministry of Agriculture states that its vision for Ethiopia is to "Create market-led modern agriculture and a society free from poverty" (http://www.moa.gov.et/web/pages/vision-mission-and-objective). The rising demand for poultry products in Ethiopia's growing and increasingly urbanized population should be a market opportunity for rural households which raise the indigenous poultry preferred by most Ethiopians. Indigenous birds are perceived as having superior taste and quality of both the eggs and meat that they produce (Dana et al., 2010), and in addition have a number of other characteristics which make them better able to survive and reproduce successfully in an extensive scavenging system. From a smallholder farmer's point of view, the profitability per unit of money invested in village chickens is higher than for commercial birds, despite the latter's greater outputs in terms of eggs and meat. Whereas feed for commercial birds must be purchased, indigenous birds find their own feed, and so have a better benefit/cost ratio (Bell, 2009).

However, the system under which village chickens are currently kept imposes many diverse constraints to their productivity. Disease, predation, lack of feed, water and shelter; and poor access to veterinary services are most frequently reported to restrict the numbers of village chickens kept (Halima et al., 2007, Dessie and Ogle, 2001, Dinka et al., 2010); whilst slow growth and low egg outputs limit their profitability (Dessie and Ogle, 2001), the problem being compounded by poor access to markets (Moges et al., 2010b). High bird, and particularly chick, mortalities mean that a large proportion of what would otherwise be potentially saleable eggs and birds are required to be hatched and raised for replacement stock. In order to take advantage of growing markets, rural households need to be able to increase the offtakes from their flocks, whilst still utilising the natural advantages of the

scavenging system. Kitalyi (1998) recommended a step-wise program starting with the basic indigenous stock, and initially developing practices including good hygiene, shelter, preferential treatment of chicks and control of the most devastating diseases, such as Newcastle disease virus (NDV). Later improvements in the management include developing feeding and other disease control programmes, followed by improving the stock by introducing high-yielding traits and developing marketing strategies.

An approach adopted by the Ethiopian Agricultural Research Institute (EIAR) has focused on the use of selective and cross-breeding programmes to improve the productivity of indigenous chickens both in terms of increased egg and meat production (Dana et al., 2011). The aim of this programme is to to produce a dual-purpose bird specifically intended for village systems by utilising the genetic merits of the indigenous breeds. However, birds in the breeding programme are maintained in an intensive system, i.e. in housed conditions with a supply of commercial feed, and reliance on vaccination for disease control. There is therefore some concern that, when reared under village conditions, the "improved" indigenous birds will continue to require unsustainable investments in terms of vaccination or feed inputs in order to sustain the higher levels of productivity. Although growth and egg yield are important characteristics, "adaptation", comprising disease and stress tolerance, flightiness (conferring ability to escape predators) and scavenging vigour, is recognised by Ethiopian farmers to be the most important trait of chickens kept in village systems (Dana et al., 2010). From this it may be seen that farmers well recognise the diverse array of pressures which act on the village chicken; disease, climate, predation and nutrition are important selection pressures in these birds as they would be in a wild population, whilst in addition the village chicken is also subject to the selection choices made by farmers over generations to fulfil their own requirements for desirable bird characteristics. These characteristics are frequently dictated by market demands, and include factors such as comb type and plumage colour, as well as factors which can be more directly linked to

productivity, such as size, broodiness and mothering ability (Moges et al., 2010a, Dana et al., 2010).

The extensive system in which village birds are kept exposes them to numerous potential sources of infectious disease; biosecurity is minimal and, as yet, vaccination programmes are not widely available for rural farmers in Ethiopia (Dana et al., 2010, Moges et al., 2010b). Management of disease clearly needs to be incorporated into the current poultry development programmes in order for them to succeed. Veterinary studies on village chickens in Ethiopia to date have tended to focus on identifying exposure to or infection with various pathogens in healthy birds, sometimes examining bird signalment, management or environmental variables as potential risk factors for infection (for examples, see Mekuria and Gezahegn (2010), Jenbreie et al. (2012), Chaka et al. (2013). Several other studies have described the village chicken characteristics and their management in various regions of Ethiopia in great detail (Dessie and Ogle, 2001, Duguma, 2009, Dinka et al., 2010), yet whilst these all mention disease as a major constraint, none have addressed factors associated with an increased disease risk.

The gap between those studies focused on pathogens and those studies focused on birds and their production system needs to be addressed, as there is a difference between "infection" and "disease", which is not considered by many researchers. The veterinary studies' identification of clinically healthy infected and seropositive birds within household flocks demonstrates that many birds are resisting or tolerating these infectious pathogens. The manifestation and outcomes of clinical disease, on the other hand, will be affected by many factors; not only the pathogens to which chickens are exposed, but also the innate and acquired characteristics of the birds themselves which dictate how the bird responds to an infecting pathogen. This interaction is, in turn, influenced by decisions the farmers

make about the management of their birds, which shape both the chicken population and how birds can interact with the production environment.

The aim of this study was to describe the village production system as an agroecological system with the potential to be inhabited by micro- and macroparasites of chickens; as many of the management practices previously reported have the potential to be risk factors for exposure to various pathogens, or to alter the interaction between chicken and pathogen. To address this, we collected data from Ethiopian farmers on the management of chickens and how they contribute to the economics of smallholder production, and also studied various characteristics of the birds themselves. Two geographically separate regions in Ethiopia, expected a priori to have some differences in their approach to chicken keeping, were studied to encompass at least some of the differences in social, cultural, economic and productivity constraints.

Objectives were:

1) To describe the diversity of the village production systems, farmer behaviour and motivators.

2) To better understand how the different local ecotypes are adapted to their specific environments and farmer requirements.

3) To hypothesise how any observed variations in the production system may have a bearing on infection dynamics or biology.

3.2 Methods

3.2.1 The study areas

This study was conducted in two geographically distinct woredas (administrative districts) within the Oromia region. Oromia is the largest administrative region in Ethiopia and includes 180 woredas, which are further divided into kebeles. These are the smallest administrative districts in Ethiopia and often encompass a number of proximate communities or villages. Kebeles may be loosely grouped within market sheds, which supply chicken or chicken products to a sub-regional market or urban centre (Tadelle et al., 2003). Market sheds are not simply the geographical areas and population surrounding one or more markets, but rather describe the flow of products along a network (FAO, 2014).

Horro woreda is located in the Horro Guduru Wellega zone, in western Ethiopia. The administrative town, Shambu, is located at 9° 34′ 0″ N, 37° 6′ 0″ E, approximately 310 km from the capital, Addis Ababa. At the time of the last census in 2007, the population was estimated to be 74,989, of which 71,320 were living in rural districts. According to unpublished data obtained from Horro woreda Office of Agriculture, the estimated current human population is 87,505, and the predominant religions, Ethiopian Orthodox and Protestant Christian, are followed by 89.5% of the population. Population density is considered moderate, and the topography of the land is predominantly undulating, with good vegetation coverage, including varied grasslands. The average annual rainfall ranges from 1200mm to 1800mm across the zone, and the main rainy season lasts from early May to the end of September. The zone is known for production of teff, niger seed (as a cash crop) and cattle, and considered an area of high agricultural potential, producing an annual food surplus (FEG, 2009).

Jarso woreda lies in the East Harerge zone, in the eastern part of the country. The administrative town, Ejerso Goro, is located at 9° 29′ 0″ N, 42° 14′ 0″ E, approximately 560km from the capital, Addis Ababa. The last census estimate of the population was 116,638, of which 112,708 were living in rural areas (CSA, 2007). According to unpublished data obtained from the woreda Office of Agriculture the estimated current human population is 134,426, and 88% of the population follow Islam. The population is described as moderately dense, and the landscape is undulating, with bushes, shrubs and forest being the primary vegetation. The average annual rainfall is only 700mm to 800mm across the zone, and most crops are grown in the main rainy season between July and September. The main crops are chat (a cash crop, chewed as a mild stimulant), vegetables, sorghum and maize, but the area has a food deficit every year (FEG, 2008).

Within each woreda, four kebeles were purposely selected in consultation with local representatives, as described in Chapter 2, which also shows a map of the study areas. Initially, a rapid rural appraisal (RRA) was conducted in four of the study kebeles; 2 each in Horro and Jarso. Focus group discussions (FGD) comprised of up to 50 farmers, of which between 20 and 50% were female. Group discussions typically took two hours. Following the FGD, field visits were conducted in each of the kebeles to directly observe village poultry production. Discussions with key informants, such as district administrators and animal extension workers, were used to collect additional information and triangulate the FGD.

Subsequently, cross-sectional surveys were used to collect further information. Lists of farmers in each kebele were compiled by the local development agents (DA). Within each kebele, a number of sub-areas were selected in order to produce a list of approximately 400-700 households. Potential participants were selected from the final list by systematic random sampling.

Each kebele was visited on four occasions; in May/June and October/November in each year of the study (2011 and 2012). These visits were timed for before and after the main rainy season, which occurs between June and September. Different households were selected to visit on each occasion to take part in a questionnaire survey and for two of their chickens to be sampled. Ethiopian staff were recruited to collect the field samples, and administer the questionnaires in the local language. Training was provided beforehand and, where necessary, in the field if problems were identified. Households were visited by the study team, and the farmers were interviewed to confirm that they had no exotic birds or vaccinations used in their flock. They also needed to own two indigenous-type birds of at least six months of age in order to be included in the study on poultry infectious diseases and genetics.

An additional 200 households, 25 in each kebele, were visited during the first round of surveys in May 2011. Selection of these households was identical to that described above, but without the inclusion criterion that required them to currently own chickens. These households were only required to complete a survey relating to the social and economic aspects of chicken-keeping, and so did not need to currently own chickens in order to take part. This survey was only carried out during one round of sampling, as it was not expected that the findings would be affected by the timing of the survey, unlike the data collected to investigate infectious diseases, where it was anticipated that findings may vary between seasons and years.

3.2.2 Questionnaires

During each round of sampling, questionnaire interviews were carried out with a member of each recruited household, in order to collect data on the management practices and the disease history of the flock. During the first round of sampling (May 2011), all households recruited, including those additional ones selected, were asked to participate in a

questionnaire on social and economic aspects of chicken keeping, and how this contributed to their livelihood. During the second round of sampling (October 2011), households were asked a number of additional questions relating specifically to aspects of reproduction and productivity of their birds. A copy of each questionnaire used is in General Appendix A.

3.2.3 Clinical examination

A form was provided with a checklist of the information which needed to be collected for each bird (General Appendix A) for fieldworkers to complete, labelled with a unique code to identify the bird and link it to the household questionnaire. Information about the clinical history of the two sampled birds was obtained by interviewing a member of the household. Hens sampled were classified by the farmer as being either currently in lay, incubating eggs, rearing chicks or not currently contributing to production. Farmers were also asked to estimate the birds' ages, and provide information as to the origin of the bird; whether bred at home or, if brought in, where it was obtained.

As part of the clinical examination and scoring for ectoparasites, as detailed in Chapter 2, information was collected on certain morphological traits, such as feathering, for the genetics study. Birds were also weighed in the baskets, and ascribed a body condition score on a 0 – 3 grading system (Gregory and Robins, 1998). In addition, birds sampled during the second round of sampling (October – November 2011) had a number of anatomical characteristics measured (General Appendix A) as part of a project undertaken by a Masters student.

3.2.4 Analysis

Birds were classified according to age and sex into the following categories: Hens and cocks were birds of breeding age (from approximately 6 months) and juvenile females and males between approximately 2 and 6 months are classified as pullets and cockerels. All birds below 2 months of age are classified as chicks.

Livestock holdings in each household were converted to a Tropical Livestock Unit (TLU) following Storck (1991) to facilitate a comparison between individuals and areas. Descriptive statistics, chi-squared tests and Mann-Whitney tests, and multilevel modelling were performed using R software (R Development Core Team, 2008). See Chapter 2.8.3 for a further description of how modelling was performed.

3.3 Results

3.3.1 Response rates

A total of 840 households were visited over the course of the study period, and their contributions to the data collected are shown in Table 3.1. Of those 640 households which answered the questionnaire relating to flock management, 60% gave a complete set of answers. There were 173 farmers (27%) who missed only one question, 156 of whom failed to report how they disposed of dead birds. This may have been to do with the layout of the questionnaire, as this question followed the section on recent experiences of disease, and so may have been omitted by the enumerator if farmers had not described any diseases in the flock. Excluding this question, 482 (75%) farmers gave a complete set of answers, 124 (19%) missed one question, 23 (3.6%) missed two questions and 11 (1.7%) missed more than two. Appendix 3.1 gives a complete list of the missing data for each question relating to flock management.

All 400 farmers asked the socio-economic questionnaire provided all data necessary to calculate their current flock sizes, the numbers of chickens and eggs sold over the previous year and the proportion of their annual income derived from poultry and poultry products. There were 13 (3%) of farmers who did not report why they kept chickens, and 29 (7%) farmers who did not report where they sold them. Only one (0.5%) of 199 farmers asked to estimate hens' reproductive parameters did not answer these questions.

Table 3.1 Contributions of households recruited in each round of sampling

Data types collected	May 2011	October 2011	May 2012	October 2012	Total
Total number of households recruited	**400**	**199**	**120**	**121**	**840**
Questionnaire on flock management and disease experiences Sampling of two birds	200	199	120	121	640
Questionnaire on social and economic aspects	400				400
Questionnaire on reproductive aspects of poultry keeping Measurements of two birds sampled		199			199

3.3.2 Demographic and socio-economic structures

The main livelihood activities in both woredas, as ranked by participants during the RRA, were crop production, livestock production, petty trading (defined as informal distribution or selling of cheap consumer goods, Hart (1973)), unskilled labour and skilled labour. The crops of main importance, as ranked by the developmental agents, in Horro were teff, maize and wheat; and maize, wheat and sorghum in Jarso. Chat (*Catha edulis*) is also very common in Jarso; its evergreen leaves are chewed for their stimulating properties and routinely sold to generate additional or continuous income. Key livestock in all areas included cattle, sheep, goats and chickens, as well as donkeys. Crop production and livestock production were reported to be practiced by all wealth categories with involvement increasing from the poorer to wealthier households.

The RRA indicated that chicken production was practised more by the medium to poor households than by the wealthier households. It also highlighted a clear difference between the regions, suggesting that in Horro, chickens were viewed as an important source of additional income for incidental expense or as continuous low-level income from the sale of eggs and birds, and that women often managed this income. In contrast, chicken production appeared to offer much less opportunity for income in Jarso, where chat

provided an important source of incidental or continuous income. These findings were supported by economic data collected during the questionnaire surveys, which showed that chicken production in both regions contributed a greater percentage of family income in poorer households, and that the income derived from poultry was positively correlated with the number of adult birds kept. However, it was also observed that in Horro, wealthier families tended to derive slightly more income from their chickens than poorer families did, even after allowing for the difference in flock size. In Jarso, income from chickens was similar across all wealth categories. Income per adult bird kept was significantly less in Jarso (Figure 3.1). The vast majority of farmers – 84% in Horro and 86% in Jarso – sold their birds locally, either to neighbours or in the local market.

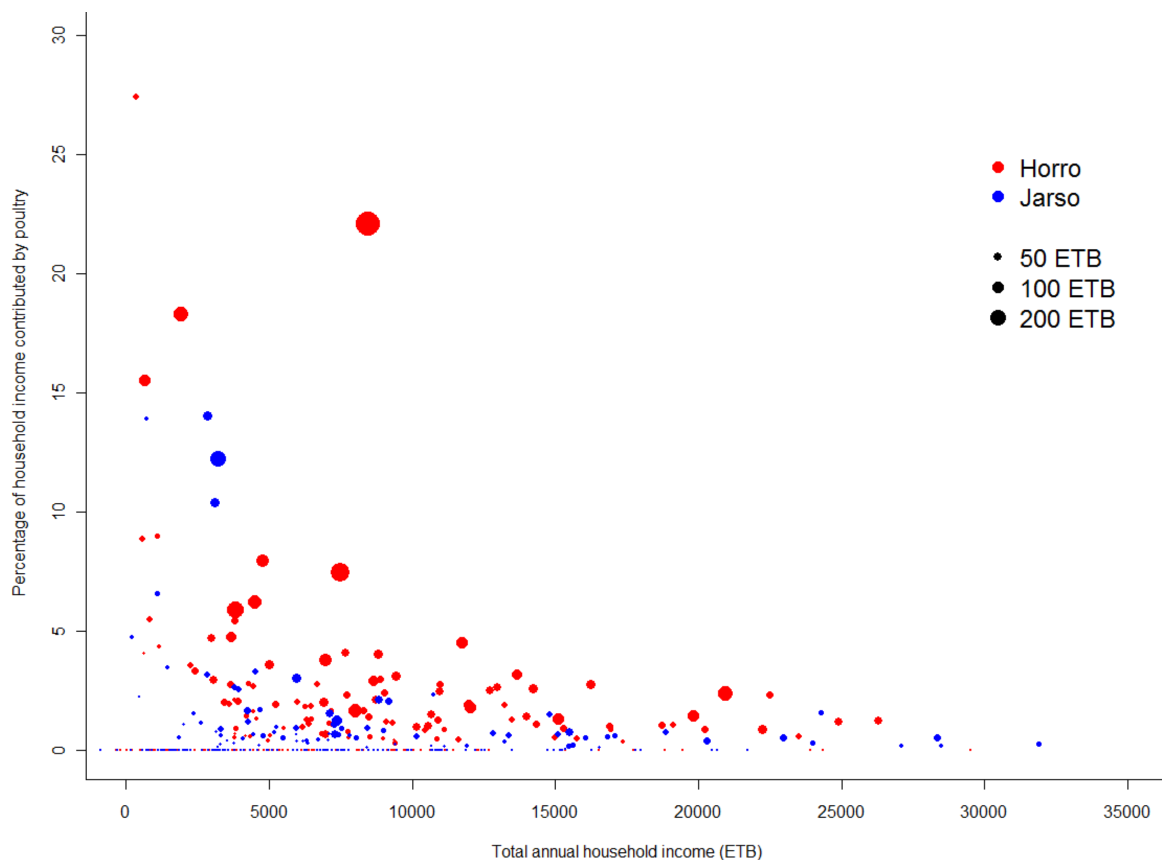

Figure 3.1 Farmer income derived from poultry compared to total annual household income. Points are scaled relative to the income generated per adult bird kept.

3.3.3 Poultry production and drivers for bird selection

Householders reported that the primary purpose of keeping chickens was either for sale, for consumption, or both, although fewer farmers in Jarso kept chickens for consumption (Figure 3.2). Fifty seven per cent of Horro farmers had sold at least one chicken within the last 12 months, and among those who had sold chickens, an estimated total of 516 birds had been sold, giving a mean of 4.5 birds per farmer. In Jarso, however, only 38% of farmers had sold a chicken within the previous 12 months, and only 237 birds had been sold between them, giving a mean of 3.1 chickens per farmer. Approximately 68% of Horro farmers but only 18% of Jarso farmers reported that they had consumed chicken in the past year, and around 17% of farmers in Horro and 23% in Jarso had consumed eggs.

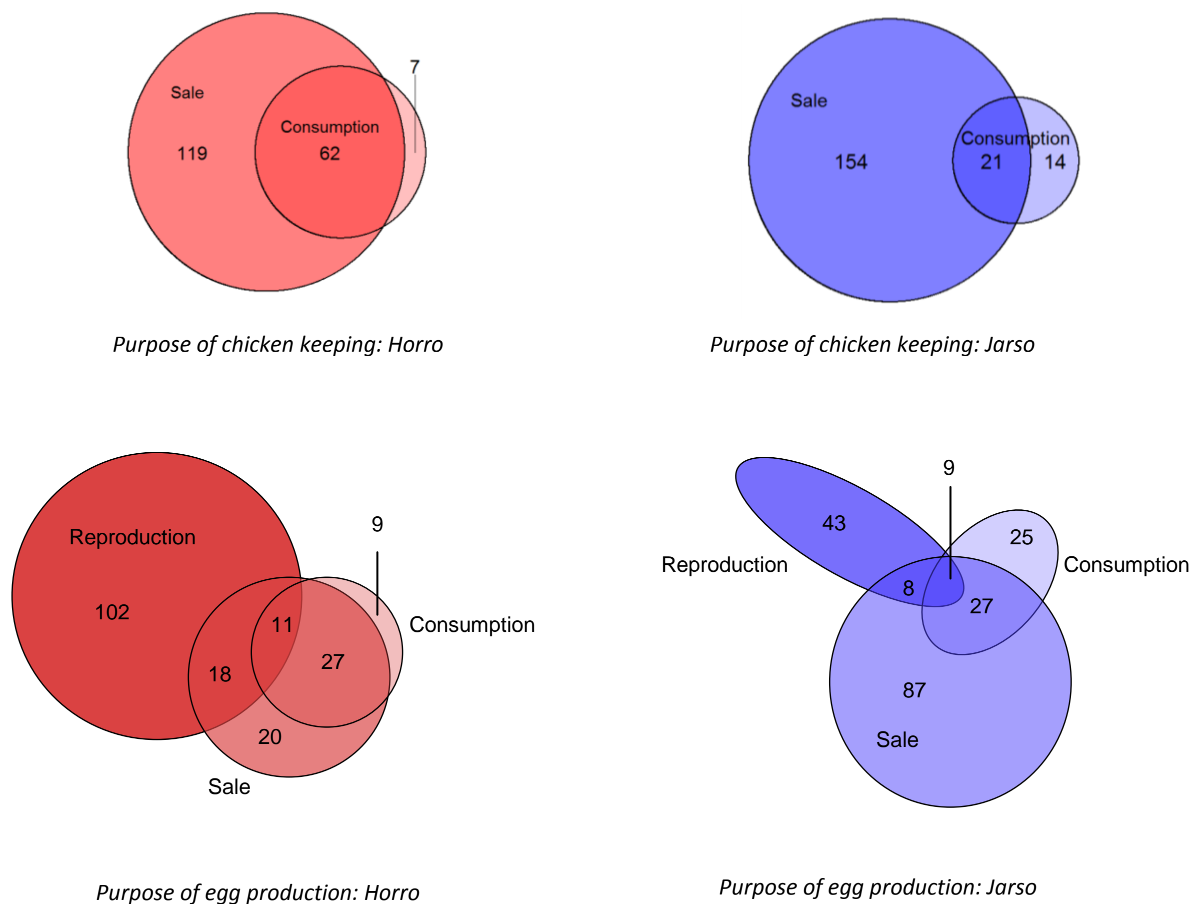

Figure 3.2: Purposes of chicken and egg production in the two study regions

Egg production, in addition for sale or consumption, was also undertaken for reproduction, and in Horro, this was the primary destination of eggs for over half of farmers questioned. In Jarso, 66% of farmers reported the sale of eggs to be among the primary reasons for undertaking egg production (Figure 3.2), although only 18.5% of Jarso farmers reported receiving any income from eggs in the past year; the mean annual income from eggs for these farmers was 40.6 Ethiopian Birr (ETB). This was comparable to a mean annual income from egg sales of 38.1 ETB among the farmers who sold them in Horro, although only 17 of the Horro farmers who were interviewed (8.5%) reported receiving income from eggs within the previous 12 months. There were 39 farmers in Horro (19.5%) and 73 in Jarso (36.5%) who, despite all owning at least one adult hen, reported that they had neither eaten nor sold chickens or eggs in the previous 12 months.

When asked during the survey to describe the most important characteristics of hens, the most frequently mentioned items in both areas were egg production followed by mothering ability. Mothering ability was mentioned as being either the most or second most important characteristic by 55% of Horro farmers, and 41% of Jarso farmers. Egg productivity was ranked first or second by 66% of Jarso farmers and 55% of Horro farmers. Other trait preferences mentioned in both areas were to do with growth or body size (18%), behavioural characteristics (15%) and appearance (16%).

Appearance, namely colour or comb type, was more important for cocks, with 52% of Jarso farmers and 38% of Horro farmers mentioning this as their first or second most important trait. Breeding requirements, such as virility and caring for the rest of the flock was also highly ranked in both areas, mentioned by 45% of Horro farmers and 38% of Jarso farmers. However, a notable difference between the two areas was the importance attached to body size or growth, mentioned by 43% of Horro farmers, compared to only 12% in Jarso.

Jarso farmers were more likely to mention other behavioural characteristics as being important, such as docility or alertness to predators.

3.3.4 Reproduction

3.3.4 (a) Hatching and rearing practices

Of 621 respondents, only 20 did not provide a nest box for hens rearing chicks, of which 18 were in the Jarso region. Households would set eggs under a broody hen to hatch chicks between 1 and 5 times per year, with the exception of 6 households, who said they had not yet hatched their own chicks. Half of all respondents (n=319) set eggs just twice a year. There was a significant difference between the regions, with respondents in Jarso being more likely to set eggs only once a year, and Horro respondents more likely to set eggs more than twice (chi squared statistic 97.8, $p<0.01$).

Data collected as part of the reproduction questionnaire in the second sampling season indicated that in Horro the median number of eggs set was 14, and the median number of chicks successfully reared by a broody hen to 8 weeks of age was 6. In Jarso, the median numbers of eggs and chicks were 12 and 5, respectively. This represented a small but significant difference between the regions (Mann-Whitney-Wilcoxon test, for both comparisons $p<0.01$).

There was also a striking difference in the preference of the timing of hatches between the two regions. Whereas the respondents in Horro preferred to set eggs between September and May, those in Jarso preferred to hatch eggs in June, July or August (Figure 3.3). The main rainy season in Ethiopia occurs between June and August, and anecdotes from farmers suggest that chick rearing was avoided during this period in Horro, as cold and mud adversely affected the chicks. However, in Jarso, chick survival was reported to be better during the rainy season, when there was more vegetation cover, which allowed chicks to avoid predators, particularly birds of prey.

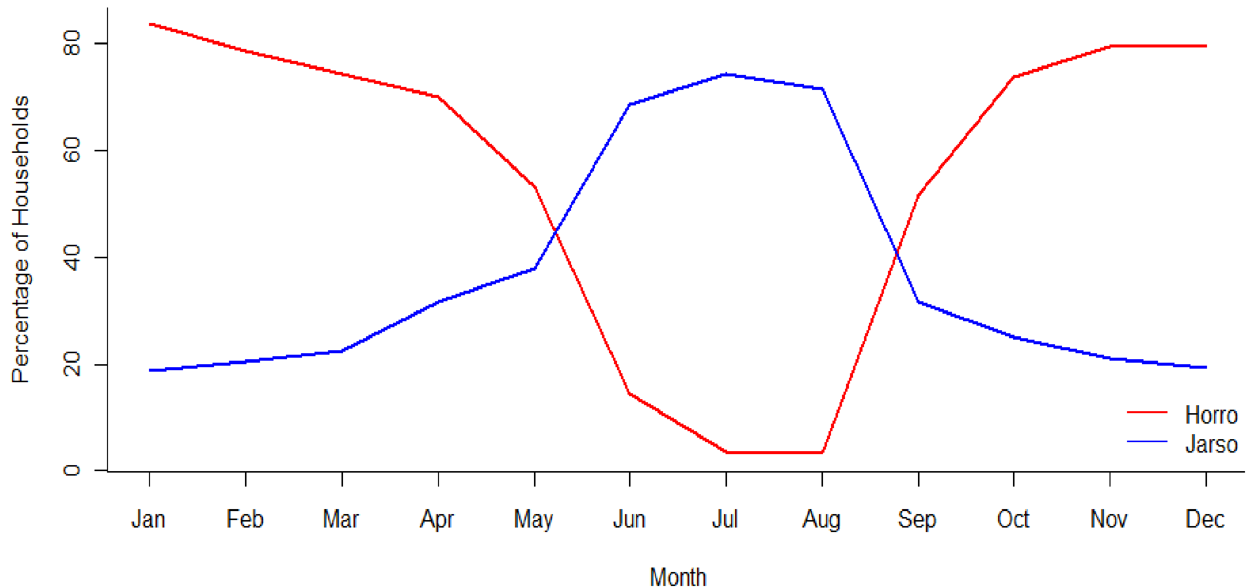

Figure 3.3   Farmer preferences for the time of year in which to hatch eggs in Horro and Jarso regions

3.3.4 (b) Reproductive status

Farmers who answered the reproduction questionnaires in the second sampling season were asked to estimate the average breeding parameters for their chickens. Although not significantly different between the two regions, farmers in Jarso tended to give slightly greater estimates of the number of clutches per year, clutch length (i.e. the number of weeks a bird is in lay) and the inter-clutch period (the time she is not productive). However, they did give lower estimates of the number of eggs per clutch (median of 12 eggs compared to 15 in Horro) (Figure 3.4).

At the time of examination, farmers reported the stage of the production cycle their hens were in. On the whole, kebeles in Jarso had higher proportions of birds which were not in production (i.e. neither in lay, nor brooding eggs or chicks) at the time of sampling.

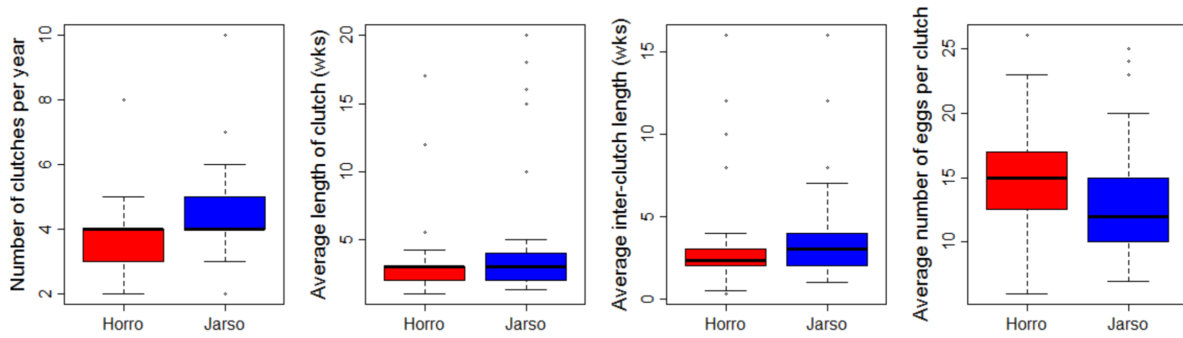

Figure 3.4 Farmers' estimates of hen reproductive parameters in each region

Numbers of birds incubating eggs were similar in the two areas, but in Horro, the random sample of hens included twice as many hens brooding chicks compared to Jarso. However, in Jarso a greater proportion of birds sampled were in lay (Figure 3.5).

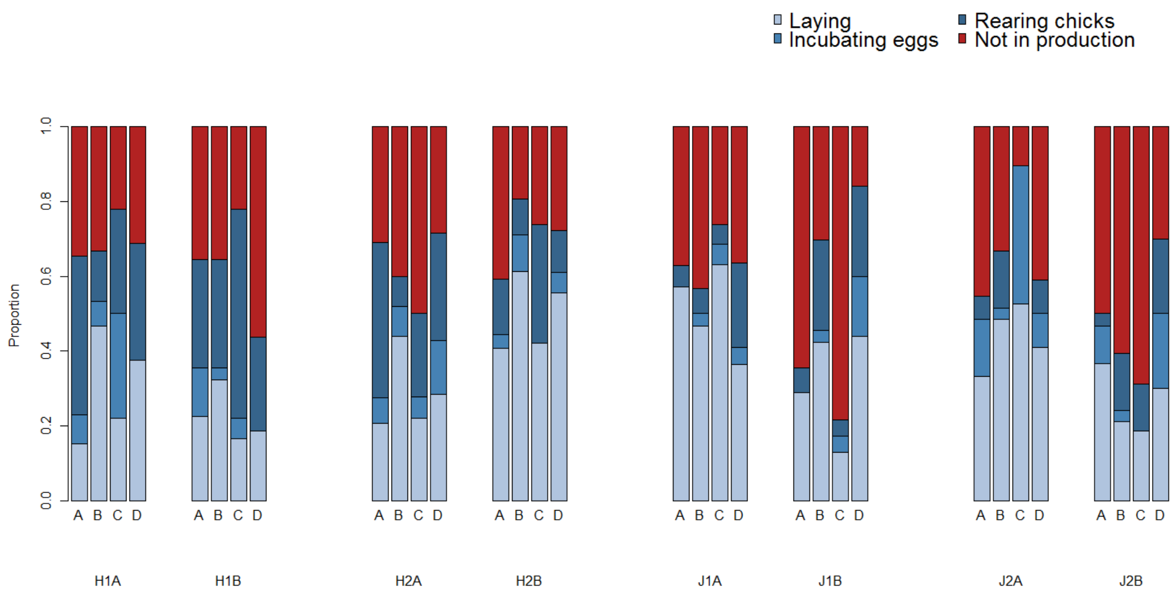

Figure 3.5 Production status of sampled hens by kebele and season of sampling

3.3.5 Flock size

3.3.5 (a) Regional differences in flock composition

Flock size has been calculated using data from those households which participated in the questionnaires on flock management and disease, and had their birds sampled. Although this introduces a bias, in that these households had to own at least 2 adult chickens in order to participate, from data collected on reasons for non-participation, we estimate that around 5% of the randomly-selected farmers who kept chickens in Horro and 8.5% of those in Jarso were excluded because they owned fewer than 2 adult birds. We have chosen to use this data, which was collected over the four seasons of the study, to capture how flock size may vary throughout the year, and between years, rather than the socio-economic data which, although it includes the small flocks, was measured at only a single time-point.

One farm in Horro was accidentally included in the survey despite owning only one adult bird, as the farmer submitted two birds for sampling, one of which belonged to his brother, who lived in the adjoining household. The birds owned by both families' households were treated as one flock, although for the purposes of the questionnaire, the sampled farmer reported only the birds which his family actually owned.

Flock size in the sampled households therefore ranged from 1 to 44 birds in Horro (median 7.5 birds), and from 2 to 37 birds in Jarso (median 5 birds), showing a clear difference between the two populations (Mann-Whitney-Wilcoxon test, $p<0.01$). The median number of cocks owned by households in both areas was 1, and the median number of hens owned was 2 and 3 in Horro and Jarso respectively.

Although Horro flocks tended to be larger, young stock represented a greater proportion of the birds owned (Figure 3.6), especially once flock sizes reached more than 8 birds, when adult hens made up, on average, less than a quarter of the flock. In Jarso, adult hens made up, on average, over 60% of the flock, and even in larger flocks still tended to comprise at

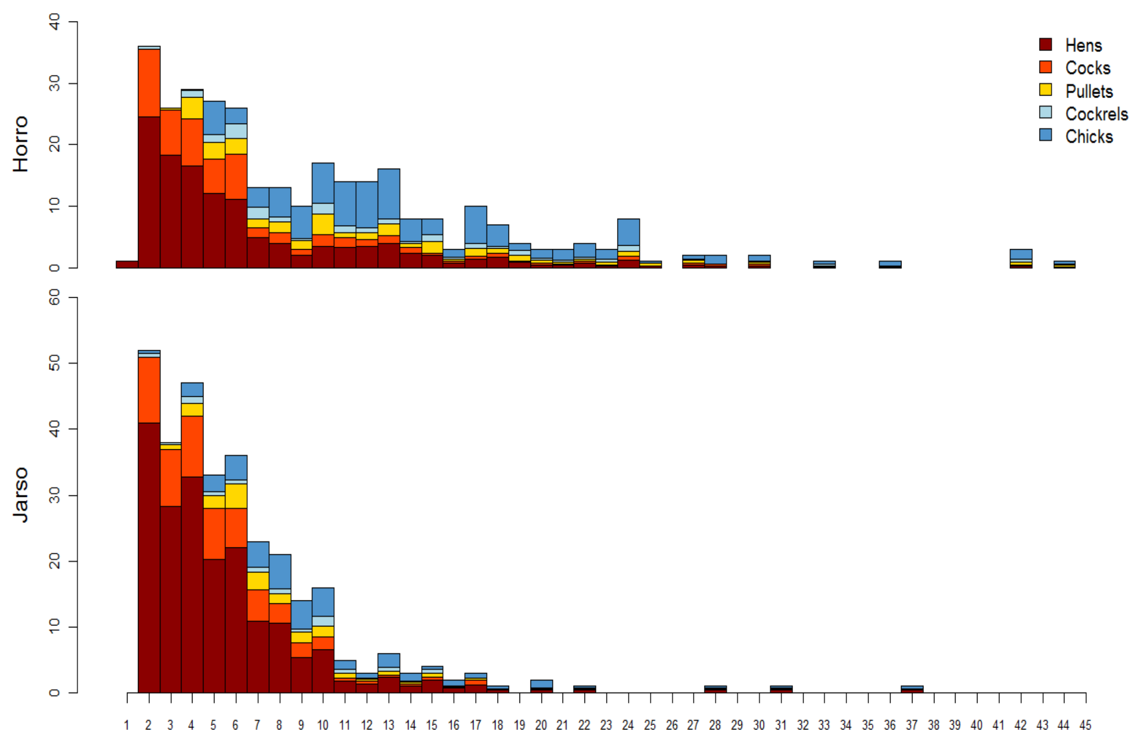

Figure 3.6 Distribution of household flock sizes in Horro and Jarso. Each bin is split into 5 segments which represent the mean proportion of bird types, classified by age and sex, for different sizes of flock.

least a third of the stock. Over 80% of the randomly-sampled adult birds had been bred by the owner, and a further 10% had been bought from neighbours in the same kebele. Only 10% had been bought in from other kebeles or markets.

3.3.5(b) Seasonal changes in flock size

In each region, there were no differences in the distribution of flock sizes between sampling rounds carried out in May to June between the two years of the study; or between those rounds carried out in October and November. However, in both areas, there were differences in the distribution of flock sizes between the spring and autumn sampling periods. In Horro, flock sizes were larger in spring, with a median flock size of 11 birds, compared to a median of only 6 birds in autumn. This represented a significant difference between the two times of year (Mann-Whitney-Wilcoxon, $p<0.01$). However, in

Jarso, flock sizes were significantly larger in autumn (Mann-Whitney-Wilcoxon, $p<0.01$), with a median flock size of 6 birds, compared to only 4 during spring samplings. In both regions there were no differences in the numbers of adult stock between spring and autumn; and the seasonal differences in the flock sizes was attributable to increasing numbers of chicks, pullets and cockerels, although the increase in the latter was only evident in Horro (Appendix 3.2).

3.3.6 Age distribution

A multilevel model (see Appendix 3.3) suggested that much of the variation in age of the randomly sampled birds was due to between-farm variation, but there were sex and regional differences with an interaction between these variables. The mean age of Horro males was just under 11 months old, whereas females were around 2.5 months older. Males in Jarso were, on average, around 14 months old. Around 15% of Jarso females were estimated to be over 2 years old, compared to just 3% of Horro females, and their mean age was around 19 months (Figure 3.7).

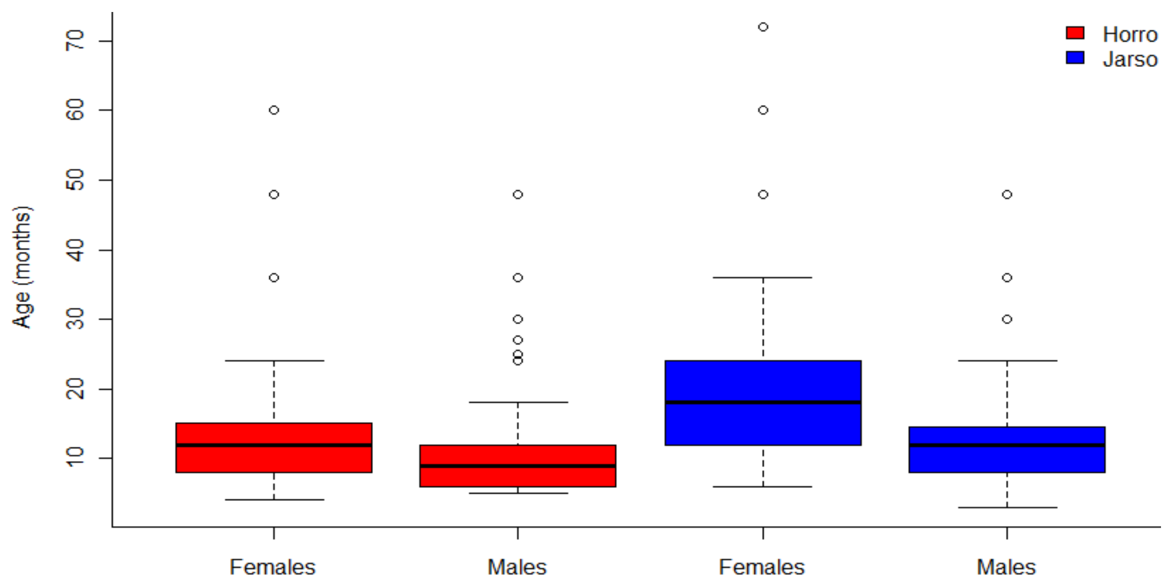

Figure 3.7 Age distributions of females and males sampled in each region

3.3.7 Growth and secondary sex characteristics

Bird weights were found to be influenced by a variety of factors, including age, sex, body condition, region and sampling period. There were also interactions between several of these variables (Appendix 3.4). Bird weights increased with age, and males grew larger than females. However, Horro birds of both sexes grew larger with increasing age than their counterparts in Jarso, and the difference in body weight between males and females of the same age was larger in Horro than it was in Jarso (Figure 3.8). In addition, the wattle length of birds was also affected by the age, sex and region, with a significant interaction between all three of these variables (Appendix 3.5). There was no difference between the females' wattles between the two regions; however, males in Horro had a larger increase in the size of their wattles with increasing age than did males in Jarso (Figure 3.8).

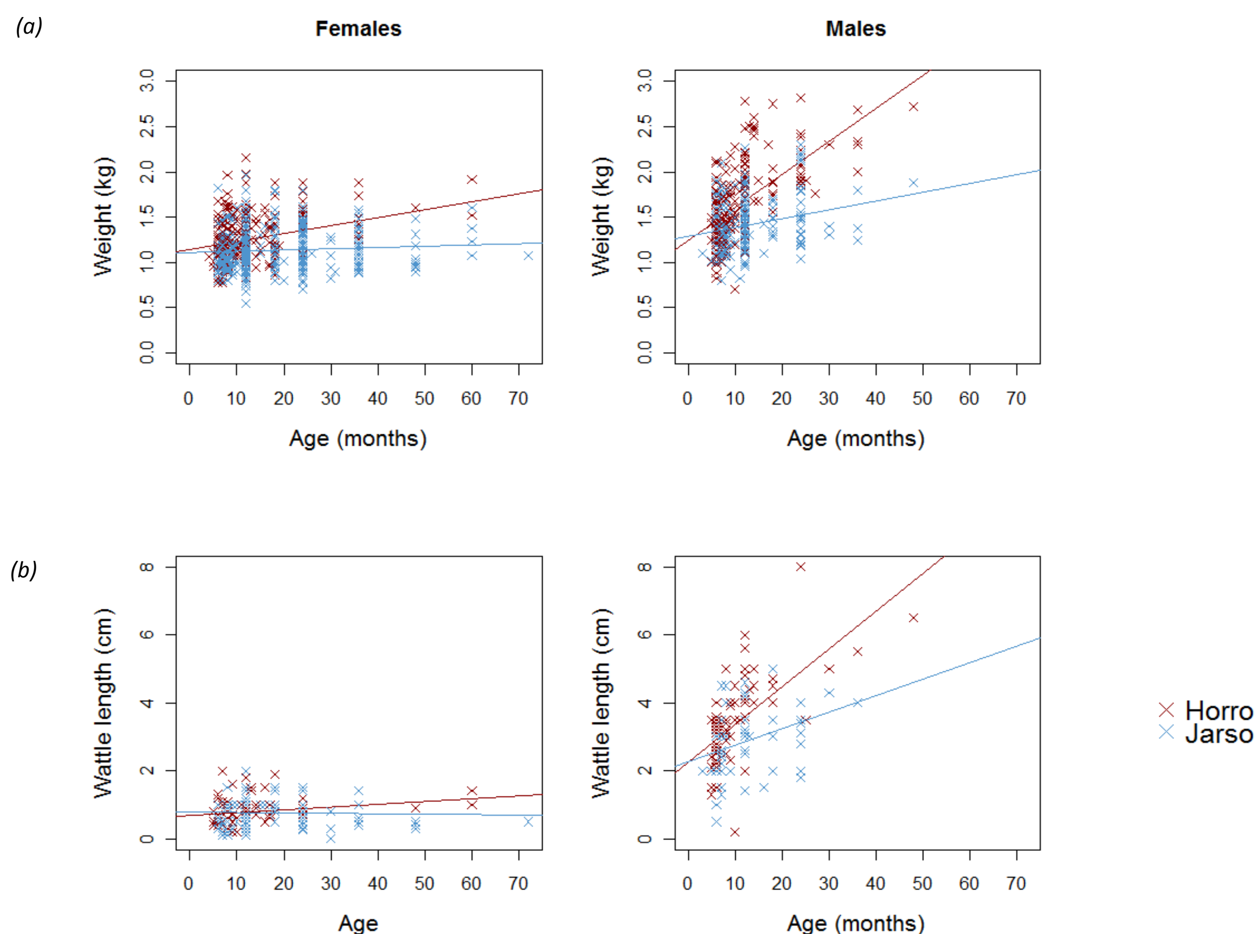

Figure 3.8 Plots of (a) weight and (b) wattle length against age for females and males in different regions

3.3.8 Bird management

3.3.8a) Night shelters

The majority of farmers kept their birds inside the family house at night. In Horro, 59% of farmers kept their birds in the house and 29% kept them in the kitchen building; 6% had constructed a separate shelter, either for chickens or for livestock in general, and 6% kept birds on the veranda overnight. In Jarso, 83% of farmers kept them in the house, 7% in the kitchen building, and 10% of farmers had constructed a separate shelter for them.

3.3.8 (b) Feeding practices

Nearly all farmers (98%) in both regions provided supplementary feed for their birds, and 98% provided supplementary feed separately for chicks. The majority of farmers (75% in Horro and 66% in Jarso) provided additional feed for birds during the rainy season. Between 10% and 20% of farmers in all kebeles provided the same amount of feed year round, and a minority of farmers reduced or stopped providing supplementary feed in the rainy season, although this differed between regions, and in Jarso, between kebeles. In three kebeles in Jarso, between 19 and 25% of respondents reported they offered less feed during the rainy season, whereas in kebele J2B only 6 farmers (7.6%) gave less feed.

3.3.8 (c) Confinement

Birds were generally allowed to scavenge freely during the day. However, some farmers would confine their chickens for some periods. There were differences between the two regions, and also between kebeles within a region. Jarso farmers were, in general, more likely to confine birds, with 58% of respondents reporting that they confined their birds for some part of the year, compared to 36% in Horro. In one Jarso kebele, J1A, only 36% of farmers confined their birds; however, those that did were more likely to confine them for between 3 and 6 months of the year. This contrasted with the other kebeles in the region, where farmers generally confined birds for only one or two months, coinciding with the planting season in April / May. In both regions, farmers were generally least likely to

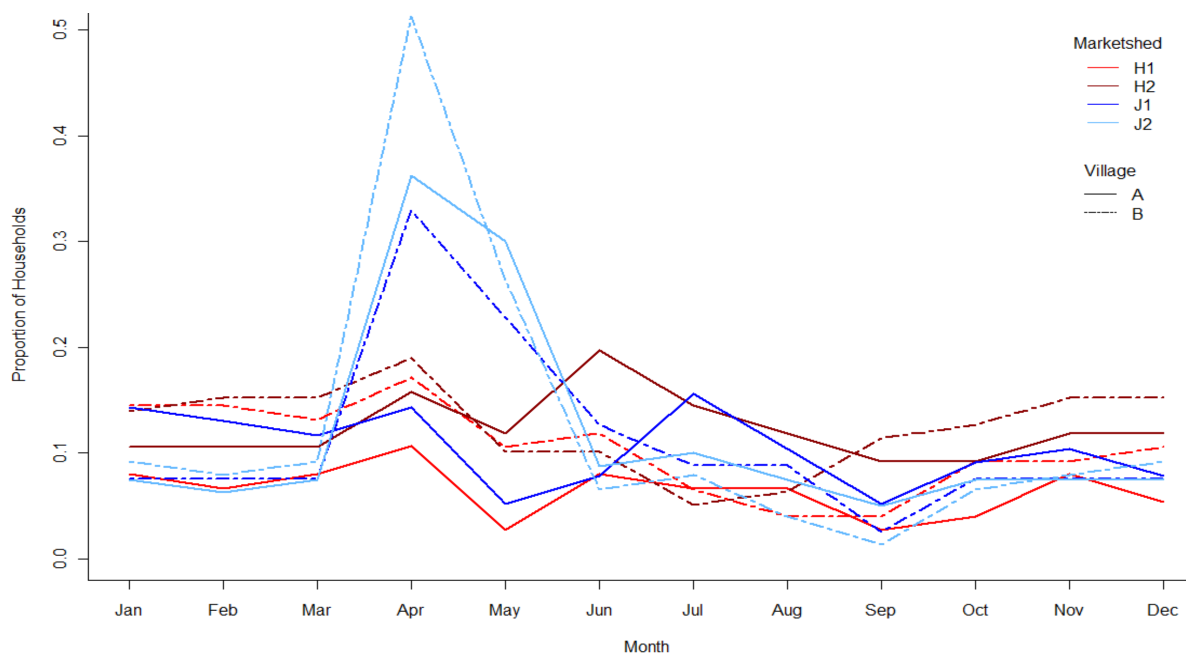

Figure 3.9 Proportion of farmers who confine birds in each month of the year.

confine their birds towards the end of the rainy season, between August and October (Figure 3.9).

3.3.8 (d) Mixing between household flocks

Farmers were asked to grade the amount of time their birds were able to mix with chickens from other households on a scale ranging from 0 to 5, where 0 was "never" and 5, "every day for more than an hour". They were asked to do this for both the wet and dry seasons. The amount of time farmers estimated their birds mixed with other flocks is shown in Figure 3.10. Only 5 farmers (0.7%) estimated that their birds' contacts increased during the wet season, whereas 33% of Horro farmers and 22.6% of Jarso farmers estimated a decrease in birds' contacts from the dry to the rainy season.

3.3.9 Parasite control

Farmers in both regions took a variety of approaches to parasite control. There were 36% of farmers in Horro and 42% in Jarso who stated that they used no preventive treatment against parasites. In Horro, 24% of farmers used an insecticide spray on the housing, and a

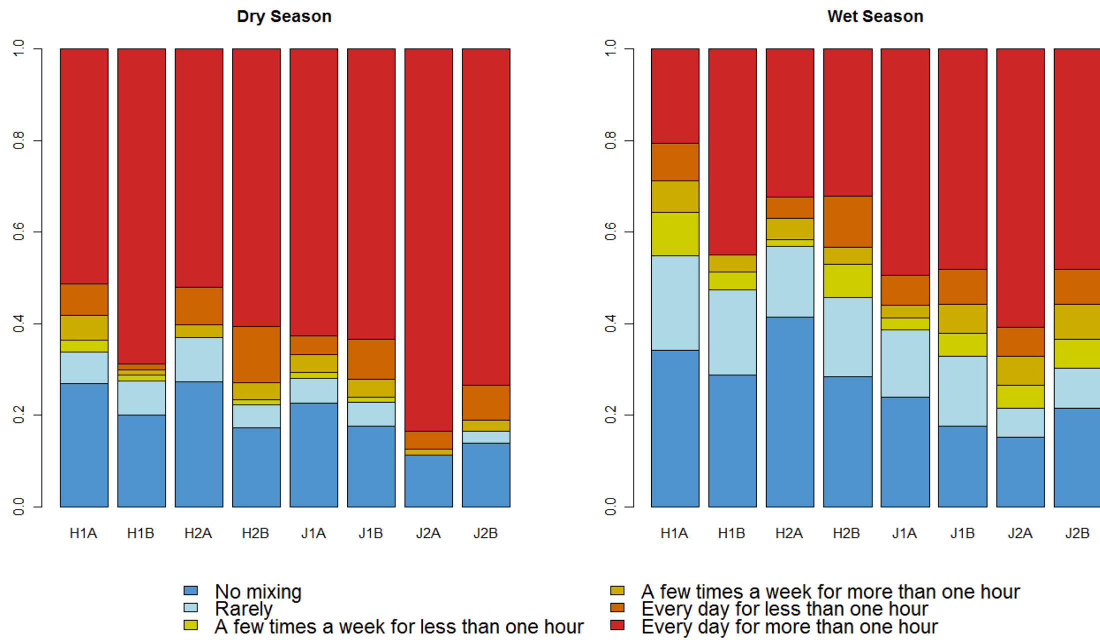

Figure 3.10 Farmers' estimates of the amount of time their chickens spend mixing with those from other households

further 7% used a spray on the birds. However, in Jarso, only 3% used a spray in the housing and less than 1% of farmers interviewed used a spray on the birds. Chemicals mentioned included malathione and DDT.

Ethnoveterinary (referring to people's knowledge, beliefs, practices, technology and resources pertaining to animal health; McCorkle (1986)) techniques were used commonly against parasites. These included changing the perches and the nest box, or cleaning, either with hot water, or sometimes with local plants. Singeing the perches, putting the nest in the sun and burning the nest were also mentioned as ways of reducing parasites in the housing. Fumigation using various local leaves, roots and barks or manure was used for both the housing and the birds themselves. Other external treatments mentioned included rubbing or cleaning the birds with ash, various types of leaf, lemon juice or manure. Enteric treatments included combinations of red or green chilli peppers, onion and garlic, either mixed in water or feed, or given with oil as a drench. Penicillin was also mentioned as a parasite treatment by one householder.

3.4 Discussion

Although the production environments of village chickens may, at first glance, appear similar across the areas studied here, we found a number of differences both between the two regions and between kebeles and households within these regions. The chickens are only one of many enterprises in the mixed crop-livestock system practised by these farmers, and their contribution to income, especially in wealthier households, is relatively small. Here we examine some of the forces that may be shaping these two chicken populations to allow them to meet the various roles that farmers demand of them.

3.4.1 Economic and cultural importance of chickens

In both areas, the primary purpose of keeping chickens was to derive monetary benefits from them, although some families also used them for home consumption. Income derived from chickens tended to be low, both in terms of the contribution to the total household income and the actual monetary income per bird kept. Whilst a small number of households derived regular income from birds, they were frequently more important as an asset which could be sold occasionally to meet incidental expenses. Previous studies have reported seasonal fluctuations in demand for and price of chickens, based around the Orthodox Church calendar (Moges et al., 2010b), and lack of organised marketing systems (Dinka et al., 2010), which preclude many smallholders from deriving regular income from chickens. Similar constraints are likely to exist in the regions in our study.

Eating chicken was much more common in Horro, and anecdotes from Jarso families suggested that many of them did not know how to prepare chicken for consumption. Egg consumption was similar in both areas, both in the percentage of families who consumed eggs and the numbers of eggs they consumed annually. Strategies and potential for income generation from chickens thus appeared to vary between the two areas. Given that the

vast majority of farmers only sell their birds locally, the cultural differences, which make chicken consumption more common in Horro, are likely to provide greater marketing opportunities for live birds in this region. Therefore most families tend to sell live chickens, and use eggs primarily for reproduction. This may be a factor in allowing Horro farmers to derive more income from each adult bird kept, due to the higher prices commanded by live birds, compared to eggs. In Jarso, fewer families sold live chickens, and those that did sold fewer chickens, on average, than farmers in Horro. More families sold eggs than those in Horro, but this still involved only a low proportion of households overall. A substantial proportion of families in both regions had not sold or eaten chicken or eggs within the last 12 months, suggesting that, for them, chickens fulfilled the "safety net" role (McLeod et al., 2009).

The marketing potential also appeared to influence the characteristics that farmers found desirable in their chickens. Horro farmers gave a greater weight to mothering ability, rather than simply egg production in hens; whilst in cocks they gave growth rates and body size an almost equal importance with breeding ability, and appearance was the third most frequently mentioned attribute. Jarso farmers, in contrast to this, rarely mentioned body weight as having any importance, but were predominantly concerned with appearance. Dana et al. (2010) reported of farmers interviewed in five Ethiopian woredas that weight was one of the most important characteristics to them, due to the higher prices commanded by heavier birds. However, this may not hold true for the farmers in Jarso, where there would appear to be little opportunity for marketing birds for consumption.

We found Horro birds, especially the cocks, to have larger body sizes and better body condition scores. This may partly be a reflection of diet, as more Horro farmers provided extra feed in the rainy season, when food was scarce. However, it may also be a result of selection by farmers over many generations for larger birds, so that, despite the apparently

uncontrolled natural mating within the village chicken population, larger birds have been favoured and retained for breeding, whereas smaller ones have been culled. Local markets are thought to be the principal dictators of selection criteria in Ethiopia (Dana et al., 2010), and this weight difference in birds between the two regions may well be a reflection of the differences in local cultures and frequency of chicken consumption.

3.4.2 Reproduction and dynamics of the "Village Flock"

The village flock population is not static, but fluctuates with birds entering and leaving the population continuously. Our results, in agreement with other surveys (Guèye, 1998, Dessie and Ogle, 2001) suggested that, although the village chicken population is not closed, the majority of birds are born and raised within the kebele, rather than entering it from outside. Losses from the population occur as birds are either sold or eaten (which may be considered beneficial for farmers) or die, often from disease, predation or accidents (which represent a loss for farmers, both in terms of invested resources into each bird reared, and loss of the potential to benefit from the bird). The adult bird population size in both areas was apparently stable at all the time points sampled, but the population of young birds was found to be different between regions, and between spring and autumn samplings. The choice of the majority of Jarso farmers to set eggs predominantly during June, July or August led to an increase of young stock, and consequently flock size, during the autumn months at the start of the dry season. In Horro, the reverse was true, and with more than 50% of farmers setting eggs for the remaining 9 months of the year, and a tendency to hatch more clutches per year than Jarso farmers, flock sizes, proportions of young stock and the number of hens rearing chicks were consistently greater in Horro at each time point sampled, and particularly so towards the end of the dry season. Within each region there was no difference in the distributions of flock sizes observed between the two years of the study for comparable seasons, perhaps suggesting these seasonal fluctuations are

relatively consistent in the absence of any major perturbation, such as a disease outbreak, which was never reported during the study period.

The fact that the adult population in both regions appears relatively stable, despite the difference in the numbers of young birds, suggests that the population turnover is more rapid in Horro. This is reinforced by the difference in age distributions between the two areas, with females in particular being relatively long-lived in Jarso. In ecology terms, this could be interpreted as a difference in life-history choices, with Horro birds utilising more of a “live fast, die young” strategy. Organisms which adopt such a strategy invest more resources into reproductive rather than maintenance functions, such as immunity (Møller, 1997, Zuk and Stoehr, 2002). Reproductive effort per se in village chickens is difficult to quantify, as not all eggs laid will be incubated, hatched or reared due to human intervention. Therefore the resources allocated by an individual bird into egg production do not directly translate into reproductive effort. In addition, she must also hatch and rear the chicks, requiring energy to be diverted into protecting them and helping them find food.

Some reproductive differences were observed between the two populations. Hens in Horro reportedly laid slightly more eggs per clutch, and clutch length was slightly shorter, implying a higher rate of lay. The inter-clutch period, when hens are not in production, was also shorter, and more Horro birds were found to be in production than their Jarso counterparts. Although Horro farmers reported that their hens laid fewer clutches of eggs per year, this will be influenced by the additional amount of time these hens must spend rearing chicks. Although we did not collect estimates on the number of eggs per hen per year, it might be predicted that Jarso birds may come back into lay more quickly if they are not required to brood, thus allowing them to lay more eggs overall. Horro birds were also ascribed a greater propensity for broodiness, which will decrease egg production. One further indicator that Horro birds may make greater investments into reproduction is the

finding that cocks in Horro have more pronounced secondary sex characteristics, as typified by wattle length. Horro birds are also believed to have brighter plumage colours (Desta, personal communication).

Horro birds were clearly rearing more chicks, but whether this was a natural trait exploited by farmers to produce more young stock in as short a time as possible, or whether it has evolved in response to farmer selection cannot be determined. Although differences are not as extreme as in intensive farming systems, the two regions do appear to be following different business models, with Horro birds more akin to a broiler breeder flock, and Jarso playing more of a layer role. This again may be a consequence of the differing market opportunities and production strategies adopted by farmers in the two regions, with Horro farmers producing more birds for the live market, whilst Jarso farmers have less potential to sell birds, and so rear fewer broods of chicks. The mystery remains as to what farmers in Jarso do with their eggs during the remainder of the year, given that so few families reported consuming them, and even fewer reported deriving any income from them. One possibility is that eggs are bartered rather than sold, and the questionnaire, designed to elicit the monetary income from eggs, has not captured this information adequately. Guèye (1998) has also suggested that, since the priority of most village poultry keepers is to maintain a small flock, many eggs are simply stored for several weeks as an insurance against the high mortality rates in young birds.

3.4.3 Potential risk factors for disease

Husbandry practices showed only minor differences between the regions in terms of housing, feeding and scavenging management. Nearly all farmers provided some protective shelter for their chickens at night; although very few had invested in a shelter specifically for this purpose, and more commonly provided perches or roosting areas within existing buildings. Nest boxes were almost always provided for hens to incubate eggs and rear

young chicks. The importance of cleanliness and hygiene of the poultry accommodations was clearly recognised by a number of farmers, who frequently mentioned cleaning these areas as a key component of parasite control.

Although confinement for limited periods of the year was practiced by many farmers, particularly to protect young crops during the planting season, birds were generally allowed to scavenge freely during the day, which allows them to come into contact with birds from other households. The degree of contact with other birds was generally greater in Jarso. This is likely to be related to the fact, made from personal observations and corroborated by evidence gathered during the PRA and as part of the socio-economic questionnaire (Terfa, personal communication), that, in Jarso, farmer landholdings are smaller and households are closer together. In both areas, farmers estimated that birds tended to contact other flocks less frequently in the rainy season, although this was reportedly the time when farmers were least likely to confine their birds. Whether this reduction in roaming is a choice made by birds in order to avoid bad weather, or whether there is less necessity for them to scavenge due to the tendency of most farmers to provide extra feed to compensate for the decrease in scavenging resources in the rainy season is unclear. It is interesting that farmers note a decrease in contact frequency during the months when disease outbreaks have been reported to be more likely to occur (Dessie and Ogle, 2001, Halima et al., 2007, Dinka et al., 2010), perhaps emphasising the importance of indirect transmission and environmental survival of pathogens in the village situation. Alternatively, for directly transmitted pathogens, mixing of flocks may not be sufficient to initiate an outbreak, but it may be that other seasonal factors alter birds’ susceptibility to disease.

The large number of young stock and mixed age groups of birds in Horro would be predicted to be a risk factor for a number of diseases, as the constant influx of young,

susceptible birds into the population over most of the year should increase the potential for successful transmission of infections to occur, and for pathogens to be maintained within the population. In Jarso, where it appears that most young stock is reared during a more restricted time period, this may limit the number of susceptible hosts to certain diseases for much of the year, which may then be expected to follow more of a classical epidemic pattern.

3.5 Conclusions

Chickens raised under a village situation are likely to be under very different pressures compared to intensively farmed birds, upon which most of our knowledge of poultry disease and immunity is based, and as such may be more akin to wildlife populations, which have only limited energy resources available. Ecological trade-off theory suggests that the maintenance of an immune system is a costly investment for individuals, and that the allocation of energy resources to other life history choices, such as increased growth or reproductive effort may be made at the expense of an individual's immune function (Møller, 1997, Zuk and Stoehr, 2002). The observed phenotypic differences in growth and bodyweight between our two populations may well be market-driven, and a result of human selection pressures. The rate of population turnover may also be partially human-driven, as farmers dictate how many eggs will be hatched and how many birds eaten or sold.

Whether this enforced life-history choice for Horro birds to grow larger and rear more young has implications for the maintenance requirements for individuals is not clear, although their shorter lifespan compared to their Jarso counterparts might suggest this to be the case. If this hypothesis is true, it may be predicted that Horro birds would suffer increased morbidity and mortality, which will be examined in subsequent chapters of this

thesis. These life-history choices may not always be in concordance with the direction that other selection pressures, in particular those from infectious diseases, may exert on the population, and as such may be of considerable importance when we come to consider how these birds interact with the pathogens in their environment.

Appendix 3.1 Missing data from bird management questionnaire

Table 3.2 Number of cases of missing data for each question relating to bird management

Question	Horro	Jarso	Total	(%)
Number of birds currently owned by age and sex	5	6	11	(1.7)
Housing type	4	1	5	(0.8)
Waste disposal method	0	2	2	(0.3)
Nest Box provision	12	7	19	(3.0)
Supplementary Feed provision	1	2	3	(0.5)
Feed provision separately for chicks	6	6	12	(1.9)
Alteration in feed provision during the rainy season	4	3	7	(1.1)
Confinement of birds for part of the year	4	3	7	(1.1)
Which months confined	15	7	22	(3.4)
Contacts with other birds	22	7	29	(4.5)
Length of time since last bird brought into the flock	15	16	31	(4.8)
Number of times eggs set annually	14	13	27	(4.2)
Which months egg setting is preferred	5	6	11	(1.7)
Which months egg setting is avoided	4	6	10	(1.6)
Method of disposal of dead birds	66	90	156	(24.4)
Use of parasite treatment	11	8	19	(3.0)

Appendix 3.2 Seasonal flock size distribution by region

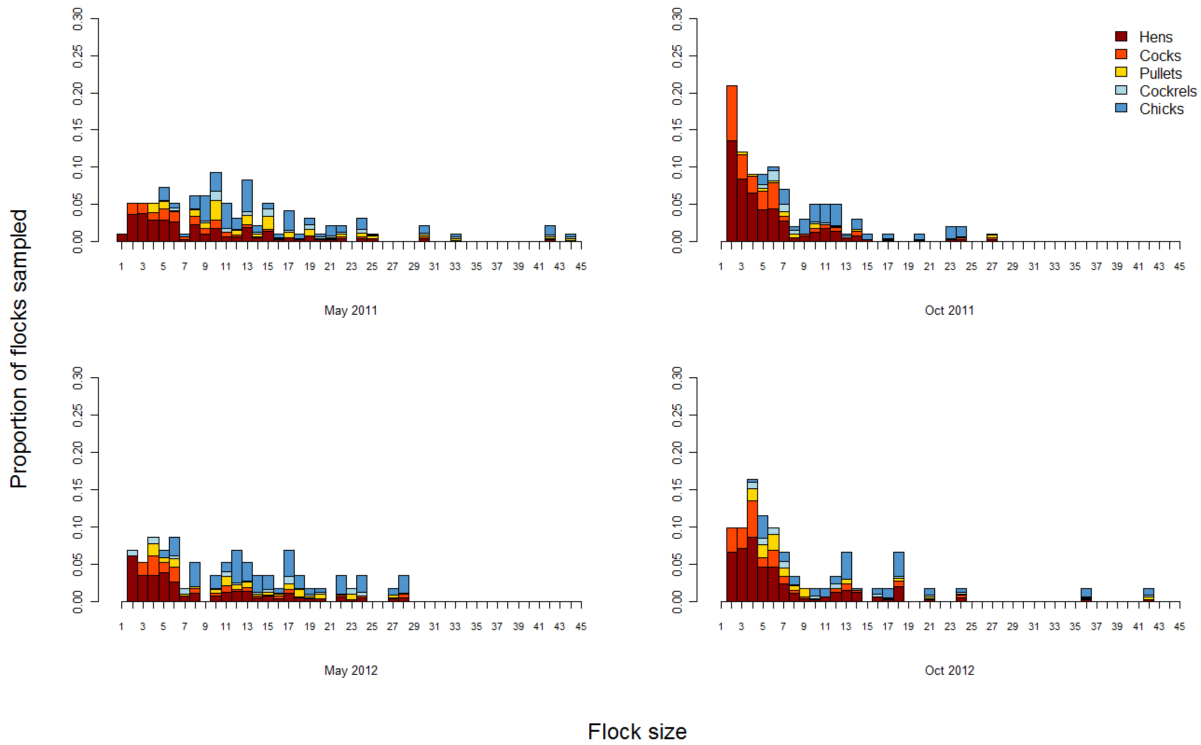

Figure 3.11 Distribution of flock sizes in Horro for different sampling seasons

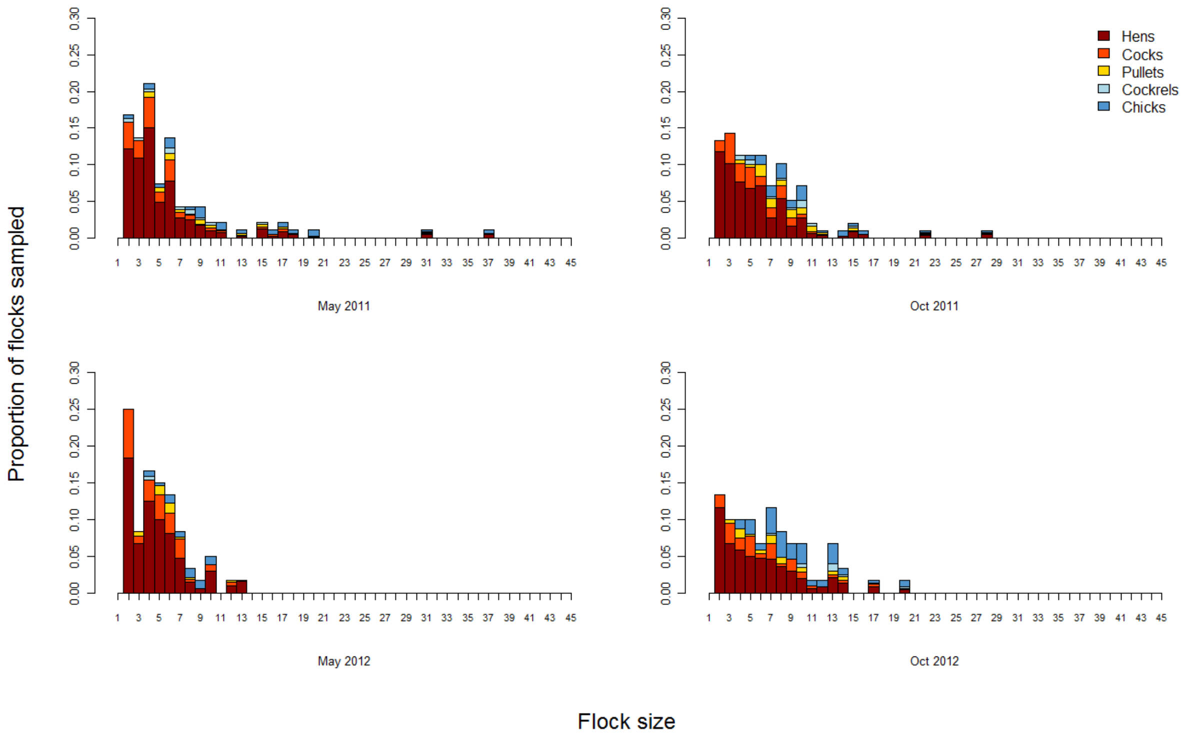

Figure 3.12 Distribution of flock sizes in Jarso for different sampling seasons

Appendix 3.3 Linear model of bird age

Mixed-effect linear regression model predicting age as a function of sex and region, with household fitted as a random effect.

```
Linear mixed model fit by REML
Formula: Age ~ RegionID * relevel(Sex, "M") + (1 | FarmUnID)
   Data: birds
  AIC  BIC logLik deviance REMLdev
 8747 8778  -4368     8737    8735
Random effects:
 Groups   Name        Variance Std.Dev.
 FarmUnID (Intercept) 25.931   5.0923
 Residual             47.374   6.8829
Number of obs: 1237, groups: FarmUnID, 626
```

Table 3.3 Coefficients for linear model of bird age

Fixed effects	Coefficient	SE	lci	uci	z	P
(Intercept)	10.70	0.54	9.64	11.76	19.80	<0.01
Region Horro	Reference					
Region Jarso	**2.99**	**0.81**	**1.40**	**4.58**	**3.69**	**<0.01**
Sex Male	Reference					
Sex Female	**2.47**	**0.60**	**1.31**	**3.64**	**4.15**	**<0.01**
Region Jarso : Sex Female	**2.77**	**0.88**	**1.04**	**4.50**	**3.13**	**<0.01**

Appendix 3.4 Linear model of bird weight

Mixed-effect linear regression model predicting weight as a function of age, sex, body condition, region and season, with household fitted as a random effect.

```
Linear mixed model fit by REML
Formula: BirdWt ~ SeasonID * RegionID + Sex * Age * RegionID +
relevel(as.factor(BCS),"3") + (1 | FarmUnID)

Data: birds
    AIC   BIC logLik deviance REMLdev
 -33.26 63.48  35.63   -189.7  -71.26

Random effects:
 Groups   Name        Variance Std.Dev.
 FarmUnID (Intercept) 0.016436 0.1282
 Residual             0.036786 0.1918

Number of obs: 1202, groups: FarmUnID, 624
```

Table 3.4 Coefficients for linear model of bird weight

Fixed effects	Coefficient	SE	lci	uci	z	P
(Intercept)	1.35	0.04	1.28	1.43	34.73	<0.01
Continuous variables						
Age	**0.01**	**0.00**	**0.00**	**0.01**	**4.42**	**0.00**
Categorical variables						
Season A	Reference					
Season B	**0.07**	**0.03**	**0.02**	**0.12**	**2.57**	**0.01**
Season C	0.02	0.03	-0.04	0.09	0.77	0.44
Season D	0.03	0.03	-0.03	0.09	0.88	0.38
Region Horro	Reference					
Region Jarso	**0.08**	**0.04**	**0.00**	**0.16**	**2.01**	**0.04**
Sex Female	Reference					
Sex Male	**0.12**	**0.04**	**0.05**	**0.19**	**3.39**	**<0.01**
Body condition score 3	Reference					
Body condition score 2	**-0.17**	**0.03**	**-0.22**	**-0.12**	**-6.77**	**<0.01**
Body condition score 1	**-0.31**	**0.03**	**-0.36**	**-0.25**	**-11.16**	**<0.01**
Body condition score 0	**-0.55**	**0.07**	**-0.69**	**-0.41**	**-7.54**	**<0.01**
Interactions						
Region Jarso : Season B	**-0.19**	**0.04**	**-0.26**	**-0.11**	**-4.86**	**<0.01**
Region Jarso : Season C	**-0.22**	**0.04**	**-0.31**	**-0.13**	**-5.00**	**<0.01**
Region Jarso : Season D	**-0.22**	**0.04**	**-0.30**	**-0.13**	**-4.97**	**<0.01**
Sex Male : Age	**0.02**	**0.00**	**0.02**	**0.03**	**8.94**	**<0.01**
Region Jarso : Sex Male	0.09	0.05	-0.02	0.19	1.61	0.11
Region Jarso : Age	**-0.01**	**0.00**	**-0.01**	**0.00**	**-2.68**	**0.01**
Region Jarso : Sex Male : Age	**-0.02**	**0.00**	**-0.02**	**-0.01**	**-4.84**	**<0.01**

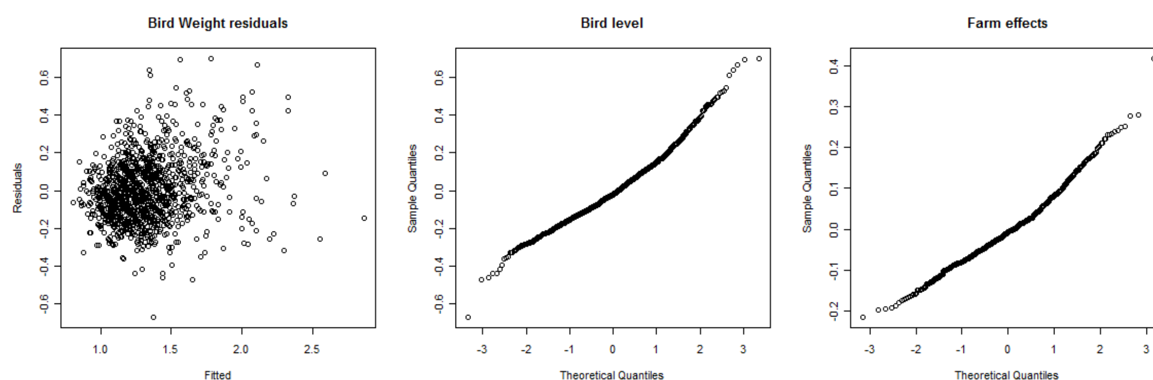

Figure 3.13 Diagnostic plots of residuals from regression model of bird weight

Appendix 3.5 Linear model of bird wattle length

Mixed-effect linear regression model predicting wattle length as a function of age, sex, and region, with household fitted as a random effect.

```
Linear mixed model fit by REML
Formula: WattleL ~ RegionID * Sex * Age + (1 | FarmUnID)

Data: Esk
   AIC   BIC logLik deviance REMLdev
 793.2 832.3 -386.6    728.1   773.2

Random effects:
 Groups   Name        Variance Std.Dev.
 FarmUnID (Intercept) 0.026949 0.16416
 Residual             0.402047 0.63407

Number of obs: 370, groups: FarmUnID, 192
```

Table 3.5 Coefficients for linear model of bird wattle length

Fixed effects	Coefficient	SE	lci	uci	z	P
(Intercept)	0.70	0.11	0.49	0.90	6.54	<0.01
Continuous variables						
Age	0.01	0.01	0.00	0.02	1.22	0.22
Categorical variables						
Region Horro	Reference					
Region Jarso	0.10	0.16	-0.21	0.40	0.61	0.54
Sex Female	Reference					
Sex Male	**1.58**	**0.17**	**1.24**	**1.92**	**9.13**	**<0.01**
Interactions						
Region Jarso : Sex Male	-0.10	0.28	-0.64	0.44	-0.37	0.71
Region Jarso : Age	-0.01	0.01	-0.03	0.01	-1.12	0.26
Sex Male : Age	**0.10**	**0.01**	**0.08**	**0.13**	**8.18**	**<0.01**
Region Jarso : Sex Male : Age	**-0.05**	**0.02**	**-0.09**	**-0.02**	**-2.92**	**<0.01**

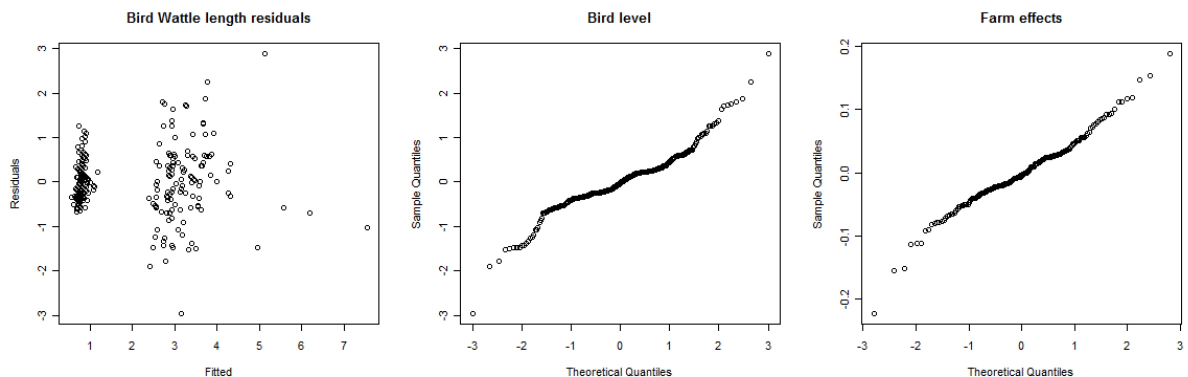

Figure 3.14 Diagnostic plots of residuals from regression model of bird wattle length

Chapter 4

Quantifying disease in Ethiopian village chickens

4.1 Introduction

There is widespread recognition of the importance of poultry-keeping to poor rural households in developing countries. Birds are typically indigenous breeds which require few inputs and have low outputs of both eggs and meat. McLeod et al. (2009) described the "safety net" flock; which usually contribute little in terms of a family's economic income, but may be sold or given away as required, and thus contribute both to poor families' livelihoods and to their social dynamics. The "asset-builder" flock is seen as a potential route out of poverty, and here poultry may represent a much greater proportion of a household's assets and income, but such an enterprise carries a high risk. In shifting village poultry production from the safety-net to the asset-builder flock, disease is reported as one of the greatest constraints (Guèye, 1998; Mack et al., 2005). A study in 13 African countries concluded that vaccination against Newcastle disease virus (NDV) was the most critical intervention in all cases, and that other interventions such as improving feed, housing or controlling parasites were only effective if used in conjunction with vaccination. All 13 country projects demonstrated an economic return on the investment in NDV vaccines (Dwinger and Unger, 2006). However, as Bell (2009) points out, the key to improving profitability via disease control is to know which diseases are prevalent in the area before starting.

Numerous studies looking at the seroprevalence of antibodies to NDV have been carried out in Ethiopia with reported values ranging from 6 – 64% (Tadesse et al., 2005; Zeleke et al., 2005b; Regasa et al., 2007; Mazengia et al., 2010; Chaka et al., 2012). However, NDV is not the only problem; other surveys in village chickens have identified the presence of infectious bursal disease (Mazengia et al., 2010; Chaka et al., 2012; Jenbreie et al., 2012), salmonellosis (Berhe et al., 2012; Alebachew and Mekonnen, 2013), pasteurellosis and

mycoplasma infection (Chaka et al., 2012). Marek's disease has also been identified in village chickens kept under intensive management (Duguma et al., 2005). Parasitic diseases, including coccidiosis (Ashenafi et al., 2004b; Luu et al., 2013), helminths (Tolossa et al., 2009; Molla et al., 2012) and ectoparasites (Belihu et al., 2009; Tolossa et al., 2009) have also been demonstrated to be highly prevalent in the country. The main problem with prevalence surveys is that they demonstrate the presence of the infection without necessarily providing much information about its impact on the population. A high seroprevalence indicates that a large number of animals have been exposed, but does not necessarily correlate with a high impact on that population – for example, infectious bursal disease in a flock of adult chickens may pass unnoticed, as birds will not show clinical signs (Eterradossi, 2008). Conversely, a low seroprevalence may be found both in cases where the exposure to a pathogen is low, but also where a highly virulent pathogen, such as velogenic NDV, has led to high mortality rates in infected animals.

Estimating the relative importance of various diseases in a population is therefore complex. Alamargot (1987) performed a series of post-mortem examinations in order to determine which diseases were causing the most losses in Ethiopian poultry. However 193 out of the 198 necropsies were birds from commercial farms, and as such his findings cannot necessarily be extrapolated to village poultry. Investigating field outbreaks in village poultry is challenging in Ethiopia, due to both a lack of knowledge of poultry disease in local veterinary extension services (Mammo et al., 2011), and the lack of supporting diagnostic services, which prevents the identification of the aetiological agents (Halima et al., 2007). Although Ethiopian farmers can recognise and describe a variety of diseases (Sambo, 2012) they tend to designate any acute disease with high mortality as *"fengele",* which was described by Dessie and Ogle (2001) as a syndrome comprising of inappetance, watery, yellowish droppings, paralysis and eventually death. However, it has also been translated by Nega et al. (2012) to mean "sudden, dorsal prostration". The term has, in many

participatory and questionnaire-based studies without laboratory confirmation, become synonymous with Newcastle disease, even though the syndromic description could in fact include a number of other disease conditions.

Studies based on interviews or PRA work report outbreaks of "*fengele*" to be seasonal and to occur annually during the rainy season (Dessie and Ogle, 2001; Halima et al., 2007; Dinka et al., 2010). However, almost no work has been done which attempts to quantify the impact of these outbreaks. One study by Nega et al. (2012) reports that disease-related mortality rates of chickens between 1 day old to (undefined) adult age was 34% (my calculation); and that NDV vaccination reduced mortality by 82%. However, these rates appear to have been derived from farmers' estimates taken during a questionnaire survey. Chick mortality has been estimated to be around 60% up to 8 weeks of age in a participatory study in Ethiopia (Dessie and Ogle, 2001), but attributed to a variety of causes, including disease, predation, feed deficiency and a hostile environment. Mortality rates in adult chickens do not appear to have been reported in Ethiopia, although the main causes are reported to be disease and predation (Halima et al., 2007; Dinka et al., 2010; Selam and Kelay, 2013).

The aim of this study was to quantify losses in adult village poultry in two geographically distinct areas of Ethiopia and to investigate the impact of NDV in these populations. Due to the remoteness of the two locations, and the inaccessibility during the rainy season, it was unfeasible to gather these data by means of a cohort study to calculate proportional mortalities from different causes, or to actively investigate any outbreaks, due to the lack of any reporting system. We therefore chose to address the research question by measuring the 6-month survival rate in a population of randomly-sampled birds, with a series of cross-sectional serological surveys to monitor the population exposure to NDV. In

addition, we gathered detailed information on disease outbreaks from farmers through questionnaires to gain an overall picture of the seasonal patterns of disease.

Objectives were

1) To estimate the rate of losses of adult birds from disease, predation, or other causes
2) To measure any variations with respect to season or region
3) To measure population changes in the seroprevalence of NDV over the same period, and observe any correlations with disease-related mortality rates.

4.2 Methods

This study was carried out between May 2011 and November 2012 in two geographically distinct woredas (administrative districts), Horro and Jarso, within the Oromia region of Ethiopia. Woredas are divided into smaller administrative districts, called kebeles, which may comprise several villages. A group of villages which are linked by trading networks is collectively known as a market-shed (Tadelle et al., 2003)

Within each region two market-sheds and two kebele per market shed, believed locally to be representative of the woreda, were purposively selected on the basis of their willingness to participate, in consultation with local representatives of the Department of Agriculture and the communities. A list of names of all household heads in each kebele, grouped by sub-area, was obtained from the respective kebele agricultural development agents (DA). Within each kebele a number of sub-areas were selected, so that each kebele included approximately 400-700 households; finally systematic random sampling was used to select potential participants.

Each kebele was visited on four occasions; in May/June and October/November in each year of the study (2011-2012). These visits were timed for before and after the main rainy season, which occurs between June and September. Different households were selected to visit on each occasion to take part in a questionnaire survey and for two of their chickens to be sampled. Households which did not own at least two indigenous breed chickens of over 6 months of age were excluded from the study. Two chickens of at least 6 months of age were selected from the household flock using random number tables. Blood samples were collected from the brachial vein of the selected birds into sodium citrate and transported to the laboratory for testing. Sera were tested for antibodies to NDV using the haemagglutination inhibition (HAI) assay (O.I.E., 2009).

Information on disease occurrence over the previous 12 months was collected in a questionnaire interview with a member of the household. Farmers were asked to provide details of outbreaks in their flock, with details of when they occurred, how many birds were lost and which age groups were affected. An outbreak was defined as an event where several birds died from the same disease. A follow-up visit was also carried out for households interviewed during the first three rounds of sampling. This visit occurred around 6 months later, at the time of the next visit to the kebele. Farmers took part in a short interview to ascertain what further disease events they had experienced in the intervening period, and what had happened to the two previously sampled birds. Surviving birds were re-examined and matched using leg rings and photographs. If a bird had not survived, farmers were asked to describe what had happened to it.

Data were stored in an Access database (Microsoft, 2007). Statistical calculations, graphical summaries and multilevel modelling were performed using R software (R Development Core Team, 2008). Multilevel random effects logistic regression models were constructed to investigate whether there were any factors that altered the probability of farmers'

reporting of outbreaks and, given an outbreak was reported, whether this consisted of single or multiple deaths. Explanatory variables tested included region, season, time elapsed between the outbreak and the time of interview, whether it was the first or second interview, and whether the month of the outbreak was in the rainy or the dry season. For farms reporting more than one death in an outbreak, the same explanatory variables were fitted in a Poisson regression model to see whether any of these factors contributed to the number of birds lost. Only farms which could provide month-specific data were included in the model, and data were arranged to create an observation for each month a farm was asked about in the questionnaire. Thus, farms that were interviewed twice had 18 observations, and those interviewed only once had 12. Variables were created for the first or second time of interviewing, and the length of time elapsed between the recalled month and the interview. Also a categorical variable was introduced to define whether the month in question was in the wet or dry season. Stepwise selection of variables to include was performed, including interactions between variables. Full details of the model formulae, along with an example of the data, are given in Appendices 4.2 and 4.3.

4.3 Results and discussion

4.3.1 Bird mortality

In total we ascertained the fate of 946 birds over the total period of the study, 6 months after they were first recruited. A number of fates were described, which we classified into seven outcomes: Birds may have survived, or they may have been sold or consumed, which are beneficial to the farmer; or they may have been an economic loss, if they died due to disease, predation or accidents, or simply went missing. The only outcome we could verify was if the bird had survived, otherwise we relied on the farmer to report what had happened to the bird in question. However, although we confirmed the identity of 496 survivors, we were also presented at the second visit with another 46 “surviving” birds

which, when we compared photographs, were found not to be part of the original study group. There may therefore be some error in the non-surviving fates of birds reported by farmers, as it is not always possible for them to identify individuals accurately.

Deaths from disease accounted for a considerable proportion of the losses in farmers' flocks. In Horro, 32% of the birds we examined were reported to have died from disease 6 months later. In Jarso, the most common cause of death was predation, while only 9.1% had died from disease; a significantly lower proportion than that in Horro (Chi-square value = 70.8, $p<0.01$). In Horro, significantly fewer of those birds we examined (only 5.7%, compared to 14.8% in Jarso) were later reported to have been killed by predators (Chi-square value = 19.8, $p<0.01$).

Birds recruited in Seasons A and C were rechecked after the end of the wet season, whereas those recruited in Season B were only followed through the months of the dry season. There was no difference in the proportion of birds dying from disease between these two groups (Chi-square value = 1.92, $p=0.16$), although a slightly greater proportion of birds from the Season C group died from disease compared to the other two seasons. (Figure 4.1)

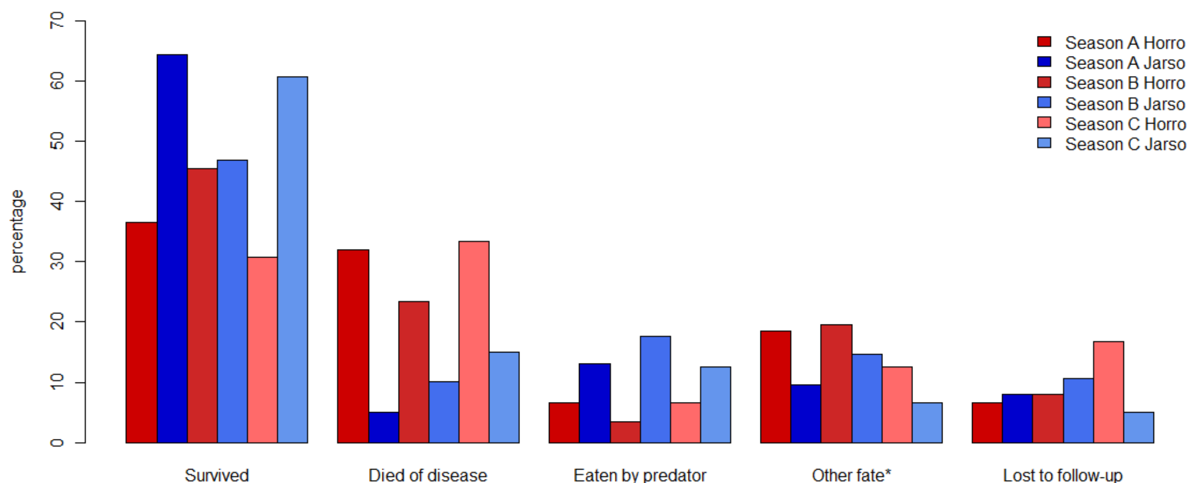

Figure 4.1 Fates of birds at the time of the follow-up visit. Other fate includes sale, consumption accident and missing.

4.3.2 NDV seroprevalence

Only nine birds over all four sampling seasons showed a positive antibody response to Newcastle disease virus by HAI. This equates to an overall seroprevalence of 0.7% (95% C.I. 0.3 - 1.3%). Two birds in village H1B in Season A were in the same household, and the others were individuals in different households in different villages. Of the 9 seropositive birds, two birds with low positive haemagglutinating titres came from households with no history of recent outbreaks or introductions of new birds into the flock, and are most likely to represent imperfect test specificity. All other households had either had an outbreak in the last 4 months, or the birds which tested seropositive were recently introduced to the household (Table 4.1).

Table 4.1 Cases of NDV seropositivity

Farm	Season	Village	Titre	Comments
HA1B22 ±	A	H1B	> 64	Outbreak 2 months previously, 26 birds died
JA1B07	A	J1B	16	No outbreaks, bird bred at home
HB1A06	B	H1A	16	No outbreaks, bird bred at home
HB1B02	B	H1B	> 64	Bird bought in within last 6 months
JB1B06	B	J1B	> 64	Bird bought in within last 6 months
JB2A23	B	J2A	> 64	Outbreak 4 months previously, 3 birds died
HC2A07	C	H2A	> 64	Bird bought in within last 3 months
HD1A14	D	H1A	32	Outbreak 1 month previously, 15 birds died

±Both birds tested in this house were seropositive.

The low seroprevalence may be due to several reasons. One possibility is that the HAI test may have had a low sensitivity. However, it was still able to detect antibodies in the positive control serum even when this was diluted to a concentration of 1 in 2048. In two of the households where seropositive birds were detected and which had had recent outbreaks, the other bird in the household tested negative, despite having reportedly been present in the flock at the time of the outbreak. Assuming that these birds were exposed, it is possible that NDV antibodies wane very quickly following infection and recovery in these birds, or were low to start with. There is little known about the factors governing the

antibody response to NDV, although it has been associated with particular genetic markers in whole-genome studies, and can be highly variable in crossbred chickens (Luo et al., 2013).

The very low prevalence of NDV antibodies throughout the study period, and the failure to detect any seropositive birds in three of the villages meant that we were unable to fulfil our original objective of determining whether mortalities were correlated with changes in NDV seroprevalence. Although more of the randomly-sampled birds in Horro had died from disease than those in Jarso, our data did not confirm that there was a strong seasonal variation. This conflicted with previous reports (Dessie and Ogle, 2001; Halima et al., 2007; Dinka et al., 2010) and evidence gathered during the PRA that high-mortality outbreaks, attributed to NDV, occurred annually during the rainy season, at least at the scale of our study. It was decided to analyse the data collected during the farmer questionnaire surveys to try and describe the farmers' experiences with poultry disease outbreaks, to look for possible explanations for this discrepancy.

4.3.3 Household questionnaire surveys

A total of 640 farms were recruited during the course of the study; their distribution by village and season is shown in Table 4.2. In addition, 494 successful revisits were carried out at the farms recruited in the first three seasons, equating to an overall follow-up rate of over 95%. The main reasons why farms were lost from the follow-up part of the study were poor weather making parts of the village inaccessible, not finding farmers at home and running out of time to complete all visits.

At the first visit, 28 farmers did not give complete details about outbreaks that had occurred in the last 12 months: Of these, 2 failed to answer the question at all; 7 recorded having an outbreak, but could not provide details of when it happened; 17 recorded when

Table 4.2 Distribution of farms recruited to the study by village and season.

Village codes beginning with an H indicate villages from the Horro region, while those beginning with a J indicate villages in the Jarso region. Numbers in parentheses show the percentage of successful follow-up visits.

Season		Village								
		H1A	H1B	H2A	H2B	J1A	J1B	J2A	J2B	Total
A	**May 2011** (Oct 2011)	25 (100%)	25 (100%)	25 (96%)	25 (100%)	25 (100%)	25 (100%)	25 (80%)	25 (80%)	**200** (94.5%)
B	**Oct 2011** (May2012)	25 (96%)	25 (100%)	25 (96%)	25 (96%)	25 (96%)	24 (92%)	25 (96%)	25 (100%)	**199** (96.5%)
C	**May 2012** (Oct 2012)	15 (100%)	15 (93%)	14 (71%)	16 (94%)	15 (100%)	15 (93%)	15 (100%)	15 (100%)	**120** (93.3%)
D	**Oct 2012**	15	15	16	15	15	15	15	15	**121**
	Total	**80** (98.5%	**80** (98.5%	**80** (90.6%	**81** (97%)	**80** (98.5%	**79** (95.3%	**80** (90.8%	**80** (92.3%)	**640** (95.2%)

they had outbreaks, but not how many birds were lost, and 2 were able to estimate when and how many birds were lost, but not the age groups.

At the second visit, 17 farmers failed to answer the question about whether they had had an outbreak in the intervening period. Of 144 farmers who had had an outbreak, 33 were unable to say in which month it occurred, and 10 did not say how many birds were lost.

Based on previous studies, which describe the majority of disease events as causing widespread losses, where morbidity/mortality can affect up to 100% of the flock, we attempted to define “outbreak” as an event where a farmer lost more than one bird to the same presumed disease, in order to try and capture information specific to epidemic infectious diseases in the population. Over the 30 month period covered by our

questionnaires, a total of 332 outbreaks were reported. However, 67 of these were single deaths, and as such they did not meet our original definition of outbreak. In addition, 10 people told us about outbreaks of disease where no birds had died. Some of these events may have been due to an endemic infectious disease or a non-infectious disease. Given the small flock sizes, it is also conceivable that the single deaths were part of an epidemic, but farmers may have only lost one susceptible bird - others may have recovered, or been immune. It is difficult to know how "outbreak" is understood by the farmers, and whether they may be incorporating other information when they decide whether they had an outbreak, such as whether neighbouring households were also affected. The interpretation therefore needs some caution, as the term "outbreak" cannot be taken to be specific to infectious diseases, but may include cases of anything a farmer considers to class as "disease", which may include nutritional or other aetiologies.

In total, there were 230 reported events which conformed to the original definition of at least two birds in the household dying, and had sufficient detail about when they occurred to be included in the analyses. Given the uncertainty over the single deaths, data have been analysed both with and without these events included.

4.3.4 Number of outbreaks

4.3.4(a) Initial questionnaires

Farmers reported between 0 and 3 outbreaks in the twelve-month period preceding the date they received the first questionnaire. However, the only farmers who ever reported having three outbreaks in the last 12 months were those in the Horro region interviewed in Season A (n=6, 6.1%). Of 220 farmers in Horro interviewed over the following 3 rounds, only 5 (2.3%) had had more than one outbreak, and no-one had had more than two. Similarly in Jarso, only 2 (0.6%) respondents over the whole study period ever reported having had more than one outbreak in the preceding 12 months.

The proportion of Horro farmers who reported having at least one outbreak is significantly greater in season A than the other 3 seasons (Chi-square value= 15.7, p< 0.01); this is also true in Jarso (Chi-square value= 14.2, p<0.01). However, the proportion of Jarso farmers who had experienced recent outbreaks is consistently lower than the corresponding proportion of Horro farmers across all four rounds of sampling. The highest percentage of Jarso farmers who had experienced an outbreak was among those sampled in Season A, at 28%: In Horro the percentage of farmers who had suffered an outbreak was never found to be less than 30%, and in Season A the percentage was 58%.

4.3.4(b) Follow-up questionnaires

In the period between the two visits 144 (30.2%) farmers said they had an outbreak, 46 (32%) of whom had reported having outbreaks previously. The lowest proportion of outbreaks during the follow-up period was in the Season B group, who were the only group followed over the dry season; the difference from the two groups followed over the rainy season was almost, but not quite statistically significant. (Chi-square value 5.88, p=0.053).

4.3.5 Timing of outbreaks

The distribution of reported outbreaks over the time period covered is shown in Figure 4.2. It appeared there was a general tendency for more outbreaks to be reported to have occurred during or just after the rainy season. However, the peaks identified by different cohorts of respondents did not quite correspond in timing or magnitude. In particular, there appeared in all cohorts of respondents other than those in Season A, a tendency to report fewer outbreaks from further back in time compared to the more recent periods. We had some concerns that there may be a tendency for people to readily report more recent outbreaks, but to either fail to report outbreaks in the more distant past or to underestimate how long ago they occurred, and to report them as having occurred more recently.

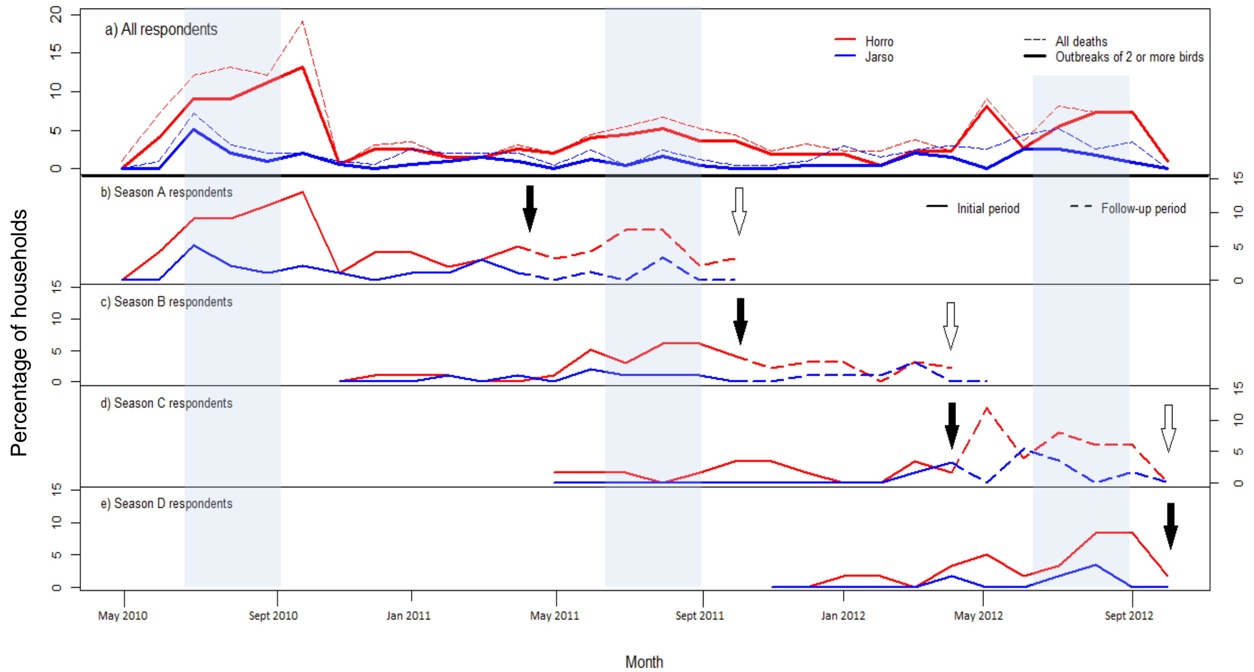

Figure 4.2 Proportion of households reporting outbreaks (with deaths of 2 or more birds) in each month for 2 regions. The graph for all respondents (a) shows an amalgamation of first round and follow-up data for each time period. Solid lines show outbreaks with deaths of 2 or more birds, and dashed lines show all households with at least one death. Graphs b) to e) show the contributions of the four cohorts of respondents. Solid lines show data collected in the initial questionnaire, the timing of which is shown by the solid arrow, where each cohort of respondents is asked to recall outbreaks in the preceding 12 month period. Dashed lines show data collected in the follow-up questionnaires, the timing of which is shown by the white arrows. Shaded areas represent the approximate timing of the main rainy season.

In order to investigate this, we began by splitting the time period covered by the initial questionnaire into two six-month blocks; Period 1 covers the time 6 to 12 months prior to the date of the questionnaire, and period 2 covers the more recent period from 0 to 6 months before the questionnaire took place. Period 3 is the 6 months covered by the follow-up questionnaire. The purpose of this was to compare data for the same time period in respondents interviewed at different times. For example, three different sets of respondents were asked to describe outbreaks which occurred in the period between May 2011 and November 2011. This included Season C respondents, first interviewed in May 2012, for whom they are period 1 outbreaks; Season B respondents, interviewed in

November 2011, for whom they are period 2 outbreaks; and Season A respondents, interviewed for the second time in November 2011, for whom they are period 3 outbreaks (Figure 4.3).

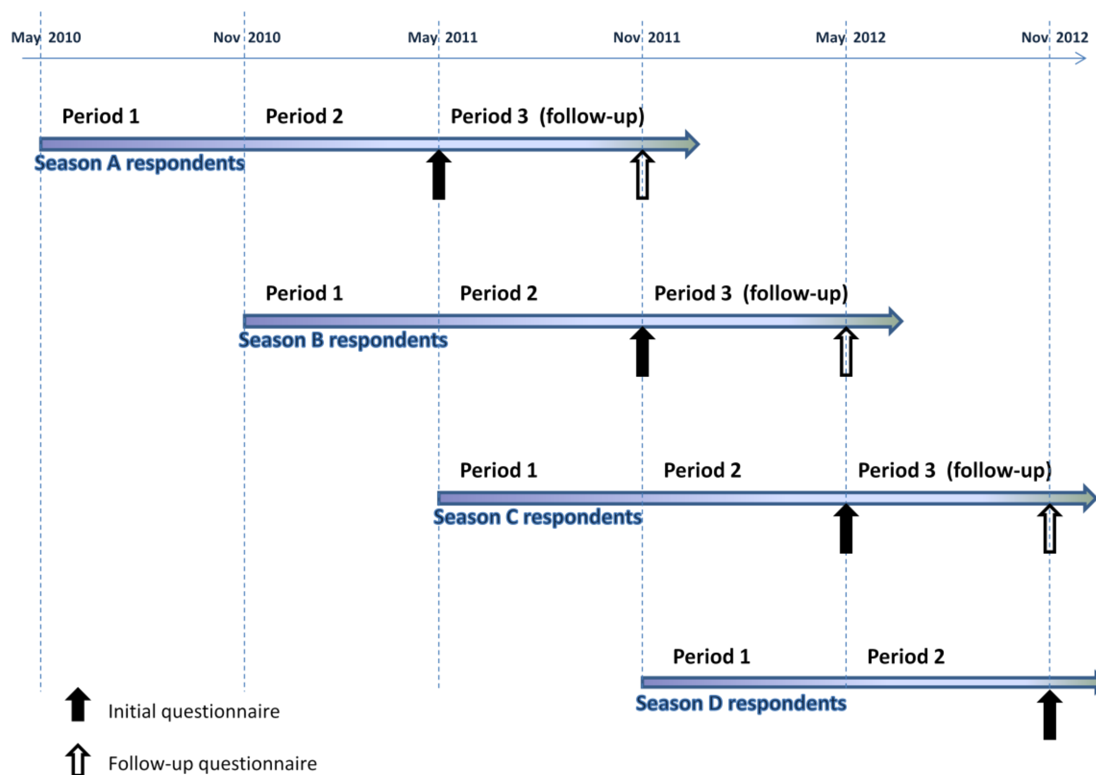

Figure 4.3 Corresponding time periods for different cohorts of respondents

We compared period 1 with period 2 and 3 responses for respondents in the same cohort, and also for respondents in different cohorts for the corresponding time-periods. We found, with the exception of Season A respondents, that only a few farmers reported outbreaks which occurred more than 6 months ago, and a higher proportion of farmers reported outbreaks in any given period if it was more recent (Table 4.3).

Table 4.3 Outbreaks by reporting period for four different cohorts of respondents.

Red cells show periods which were completely within the dry season. Blue cells show periods which include the months of the rainy season. Cells for corresponding time periods are shaded the same colour.

Time period	Respondent cohort: Season A	Season B	Season C	Season D
Period 1. 6-12 months ago	**May-Oct 2010**	**Nov-Apr 2011**	**May-Oct 2011**	**Nov-Apr 2012**
Number of households having any disease	60 (30.5%)	6 (3.0%)	6 (5.0%)	6 (5.0%)
Number of households having >1 death	46 (24.1%)	5 (2.5%)	6 (5.0%)	5 (4.2%)
Median (range) of losses, excluding single deaths	6 (2-48)	6 (2-18)	10 (2-15)	5 (2-8)
Period 2. 0-6 months ago	**Nov-Apr 2011**	**May-Oct 2011**	**Nov-Apr 2012**	**May-Oct 2012**
Number of households having any disease	34 (17.3%)	39 (19.8%)	18 (15.1%)	27 (22.7%)
Number of households having >1 death	24 (12.4%)	29 (14.7%)	9 (8.0%)	19 (16.0%)
Median (range) of losses, excluding single deaths	4 (2-42)	5 (2-45)	4 (2-24)	6 (2-42)
Initial questionnaire and examination				
Period 3. Follow-up 6 month period	**May-Oct 2011**	**Nov-Apr 2012**	**May-Oct 2012**	
Number of households having any disease	64 (34.8%)	45 (23.8%)	35 (33.0%)	
Number of households having >1 death	42 (23.9%)	26 (13.8%)	25 (23.8%)	
Median (range) of losses, excluding single deaths	3.5 (2-100)	3 (2-13)	4 (2-17)	
Follow-up questionnaire				

This effect appeared relatively consistent across villages, with the exception of Village H2B (See Appendix 4.1). The greater number of outbreaks in more recent months was partly explained by more single deaths being reported in more recent time periods – only 10 of the 67 single deaths reported occurred longer than 6 months ago – but even when these

were removed from the dataset, there were still generally more reports of outbreaks in more recent months.

We also observed a tendency for more outbreaks to be reported among respondents in the follow-up period than among respondents interviewed for the first time in the corresponding season. (i.e. comparing period 3 with period 2 for equivalent time periods: Table 4.3). This effect was consistently seen across all villages, again with the exception of Village H2B (Appendix 4.1). We therefore had some concern that we may have introduced an effect of priming in respondents interviewed twice, leading them to report more outbreaks at the second time of asking. This effect could also have been seen if we had caused iatrogenic spread of disease into their farm when visiting for the first time.

Using random effect logistic regression models to estimate which factors influenced reporting of outbreaks (detailed in Appendix 4.2), it was found that the odds of respondents in Horro reporting a disease outbreak were 4.05 (95%C.I. 2.93 – 5.60) greater than those in Jarso. The odds of reporting an outbreak in the rainy season (i.e. in the months of June to September) were 1.87 (95%C.I. 1.40 – 2.50) greater than the odds of one being reported in the dry season.

The odds of reporting an outbreak increase by 1.3 (95% C.I. 1.09 – 1.55) for each month that the time interval between the outbreak and the interview decreased, up to a limit of 4 months before the interview (Figure 4.4). If an outbreak happened within the last 4 months, there was no difference in the odds of reporting it, regardless of when in this period it happened. However, for Season A respondents, there was no change in the odds of reporting for up to 10 months before the interview. There was no difference in the odds of outbreak reporting between the first and second interviews, once factors such as time elapsed and wet/dry months were taken into account. Including the single deaths in the

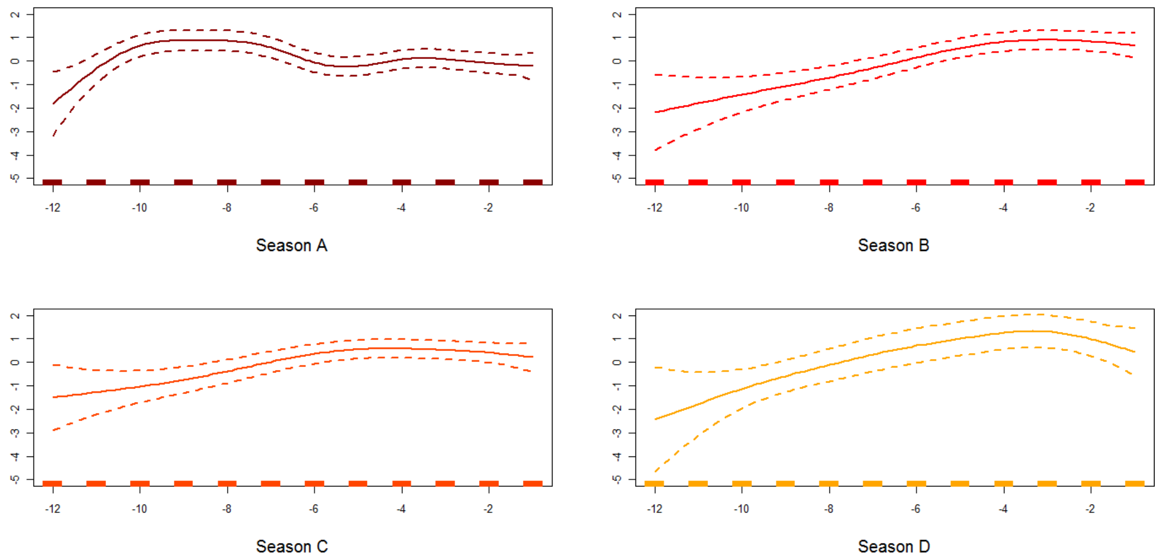

Figure 4.4 Generalised additive model plots showing the change in reporting with respect to time elapsed for the four cohorts of respondents.

model made little difference to the parameters, other than increasing the effect of the time elapsed for all but the Season A farmers; reflecting the fact that very few single deaths were reported above 6 months before the interview in any other cohort.

These results suggests a difference in recall between farmers interviewed in Season A (May 2011) and the farmers who were interviewed in other seasons. In this group there were more outbreaks reported, especially in Horro, over the rainy season of 2010, which occurred 8-11 months previous to the date of the interviews. This could reflect an unusually large number of outbreaks in that period, or a greater than normal severity, which has caused people to recall them more readily. When taken in conjunction with the increased number of farmers who had more than one outbreak and the decreased proportion who had no outbreak in this season, it seems feasible that there was an epidemic in the year preceding the commencement of our fieldwork, but that no such outbreak occurred in the following two years, during the time we were working in the

villages. If this is the case, it may be that the deaths recorded in the birds we examined, which did not show a pronounced seasonal variation, may be more a reflection of the endemic patterns of disease seen. Since the data collection finished, we have heard second-hand of another large outbreak which occurred in Horro early in 2013. This has been of particular concern to farmers, as it has occurred in the middle of the dry season, when they do not usually expect large outbreaks to occur (Terfa, personal communication).

4.3.6 Losses

In total, 303 households were able to provide month-specific data on how many birds they lost in a disease event. If a household had an outbreak in Horro, the median loss reported was 4 birds (range 1 – 100). In Jarso, the median loss was 2 birds (range 1-17). If single losses were excluded, the median values for Horro and Jarso rose to 5 and 3 birds, respectively. Although more outbreaks occur during the months of the rainy season, outbreaks causing high mortalities were reported year-round in both regions (Figure 4.5).

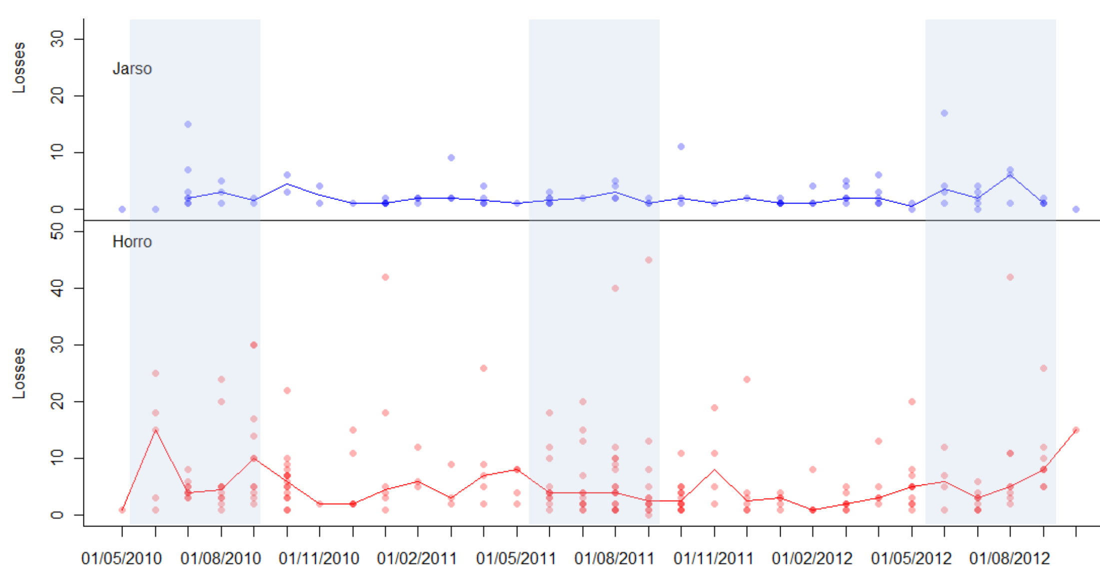

Figure 4.5 The reported numbers of birds which died on each farm by the month the farmer reported an outbreak occurred. Lines represent the median loss for each month. Two farms reporting losses of 90 and 100 birds each have been excluded from the Horro plot.

Multilevel modelling was also used to investigate the effects of memory and priming on respondents' estimates of loss, given that they reported an outbreak (Appendix 4.3). Firstly, a binomial model was used to estimate variables which contributed to a household reporting either one or more than one death. Secondly, we only considered the 230 events where two or more birds died, and investigated whether any factors contribute to households reporting greater losses, using a Poisson regression model.

The only factor which contributed significantly to farmers reporting more than one bird lost was the region, with farmers in Horro over 7 times more likely (95% C.I. 3.5 – 15.3) to report multiple deaths than those in Jarso.

Given that farmers had lost at least two birds, a number of factors were found to influence the number of birds reported lost. This included the village, season, respondent cohort, time of asking, and the interval between outbreak and interview. However, it was found that this model was heavily weighted by two observations, both from the same village and farmer cohort, where the farmers reported losses of 90 and 100 birds respectively at the second interview. These estimates were considered to be somewhat inflated, given that we never observed any flocks larger than 45 birds. The two farms in question had had flocks of 19 and 15 birds respectively at the first interview.

When these observations were excluded, the only factors which contributed to variation were region, season and whether it was the first or second interview. Farmers in Horro tended to lose slightly greater numbers of birds than those in Jarso (5.9 compared to 3.4), but farmers in both areas tended to report slightly fewer birds lost during the rainy season. This may be due to the common practice of farmers selling birds before the rainy season in anticipation of outbreaks, and thus a reduction in flock sizes over this period (Moges et al., 2010a).

Higher losses in Horro may be due, at least in part, to the larger flock sizes in this area. Unfortunately we did not collect data on the flock size at the time of the outbreak or the proportion of the flock affected, although this may have been difficult for farmers to provide over such an extended time period. However, we did collect data during the follow-up visits on the number of birds which were affected and recovered or did not become sick at the time of any outbreak reported in this period. This will be discussed in the next section.

It was also observed that there was a tendency to report fewer deaths per outbreak at the second interview, independent of region or season. It is possible that some farmers interviewed the first time may have inflated their estimates slightly. It was remarked by some of the fieldworkers that the appearance of a "*farenji*" (foreigner) in the village led many farmers to expect aid donations, and so they may have exaggerated the disease losses in order to exact sympathy. They may also have been primed by the first interview to be more observant of losses in the follow-up period; and so able to report more accurately at the second time of asking.

4.3.7 Clinical presentation

For the 144 outbreaks reported to occur during the follow-up period, owners were asked some additional questions relating to the symptoms shown, and the number of birds which had died, been clinically affected and recovered, or had shown no symptoms. The most commonly reported symptoms were diarrhoea and depression, both observed in 45% of the reported outbreaks. These two symptoms were observed together in 20% of cases. Other reported signs were paralysis (17%), sudden death (13%), respiratory problems (2%), eye problems (2%), inappetance (2%), prolapsed vent (1%), haemorrhagic nasal discharge (1%), shaking (1%) and wounds under the wings (1%).

Owners were also asked to describe the symptoms in the randomly-sampled birds which died from disease. The symptoms reported were similar to those above, with 39% of birds which had died having exhibited both depression and diarrhoea. Approximately two thirds of the randomly-sampled birds which had died had exhibited the same symptoms as other birds in the household flock.

Mortality rates in each household flock were estimated from the number of birds reported to have died, compared to the numbers which recovered or were not affected. These ranged from 0 to 100%, but showed no associations with the set of symptoms displayed, the village or the season of sampling.

4.3.8 Age affected

The majority (46.5%) of reported outbreaks affected only adult birds (over 6 months old), but outbreaks affecting all age groups were reported throughout the year (Appendix 4.4). The majority (87%) of outbreaks which only affected chicks (under 2 months) and 59% of outbreaks which only affected growers (2-6 months old) occurred between June and October, i.e. during or shortly after the rainy season.

Although farmer reports indicated a seasonal pattern of disease in village chickens, from our observations this manifested as an increase in the number of households experiencing disease losses during the rainy season, the majority of which were relatively small-scale, whereas outbreaks causing high mortalities occurred sporadically throughout the year. The seasonal pattern seen possibly reflects the additional stresses put on the birds in the wet season, when they may have to cope with cold weather and food shortages, which leads to them succumbing to one of a number of chronic or endemic infections. This would explain the disproportionate number of outbreaks in young stock, which are probably more affected by the climactic and nutritional stresses, at this time of year. It may, alternatively or in addition, reflect an increase in the infection rates, as most pathogens will survive

more readily in a cool and moist environment, and birds may have to forage more widely, increasing the opportunity for either direct or indirect transmissions. The wide variety of clinical presentations, including frequent single deaths, and highly variable mortality rates would support the role of a variety of infectious and non-infectious causes in the reported outbreaks.

4.4 Conclusions

Previous studies report outbreaks of "*fengele*" to be seasonal and to occur annually during the rainy season (Dessie and Ogle 2001, Halima 2007, Dinka 2010). The translation of "*fengele*" as Newcastle Disease by a number of researchers may be inaccurate, as the seasonal increase in mortalities associated with the rainy season, at least in our study, would seem to be largely due to an increased frequency of small-scale losses. Our data would suggest that over time, many of these small outbreaks and single deaths are forgotten by farmers, who focus on the more memorable large-scale losses. Although we cannot rule out endemic Newcastle disease as being the cause of these small-scale losses, the seroprevalence of Newcastle disease antibodies in this population was remarkably low, and we would expect the population to be highly susceptible. This may be supported by the fact that a large outbreak was reported to have occurred in Horro a few months after our last round of sampling.

Instead, we hypothesise that a number of diseases are contributing to the seasonal mortality which have been lumped together under the "*fengele*" umbrella. Our data would suggest that during the two years the study ran in these villages, there was no epidemic in these regions, thereby disputing the claim that such outbreaks are an annual occurrence (at least at the geographic scale of our study). Controlling Newcastle disease is undoubtedly important in order to prevent such outbreaks, but other diseases should not be

overlooked. Farmers incentivised to vaccinate against *"fengele"* may lose motivation if they expect a Newcastle disease vaccine to protect against a multitude of diseases, and it does not live up to their expectations. One possibility, which we were unable to investigate due to restrictions imposed by the funding bodies for this work, is that avian influenza virus, which may also cause high mortality outbreaks, could be circulating undetected in these regions. However, general and targeted surveillance measures in Ethiopia have never detected highly pathogenic avian influenza, and notifiable low pathogenic avian influenza has not been reported in Ethiopia since 2006 (O.I.E., 2013).

If what we have observed in these villages is a representation of the ongoing losses, then endemic diseases are a significant burden on the population, even before large epidemics are taken into account. Approximately one third of the adult population of Horro chickens that were randomly sampled died of disease within 6 months. Reducing the impact of endemic disease may therefore be of economic benefit to farmers, for some of whom even the death of one chicken can represent the loss of a significant proportion of the flock. However, there were clearly large differences in the impact of disease between the two regions. Therefore it cannot be assumed that any strategies for disease control will have an equal impact in different areas, but that different focuses may be required, depending on the local needs. Measures which reduce predation may be of as much or more benefit to farmers in some regions, including Jarso, to reduce losses in adult birds.

Appendix 4.1

Table 4.4 Percentage of farmers in each village reporting any disease event for each reporting period. Red cells show periods which were completely within the dry season. Blue cells show periods which include the months of the rainy season.

	Time Period	Village	A	B	C	D	Village	A	B	C	D	Village	A	B	C	D	Village	A	B	C	D
Horro	6-12 months ago	H1A	45.8	4.0	13.3	0.0	H1B	56.0	0.0	13.3	0.0	H2A	56.0	8.0	15.4	14.3	H2B	36.0	0.0	0.0	13.3
	0-6 months ago		20.8	36.0	26.7	46.7		20.0	20.8	20.0	20.0		24.0	32.0	15.4	35.7		12.0	41.7	18.8	20.0
	Initial questionnaire and examination																				
	6 month follow-up		54.2	25.0	42.9			45.8	28.0	42.9			65.2	33.3	66.7			37.5	4.6	23.1	
	Follow-up questionnaire																				
Jarso	6-12 months ago	J1A	8.7	4.0	0.0	6.7	J1B	4.2	0.0	0.0	0.0	J2B	24.0	0.0	0.0	0.0	J2B	8.0	8.0	0.0	6.7
	0-6 months ago		16.7	8.0	20.0	6.7		4.2	0.0	13.3	13.3		16.0	8.0	6.7	20.0		24.0	12.0	0.0	20.0
	Initial questionnaire and examination																				
	6 month follow-up		24.0	33.3	21.4			12.5	14.3	35.7			10.0	25.0	20.0			26.3	25.0	23.1	
	Follow-up questionnaire																				

Season

Table 4.5 Percentage of farmers in each village reporting >1 death for each reporting period. Red cells show periods which were completely within the dry season. Blue cells show periods which include the months of the rainy season.

		Season																		
	Time Period	Village	A	B	C	D	Village	A	B	C	D	Village	A	B	C	D	Village	A	B	C	D
Horro	6-12 months ago	H1A	40.9	4.0	13.3	0.0	H1B	34.8	0.0	13.3	0.0	H2A	47.8	8.0	15.4	13.3	H2B	32.0	0.0	0.0	13.3
	0-6 months ago		13.0	32.0	7.1	40.0		20.0	16.7	13.3	20.0		20.8	20.0	15.4	26.7		12.0	32.0	7.1	20.0
		Initial questionnaire and examination																			
	6 month follow-up		29.4	12.5	35.7			38.1	17.4	42.9			47.1	22.7	55.6			21.7	4.5	16.7	
		Follow-up questionnaire																			
Jarso	6-12 months ago	J1A	8.7	4.0	0.0	6.7	J1B	0.0	0.0	0.0	0.0	J2B	20.0	0.0	0.0	0.0	J2B	4.0	4.0	0.0	0.0
	0-6 months ago		8.7	0.0	14.3	0.0		0.0	0.0	7.1	6.7		12.0	8.0	0.0	7.1		8.0	8.0	0.0	6.7
		Initial questionnaire and examination																			
	6 month follow-up		12.0	9.1	8.3			0.0	0.0	8.3			5.3	9.5	13.3			0.0	9.1	15.4	
		Follow-up questionnaire																			

Appendix 4.2

In order to model factors which influenced the probability of a farmer reporting an outbreak, data were first organised to generate one observation for every month that a farm was asked about in the study. Thus, farms that were interviewed twice had 18 observations, and those interviewed only once had 12. Variables were created for the first or second time of interviewing, and the length of time elapsed between the recalled month and the interview. Also a categorical variable was introduced to define whether the month in question was in the wet or dry season.

Table 4.6 Example of data organised for random-effect models of outbreak reporting

	FarmUnID	SeasonID	RegionID	Elapse	AskTime	Rain	OB	Loss
1	HA1A01	A	H	-12	1	Dry	0	0
2	HA1A01	A	H	-11	1	Wet	0	0
3	HA1A01	A	H	-10	1	Wet	1	5
4	HA1A01	A	H	-9	1	Wet	1	3
5	HA1A01	A	H	-8	1	Wet	0	0
6	HA1A01	A	H	-7	1	Dry	0	0
7	HA1A01	A	H	-6	1	Dry	0	0
8	HA1A01	A	H	-5	1	Dry	0	0
	...	...	...	...	...	...	...	...

A multilevel model, with farm as a random effect, was fitted to the data, using the formula shown below. An interaction term between the season of asking and the time elapsed since the interview was found to be significant, but other interactions did not significantly improve the model fit.

```
Generalized linear mixed model fit by the Laplace
approximation
Formula: OB ~ relevel(RegionID,"J") + Rain + relevel(SeasonID,
"B")* lapse2 + as.factor(AskTime) + (1 | FarmUnID)

Random effects:
 Groups   Name        Variance Std.Dev.
 FarmUnID (Intercept)  0        0
Number of obs: 10219, groups: FarmUnID, 639
```

Table 4.7 Model coefficients for probability of outbreak reporting

Fixed effects		Coefficient	SE	Odds Ratio	95% C.I.	z	P
(Intercept)		-3.86	0.49	0.02	0.01 - 0.05	-7.92	<0.01
Region	Jarso	reference					
	Horro	**1.40**	**0.17**	**4.05**	**2.93 - 5.60**	**8.47**	**<0.01**
Season	Dry	reference					
	Wet	**0.63**	**0.15**	**1.87**	**1.40 - 2.50**	**4.25**	**<0.01**
Interview	B	reference					
	A	-0.73	0.52	0.48	0.17 - 1.34	-1.40	0.16
	C	-0.17	0.64	0.84	0.24 - 2.93	-0.27	0.78
	D	0.03	0.74	1.03	0.24 - 4.42	0.04	0.96
1st interview		reference					
2nd interview		0.02	0.17	1.02	0.73 - 1.42	0.10	0.92
							Continuous variables
Interval (per month for periods > 4 months)		**0.26**	**0.09**	**1.30**	**1.09 - 1.55**	**2.97**	**<0.01**
Interactions							
Season A: Interval period		**-0.26**	**0.10**	**0.77**	**0.63 - 0.93**	**-2.71**	**0.01**
Season C: Interval period		-0.07	0.12	0.93	0.74 - 1.18	-0.60	0.55
Season D: Interval period		-0.02	0.14	0.98	0.75 - 1.28	-0.15	0.88

Appendix 4.3

Logistic regression modelling the variables contributing to farmers reporting either one or more than death, given that they report an outbreak to have occurred. A stepwise selection of variables suggested that only region contributed significantly.

```
Formula: Loss > 1 ~ relevel(RegionID, "J") + (1 | FarmUnID)
   Data: ltim[which(ltim$Loss > 0), ]

   AIC   BIC logLik deviance
 292.6 303.7 -143.3    286.6

Random effects:
 Groups   Name        Variance Std.Dev.
 FarmUnID (Intercept) 1.5248   1.2348

Number of obs: 297, groups: FarmUnID, 236
```

Table 4.8 Binomial (logistic) regression model coefficients for more than one death, compared to single death events

Fixed effects		Coefficient	SE	Odds Ratio	95% C.I.		z	P
(Intercept)		0.29	0.27	1.34	0.78	- 2.30	1.08	0.28
Region	**Jarso**	**reference**						
	Horro	**1.99**	**0.37**	**7.35**	**3.54**	**- 15.25**	**5.36**	**<0.01**

A Poisson regression model was then fitted only to those households which had lost more than one bird in their reported outbreak, to estimate what factors influenced the number of birds reported lost. All the measured variables contributed significantly to the variation, with significant interactions between the farmer cohort and the season, and the farmer cohort and the time elapsed between outbreak and interview. However, this was found to be heavily weighted by two observations where two households in one season reported losing 90 and 100 birds at the second visit. When these observations were excluded, only Region, Season and the time of asking contributed significant variation.

```
Formula: Loss ~ relevel(VillageID, "J2B") + as.factor(AskTime)
+ Rain *relevel(SeasonID, "C") + relevel(SeasonID, "C")*
lapse2 +       (1 | FarmUnID)

   AIC   BIC logLik deviance
 701.5 773.7 -329.8    659.5

Random effects:
 Groups   Name        Variance Std.Dev.
 FarmUnID (Intercept) 0.55015  0.74172
Number of obs: 230, groups: FarmUnID, 183
```

Table 4.9 Poisson regression model coefficients for number of birds dying in an outbreak

Fixed effects		Coefficient	SE	Relative risk	95% C.I.		z	P
(Intercept)		0.06	0.47	1.07	0.43	-2.67	0.14	0.89
Categorical variables								
Village	J2B	reference						
	H1A	**0.75**	**0.34**	**2.11**	**1.09**	**- 4.08**	**2.21**	**0.03**
	H1B	**0.72**	**0.35**	**2.06**	**1.05**	**- 4.07**	**2.09**	**0.04**
	H2A	**0.76**	**0.34**	**2.14**	**1.10**	**- 4.16**	**2.25**	**0.02**
	H2B	0.56	0.35	1.75	0.88	- 3.49	1.60	0.11
	J1A	-0.04	0.41	0.96	0.43	- 2.12	-0.10	0.92
	J1B	0.13	0.63	1.13	0.33	- 3.91	0.20	0.84
	J2A	0.43	0.38	1.54	0.73	- 3.27	1.13	0.26
1st interview		reference						
2nd interview		**0.78**	**0.11**	**2.19**	**1.75**	**- 2.73**	**6.90**	**<0.01**
Season	**Dry**	**reference**						
	Wet	**-0.55**	**0.24**	**0.57**	**0.36**	**- 0.91**	**-2.34**	**0.02**
Interview cohort	C	reference						
	A	0.16	0.38	1.18	0.56	- 2.47	0.43	0.66
	B	**-1.13**	**0.55**	**0.32**	**0.11**	**- 0.95**	**-2.05**	**0.04**
	D	**2.83**	**1.07**	**16.95**	**2.08**	**- 138.31**	**2.64**	**0.01**
Continuous variables								
Interval period (> 4 months)		**-0.15**	**0.05**	**0.86**	**0.79**	**- 0.94**	**-3.29**	**<0.01**
Interactions								
Season A: Wet season		0.04	0.26	1.04	0.63	- 1.73	0.16	0.87
Season B: Wet season		**1.64**	**0.37**	**5.14**	**2.51**	**- 10.54**	**4.47**	**<0.01**
Season D: Wet season		-0.11	0.58	0.90	0.29	- 2.79	-0.19	0.85
Season A: Interval period		0.01	0.05	1.01	0.91	- 1.11	0.13	0.89
Season B: Interval period		-0.10	0.08	0.91	0.77	- 1.07	-1.14	0.25
Season D: Interval period		**0.39**	**0.15**	**1.47**	**1.09**	**- 1.99**	**2.52**	**0.01**

Model excluding two outlying observations

```
Formula: Loss ~ relevel(RegionID, "J") + as.factor(AskTime) +
Rain + (1 |       FarmUnID)
   Data: ltim[which(ltim$Loss > 1 & ltim$Loss < 60), ]

   AIC   BIC logLik deviance
 538.8 555.9 -264.4    528.8

Random effects:
 Groups    Name        Variance Std.Dev.
 FarmUnID (Intercept) 0.38991  0.62443

Number of obs: 228, groups: FarmUnID, 182
```

Table 4.10 Poisson regression model coefficients for the number of birds dying in an outbreak, excluding outlying observations

Fixed effects		Coefficient	SE	Relative risk	95% C.I.		z	P
(Intercept)		1.21	0.14	3.36	2.57	- 4.39	8.84	<0.01
Region	Jarso							
	Horro	**0.58**	**0.14**	**1.78**	**1.35**	**- 2.34**	**4.08**	**<0.01**
1st interview								
2nd interview		**-0.31**	**0.09**	**0.73**	**0.62**	**- 0.87**	**-3.54**	**<0.01**
Season	**Dry**							
	Wet	**0.17**	**0.08**	**1.18**	**1.00**	**- 1.39**	**2.01**	**0.04**

Appendix 4.4

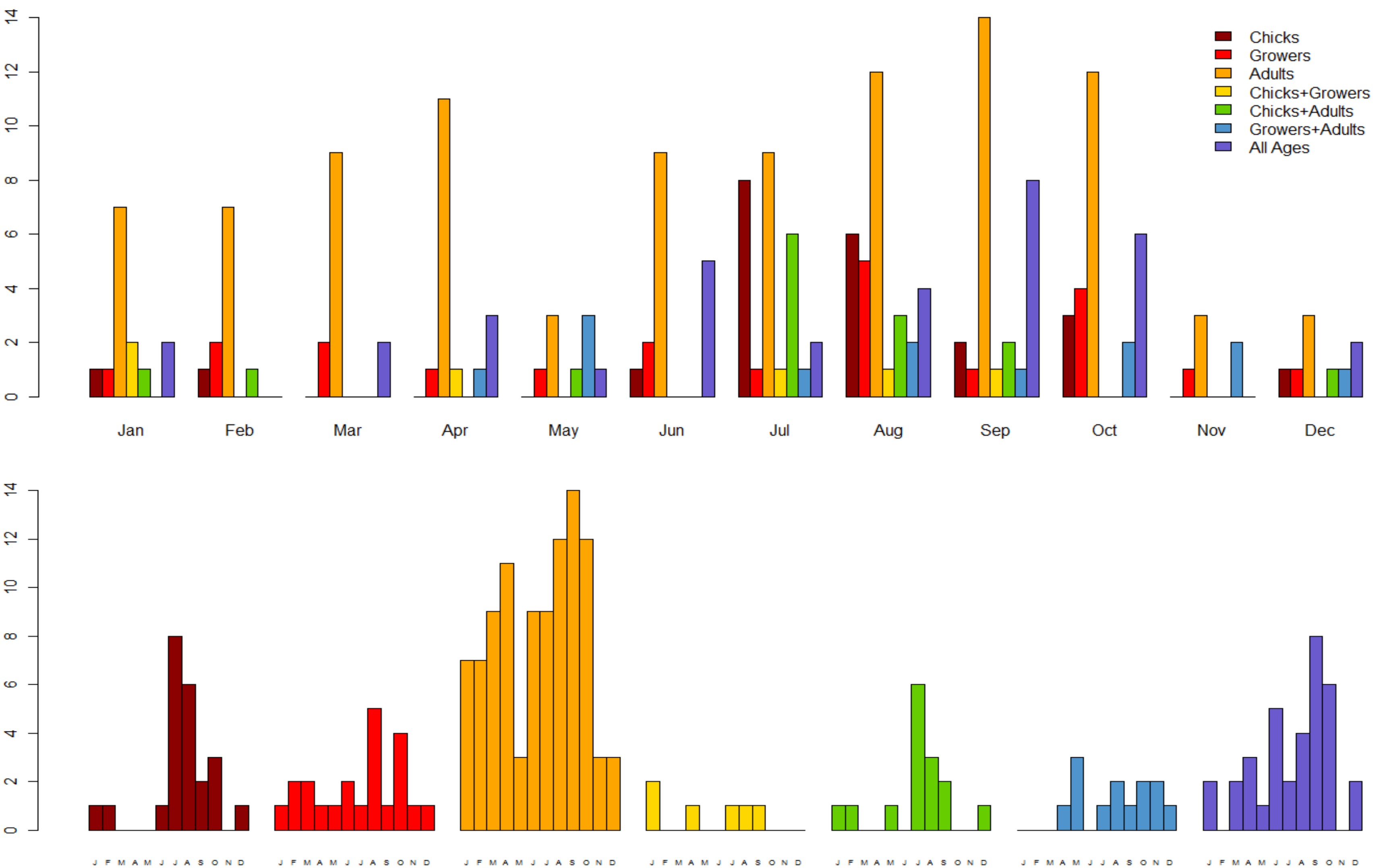

Figure 4.6 Reported outbreaks showing age groups affected across different months

Chapter 5

Determining risk factors for 6-month survival and alternative outcomes

5.1 Introduction

Disease, and in particular Newcastle disease (ND), is widely reported to be one of the major constraints to village chicken production, and it is well established that vaccination to control ND can bring economic benefits to rural producers (Dwinger and Unger, 2006, Bell, 2009, Alders et al., 2010). Although epidemic outbreaks may have a devastating impact on chicken stocks, ongoing losses of birds to endemic diseases and predation represent another drain on farmers' resources. These losses also deserve consideration, as the small flock sizes kept can mean that loss of even one bird may represent a considerable proportion of the chicken resources owned. Furthermore the necessity of having to replace these ongoing losses reduces the availability of chickens and eggs which would otherwise be valuable for income, nutritional or social purposes (Dessie and Ogle, 2001).

Although Ethiopian farmers interviewed in peri-urban villages can recognise and describe a variety of diseases (Sambo, 2012), they tend to designate any acute disease with high mortality as "*fengele*", which was described by Dessie and Ogle (2001) as a syndrome comprising of inappetance, watery, yellowish droppings, paralysis and eventually death. Outbreaks of "*fengele*" are reported to be seasonal and to occur annually during the rainy season (Dessie and Ogle 2001, Halima 2007, Dinka 2010). Newcastle disease has certainly been reported to show seasonal patterns in several other countries, often associated with seasonal change or periods of climactic stress (Martin, 1991). Other factors may also contribute to a seasonal appearance of epidemics, such as holidays which increase trading activity, seasonal peaks in breeding which increase the susceptible population (Awan et al., 1994) and seasonal alterations in the scavenging resources available (Moges et al., 2010), placing birds under nutritional stress and potentially altering the distances they must travel to find food, which can alter their exposure to direct and indirect sources of transmission.

These seasonal factors will not only affect ND epidemiology, but could impact on the biology of a range of micro- and macroparasite infections by altering either exposure or tolerance. A number of pathogens survive better in cool, moist environments, including the influenza and ND viruses and the protozoan parasite, *Eimeria* (Swayne and Halvorson, 2008, Martin, 1991, McDougald, 2003), whilst the long dry season can impede the development of nematode larvae (Weaver et al., 2010). For those parasites with an indirect lifecycle, seasonal fluctuations in rainfall and temperature may alter the abundance or behaviour of the intermediate hosts, such as snails, earthworms, beetles and flies. Seasonal changes can affect how pathogens interact with their hosts; for example, mycoplasma infections affect birds more severely and for longer periods during cold weather (Ley, 2008). Physical stresses, such as chilling, overheating, nutrient or water deficiency, or concurrent infections can reduce birds' ability to tolerate even organisms normally considered to be commensal or of low pathogenicity (Bermudez, 2008). As such, seasonal changes might be expected to be associated with the recrudescence of chronic infections, such as *Pasteurella* or *Salmonella*, or add to the immunosuppressive effects of viruses such as Marek's disease virus (MDV) or infectious bursal disease virus (IBDV).

Our observations of a very low seroprevalence of ND antibodies across the entire time period of this study, described in Chapter 4, together with an absence of any reports of large-scale focal mortalities, are consistent with this study having taken place during an inter-epidemic phase. However, farmers still reported a seasonal increase in mortalities over the rainy season, which were frequently described as "*fengele".* We hypothesise that a number of infections are contributing to the mortalities which have been aggregated within the umbrella of the annual "*fengele"* problem, and that pathogens other than ND virus (NDV) may be a cause of significant loss either in their own right or in combination. Due to the difficulty in accessing the remote areas in which the study villages were situated during the rainy season, it was not feasible to investigate the chicken deaths via necropsies

and pathogen isolation, especially since the lack of reported large outbreaks would have necessitated a considerable time period spent in each village to carry out post mortems on a sufficient number of birds. Therefore in order to explore our hypothesis we used the data we collected at the time of the birds' recruitment to the study to see if previous exposure to various infections was associated with survival or loss at a follow-up time point, approximately 6 months later.

The pathogens chosen for investigation have all been previously identified in village chickens in Ethiopia. In addition, several have features which may make them of particular relevance to a bird's longer-term survival, such as the ability to cause immunosuppression (MDV and IBDV); a variable latent period, with clinical signs appearing weeks to months after initial infection (MDV); or chronic, inapparent or mildly debilitating infections, which may become of greater relevance when other stressors are present (*Salmonella, Pasteurella*, macroparasitic infections). Although our main interest was in whether these infections might be associated with later death from disease, it is also possible that some may be sufficiently debilitating to put a bird at greater risk of predation. Modelling the risk of death is further complicated by the fact that removal of birds by sale, consumption or culling would censor them from other causes of death, including disease or predation, and that these outcomes are influenced by human decisions. Such decisions may have little to do with the biological factors we have measured or alternatively may be influenced (consciously or inadvertently) by characteristics of individual birds. Some characteristics which might be anticipated to influence the decision to sell, such as weight, which affects the market price (Dana et al., 2010) can potentially be affected by a bird's infection status. The aim of this study was therefore to develop a series of models to try and describe the contribution of various risk factors to a bird's fate over a six-month period.

5.2 Methods

Sampling was conducted between May 2011 and November 2012 in two geographically separate woredas (administrative districts) in the Oromia region of Ethiopia; Horro, in the western highlands, and Jarso in the east. Two market sheds (a group of villages linked by trading networks, as described by Tadelle et al. (2003)) were purposely selected within each region, and two kebeles (smaller administrative districts, roughly equivalent to a ward) were selected within each of the four market sheds. For each of the eight kebeles, lists of farmers were compiled by the local development agents (DAs) and potential participants were selected from the final list by systematic random sampling. Selected households were invited to take part in a questionnaire survey and for two of their chickens to be sampled. A questionnaire interview was conducted in the local language either with the person with main responsibility for caring for the birds or with the head of the household, in order to collect data on the management practices and the disease history of the flock.

Two randomly selected birds from the household flock were examined for signs of clinical disease and ectoparasite infestation, and blood samples were collected from a wing vein into sodium citrate. Wherever possible, a faecal sample was collected from the basket where the bird had been confined, or where the bird was observed to defecate after release. If a faecal sample could not be collected from the bird, a fresh sample was taken from the household environment. A series of photographs were taken of each bird, comprising a lateral, dorsal and frontal view, and close-up shots of the head, feet and skin, and birds were fitted with a leg ring before release.

Blood and faecal samples were stored and transported in a refrigerated container to the laboratory in Debre Zeit, where the plasma was separated from the cell pellets and stored

at -20°C. The faecal samples were kept at 4°C until processed. Blood samples were tested by ELISAs for antibodies to Infectious bursal disease virus, Marek's diseases virus, *Pasteurella multocida* and *Salmonella O9* serotypes. Modified McMaster's egg counts were performed on faeces, and *Eimeria* oocysts and different species of nematode and cestode eggs were counted separately.

A follow-up visit was carried out to the recruited households around 6 months later to determine the fate of the two previously sampled birds. Three cohorts of farms were recruited over the study period; two cohorts recruited in May of the two consecutive years of the study were revisited in October after the rainy season. One cohort recruited in October was followed only through 6 months of the dry season and revisited the following May. Surviving birds were re-examined and their identification confirmed using leg rings and photographs. If a bird had not survived, farmers were asked to describe what had happened to it.

Farmers described a variety of different outcomes which were grouped into four categories: Survival, Death from disease, Gain (which comprised of outcomes which were beneficial to the farmer, namely either sale or consumption of the birds) and Loss (which was predominantly made up of birds which were predated, but also those which went missing, or died due to some other accident).

All models and graphical summaries were performed using R (R Development Core Team, 2008). For each of the four outcomes (Survival, Disease, Gain and Loss), intercept-only random effect regression models were used to estimate the variance partitioning using the binary linearization and the latent variable methods (Goldstein et al., 2002). This partitioning, also known as the intraclass correlation coefficient (ICC), provides an estimation of the distribution of the observed variation that is attributable to the different levels (birds within farms within villages within market sheds within regions). Multilevel

logistic regression and Poisson log-linear models were used to examine the potential associations of variables measured at the time of the first visit with the different bird outcomes recorded at the second visit. Additional details of how each of the models was constructed can be found in Chapter 2. Explanatory variables were fitted stepwise for each model, such that only those which significantly improved the fit of the model ($p<0.05$) were retained. A list of explanatory variables with their definitions is given in Appendix 5.1. Different structures of nested models were used to explore some particular results, due to the multinomial nature of the response, and the difficulty with interpreting some of the model outputs.

The following nest structures were used (Figure 5.1):

1) A two-step approach based on survival, first separating surviving birds from the non-survivors with a logistic regression model, and then using a multinomial approach to assess the odds of each non-surviving outcome with a Poisson log-linear model.
2) A two-step approach based on human agency, which used a nested series of logistic regression models, initially assigning birds to either being lost to natural causes (Disease or Loss outcomes), or available for human uses (Survival or Gain outcomes). A second model was then used within each category to estimate the odds of the outcomes within it.
3) A four-way multinomial approach, using a Poisson log-linear model to investigate all outcomes simultaneously.

Mapping of model residuals was performed using the RgoogleMaps package (Loecher, 2011), and spatial tests were performed using the spatial test function (Eagle, unpublished) as described in Chapter 2.

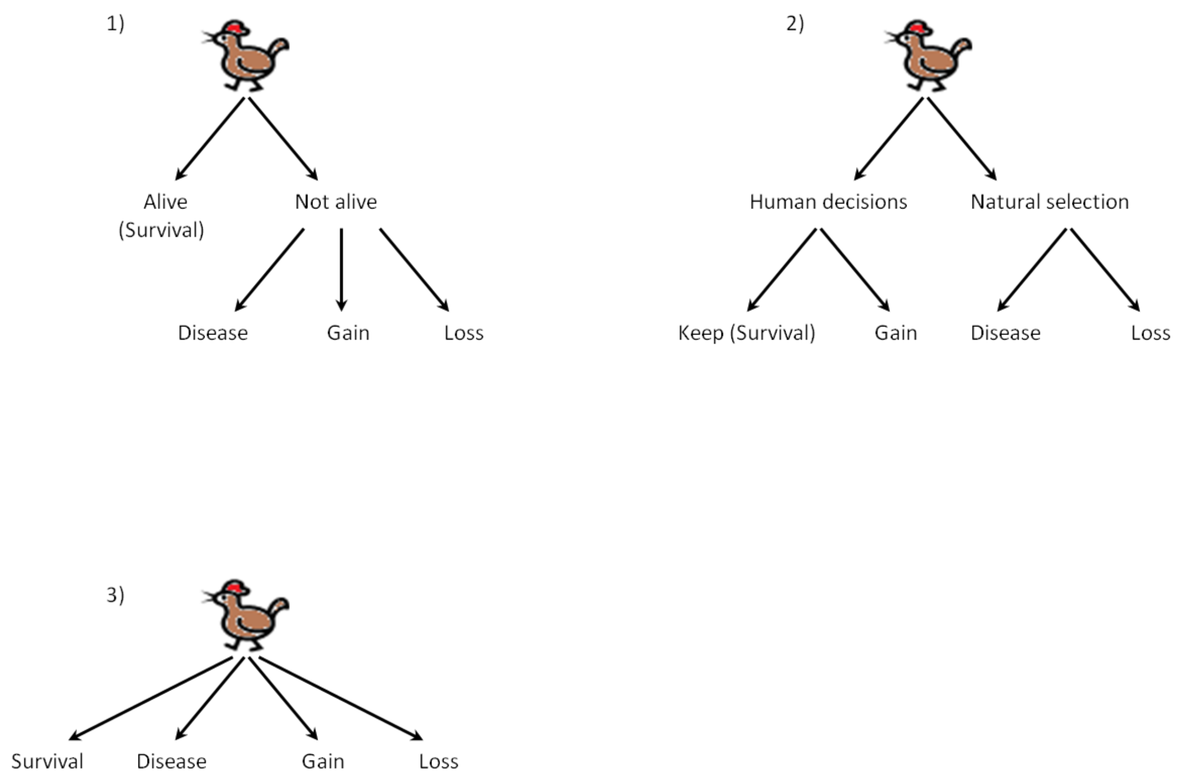

Figure 5.1 Three different nested model structures to explore the multinomial outcome bird fate

Results

In total, the fate of 946 birds over the total period of the study was ascertained 6 months after they were first recruited. The only verifiable outcome was if the bird had survived, otherwise the farmer was relied on to report what had happened to the bird in question. However, although the identity of 492 survivors was confirmed, another 46 "surviving" birds were also presented at the second visits which, when photographs were compared, were found not to be part of the original study group. There may, therefore, be some error in what farmers reported the non-surviving outcomes of their birds to be, as it may not always have been possible for them to identify/recall individuals accurately.

Calculations of the ICC for each outcome suggested that the majority of variation was at bird level, with the exception of death from disease. This outcome was highly clustered within farms, with the differences between farms estimated to contribute over 43% of the variance using the binary linearization method, and around 93% of the variance using the latent variable method. Whilst this latter approach is perhaps more appropriate for binary variables derived from an underlying continuous variable (Goldstein et al., 2002), and the model gave warnings of errors with the likelihood estimation, it supports our conclusion that farm-level variation is of particular importance in disease outcomes. Market shed did not contribute significantly to the variation for any outcome (Table 5.1). All model fits were significantly improved by including region as a fixed effect, whilst for all of the non-survival outcomes (i.e. death from disease; sale/consumption; predation) model fits were further improved by incorporating village instead of region. This suggests that, whilst survival odds are relatively similar for villages within a region, the reasons for non-survival, especially death from disease, may differ between villages in the same region (Figure 5.2).

Table 5.1 Variance partitioning estimates (%) using two methods.

Binary linearisation	Survival	Disease	Loss	Gain
Farm	13.06	43.63	7.97	11.54
Village	0.93	5.62	0.75	1.53
Market-shed	<0.01	<0.01	0.94	<0.01
Region	6.96	13.07	2.61	1.85
Residual	79.05	37.67	87.73	85.08

Latent variable	Survival	Disease*	Loss	Gain
Farm	9.32	93.05	8.82	14.72
Village	1.27	<0.01	3.96	4.87
Market-shed	<0.01	<0.01	0.45	<0.01
Region	4.29	<0.01	2.82	0.68
Residual	85.12	6.95	83.96	79.73

*This model gave warnings of false convergence

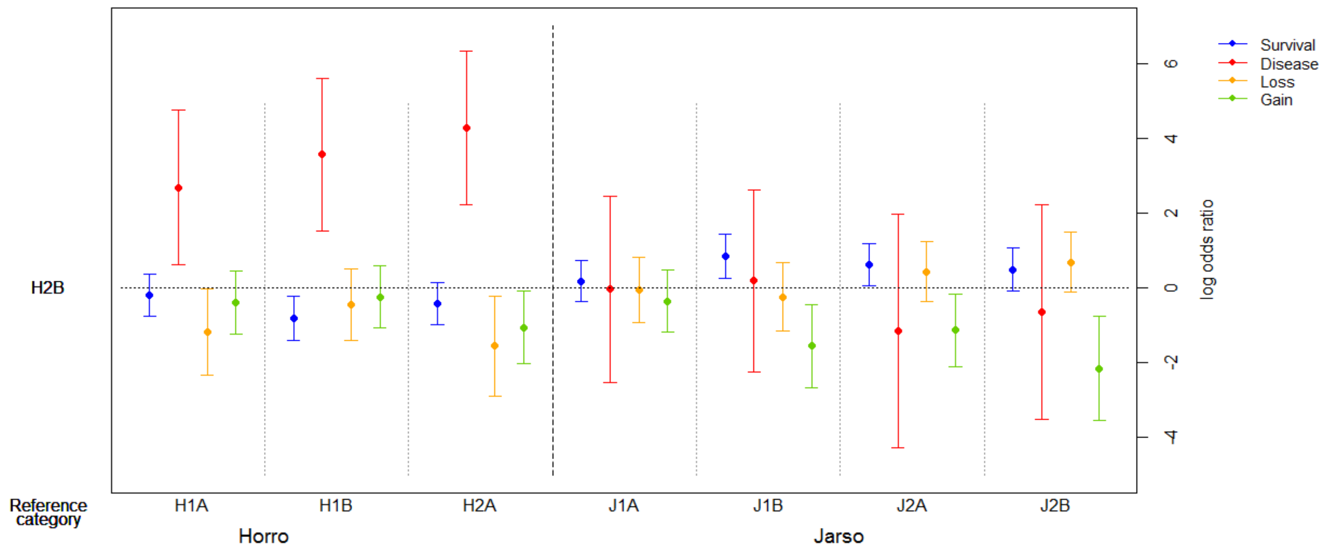

Figure 5.2 Log odds ratios for four univariable logistic regression models, comparing the effect of village on each outcome (Survival, Death from Disease, Loss or Gain). All models include farm as a random effect.

Model fits for all outcomes except death from disease were significantly improved by including season as an explanatory variable, and by fitting an interaction term between the season and the village or region. The model fit for death from disease was improved by fitting farmer cohort, which suggested that there was not only variation in the risk of dying from disease between the birds sampled in the wet and dry seasons, but also variation in the risk between the wet seasons in two consecutive years.

The results of the models suggested that the outcomes not only varied between villages, but also according to seasonal patterns. Proportions of bird outcomes by village and season are illustrated in Figure 5.3. The model results indicated that whereas birds in Horro were more likely to survive during the dry season, birds in Jarso had greater odds of survival over the wet season. This appeared to be both due to fewer incidences of predation and fewer birds being sold and consumed in this period, and was relatively consistent between villages. However, in Horro there was more variation between the villages. The reference village, H2B, was the only one in Horro which lost more birds to predation than disease;

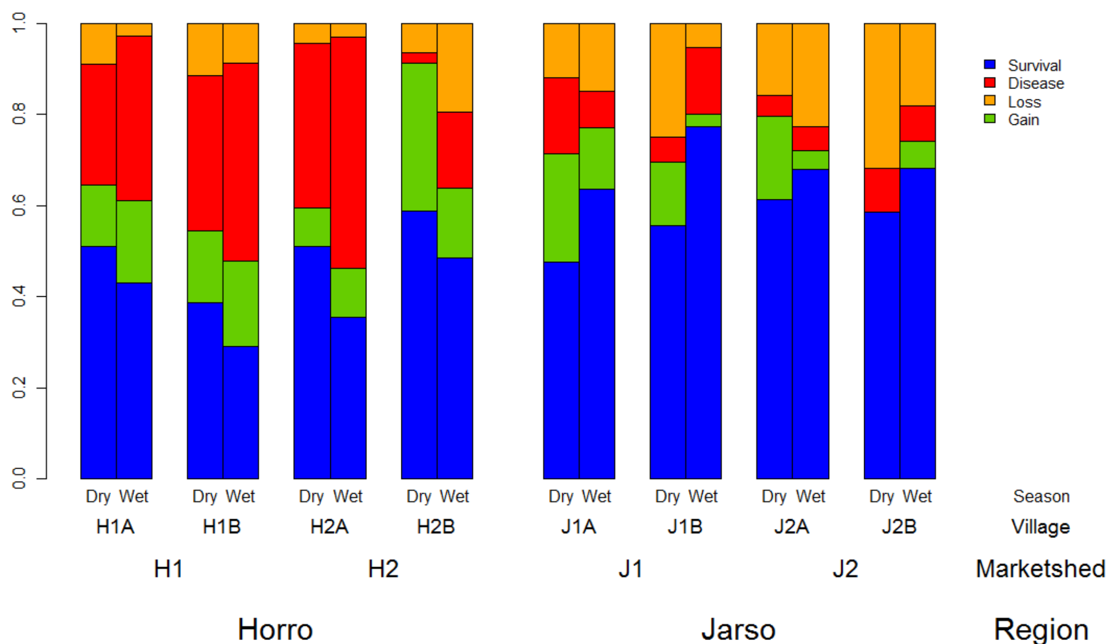

Figure 5.3 Proportions of bird fates 6 months after sampling for each village and season

but unlike Jarso, predation losses were worse in the wet season. In all Horro villages there were more losses to disease during the wet season compared to the dry season, but in market shed 1, the wet season was also the time when farmers tended to sell more birds, anecdotally so they could avoid the disease losses. However, in village H2B, considerably more birds were sold during the dry season. Therefore the fate of non-surviving birds depended on both the village and the time of year, but resulted overall in relatively minor differences in the odds of survival for birds sampled within the same region and season.

When multivariable models were constructed for each outcome it was found that there were several interactions between the region and other variables, suggesting some risk factors were more important in one region than another. It was therefore decided to construct different models for the two regions.

5.3.1 Approach 1

The initial approach taken was to first model surviving and non-surviving birds using a logistic regression model, then estimate the three non-surviving outcomes with a 3-way Poisson regression model. This is a standard approach, which is easy to comprehend. However, it was not always clear how the explanatory variables contributed to each outcome. For example, whilst male birds in Horro were less likely to survive than females, this variable did not predict which of the non-surviving outcomes might occur. Male birds in Jarso were also less likely to survive, but in this case being male increased the odds of being sold or consumed, whilst decreasing the odds of being predated.

Survival in Horro was also influenced by a bird's age, with older birds having increased odds of survival up to the age of around 3 years. However, there was an interaction between age and *Pasteurella* antibody levels, so that in young birds high levels were protective, whereas in birds over the age of 2 years they were found to decrease the odds of survival (Figure 5.4). Despite this, *Pasteurella* antibody level and age did not appear to predict which of the non-surviving outcomes would occur.

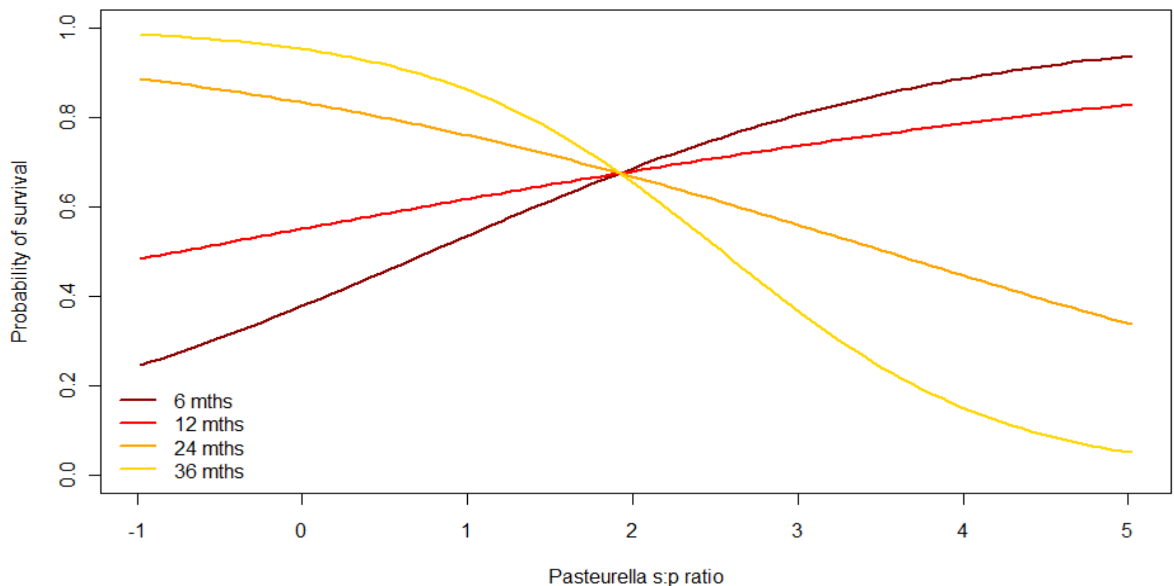

Figure 5.4 Model predictions for the probability of survival versus non-survival for birds in Horro, showing the interaction between age and Pasteurella s:p ratio.

Table 5.2 Horro model: Approach 1. Binomial separation of survival from non-survival outcomes, followed by multinomial regression on non-survival outcomes

Level 1	Binomial regression		Number of birds				
	1= Survival		200				
	0= Any other outcome		260				
Random effects				Variance	Std.Dev.		
	Farm			0.70	0.84		
		coefficient	se	OR	95% C.I.		P
(Intercept)		-0.17	0.31	0.84	0.46 - 1.55		0.58
Village	H2B	reference					
	H1A	-0.29	0.33	0.75	0.40 - 1.42		0.38
	H1B	**-1.06**	**0.35**	**0.35**	**0.18 - 0.69**		**<0.01**
	H2A	-0.63	0.34	0.53	0.27 - 1.04		0.07
Sex	**Female**	**reference**					
	Male	**-0.66**	**0.23**	**0.52**	**0.33 - 0.81**		**<0.01**
***Pasteurella* OD**		**1.01**	**0.35**	**2.73**	**1.36 - 5.47**		**<0.01**
Age (months)		**0.05**	**0.02**	**1.06**	**1.02 - 1.09**		**<0.01**
***Pasteurella*: Age interaction**		**-0.06**	**0.02**	**0.94**	**0.90 - 0.99**		**0.01**
Level 2	3-way Poisson regression		Number of birds				
	Disease		137				
	Sale/Consumption		73				
	Predation / Accident		37				
Random	effects:						
			Variance	Std.Dev.	Corr		
	Farm	Disease	0.00	0.00			
		Sale/Consumption	0.00	0.00	-0.11		
		Predation / Accident	0.00	0.00	-0.04	-0.37	
		coefficient	se	OR	95% C.I.		P
Disease	(Intercept)	-1.37	0.28	0.25	0.15 - 0.44		<0.01
Village	**H2B**	**reference**					
	H1A	**0.96**	**0.32**	**2.62**	**1.39 - 4.95**		**<0.01**
	H1B	**0.98**	**0.32**	**2.67**	**1.44 - 4.96**		**<0.01**
	H2A	**1.26**	**0.32**	**3.52**	**1.89 - 6.56**		**<0.01**
New bird bought in:	**No**	**reference**					
	Yes	**-0.49**	**0.21**	**0.61**	**0.40 - 0.93**		**0.02**
Sale/Consumption	(Intercept)	-0.83	0.21	0.44	0.29 - 0.65		<0.01
Village	H2B	reference					
	H1A	-0.45	0.31	0.64	0.35 - 1.17		0.15
	H1B	**-0.58**	**0.30**	**0.56**	**0.31 - 1.01**		**0.05**
	H2A	**-1.10**	**0.39**	**0.33**	**0.16 - 0.71**		**0.00**
New bird bought in:	No	reference					
	Yes	0.26	0.25	1.30	0.79 - 2.13		0.30
Predation / Accident	(Intercept)	-1.52	0.28	0.22	0.13 - 0.38		<0.01
Village	**H2B**	**reference**					
	H1A	**-1.16**	**0.48**	**0.31**	**0.12 - 0.80**		**0.02**
	H1B	**-0.92**	**0.40**	**0.40**	**0.18 - 0.87**		**0.02**
	H2A	**-1.62**	**0.56**	**0.20**	**0.07 - 0.59**		**<0.01**
New bird bought in:	**No**	**reference**					
	Yes	**1.02**	**0.33**	**2.78**	**1.45 - 5.32**		**<0.01**

Table 5.3 Jarso model: Approach 1. Binomial separation of survival from non-survival outcomes, followed by multinomial regression on non-survival outcomes

Level 1	Binomial regression	Number of birds						
	1= Survival	235						
	0= Any other outcome	126						
Random	effects:							
			Variance	Std.Dev.				
		Farm	0.47	0.68				
		coefficient	se	OR	95% C.I.			P
(Intercept)		1.22	0.37	3.38	1.62	-	7.05	<0.01
Production	In lay	reference						
status	rearing chicks	-1.11	0.59	0.33	0.10	-	1.04	0.06
	incubating eggs	**-1.78**	**0.68**	**0.17**	**0.04**	**-**	**0.63**	**0.01**
	not in production	-0.16	0.41	0.85	0.38	-	1.92	0.70
	Male	**-1.74**	**0.38**	**0.18**	**0.08**	**-**	**0.37**	**<0.01**
Season	**Dry**	**reference**						
	Wet	**0.92**	**0.29**	**2.52**	**1.42**	**-**	**4.48**	**<0.01**
MDV antibody ELISA OD		-0.53	0.31	0.59	0.32	-	1.07	0.08
***Eimeria* oocysts per 1000 (up to 3000 epg)**		**-0.59**	**0.17**	**0.55**	**0.40**	**-**	**0.76**	**<0.01**
Level 2	3-way Poisson regression	Number of birds						
	Disease	41						
	Sale/Consumption	42						
	Predation / Accident	78						
Random	effects:							
		Name	Variance	Std.Dev.	Corr			
		Disease	0.76	0.87				
		Sale/Consumption	0.04	0.20	-1			
		Predation / Accident	0.10	0.31	-1	1		
		coefficient	se	OR	95% C.I.			P
Disease	(Intercept)	-1.50	0.39	0.22	0.11	-	0.48	<0.01
Village	J1A	reference						
	J1B	0.44	0.50	1.55	0.58	-	4.13	0.38
	J2A	-0.60	0.61	0.55	0.17	-	1.81	0.32
	J2B	-0.19	0.55	0.83	0.28	-	2.42	0.73
Sex	Female	reference						
	Male	-0.47	0.38	0.63	0.30	-	1.31	0.22
Sale/Consumption	(Intercept)	-2.36	0.49	0.09	0.04	-	0.24	<0.01
Village	J1A	reference						
	J1B	-0.59	0.45	0.55	0.23	-	1.34	0.19
	J2A	-0.37	0.38	0.69	0.33	-	1.47	0.34
	J2B	**-1.32**	**0.56**	**0.27**	**0.09**	**-**	**0.80**	**0.02**
Sex	**Female**	**reference**						
	Male	**1.97**	**0.48**	**7.20**	**2.79**	**-**	**18.56**	**<0.01**
Predation / Accident	(Intercept)	-0.89	0.28	0.41	0.24	-	0.71	<0.01
Village	J1A							
	J1B	0.15	0.39	1.17	0.54	-	2.52	0.69
	J2A	0.56	0.34	1.75	0.90	-	3.41	0.10
	J2B	**0.70**	**0.34**	**2.02**	**1.04**	**-**	**3.92**	**0.04**
Sex	**Female**	**reference**						
	Male	**-0.59**	**0.25**	**0.56**	**0.34**	**-**	**0.90**	**0.02**

In Jarso, increasing levels of two disease measures – MDV and *Eimeria* - were associated with decreased odds of survival, as were females who were incubating eggs at the time of sampling. However, none of these variables were associated with any of the non-surviving outcomes. This lack of association of any of the infections with subsequent death from disease may suggest birds are generally tolerant of these infections, but may also be a Type II error, as only a relatively small number of birds were available for inclusion in the 3-way Poisson regression models.

Other than village, the only variable found to have an impact on whether a bird died from disease was whether the household had bought in a new bird in the 12 months prior to the initial questionnaire. This apparently decreased the odds of an individual dying from disease, but only for birds in the Horro region. Since it is unlikely that the introduction of a new bird would impact directly on the likelihood of an individual contracting an infection up to 18 months later, one possible explanation for this finding could be that this variable is a proxy for some other household practice, such as how much importance they place on poultry. It is possible that households which make a financial investment in their flock, in the form of buying in birds, may pay more attention to other husbandry factors which reduce disease risk.

5.3.2 Approach 2

Whilst dividing surviving from non-surviving birds initially appeared to be a logical way to approach the models, it did not adequately explain how some of the risk factors were contributing to the outcomes. This may be to do with the fact that a bird's fate is determined by interdependent biological and human processes, which are of greater or lesser relevance to certain outcomes. For example, for a bird to survive, it must both avoid death by disease or predation, but also be selected for retention in the flock by the farmer. Characteristics such as sex or age might be expected to influence both the human decisions

and the biological processes which result in survival, but not necessarily in the same direction.

The second approach to modelling the outcomes used a nested series of logistic regression models. The first stage (Level 1 models) separates the “bad” outcomes presumed to be beyond farmer control (“Disease” or “Loss”) from those which are “good” outcomes for the farmer – namely retention of the bird in the flock (“Survival”) or an outcome which brings an economic, agricultural or nutritional benefit (“Gain”) to the farmer, such as when the bird is sold or consumed. Within the “bad” and “good” categories, a second logistic regression model (Level 2 model) then further differentiated birds into the two outcomes which go to make up that category. Again, Horro and Jarso were modelled separately, due to the number of interactions between the region and other explanatory variables.

5.3.2(a) Horro

The set of explanatory variables found to contribute significantly to the fit of the models were the same as those found in the first model structure, but with the addition of the scaly leg scores. Birds were more likely to have a “bad” outcome in all villages other than the reference village, and in villages H2A and H2B, this was more likely to occur in the rainy season. Again, birds in households which had recently acquired a new bird had improved odds of “good” outcomes, as did older birds. The interaction between age and *Pasteurella*, with lower odds of survival in older birds with higher titres, as noted in the first model structure (Figure 5.4), was still significant in this model series. This was not associated with dying from disease but, unexpectedly, in the comparison between survival and sale. One possible explanation is that whilst owners are generally more likely to sell young birds which, on the whole, tend to have slightly lower *Pasteurella* titres, they may also purposely cull birds with characteristics associated with high *Pasteurella* titres. Whereas a high antibody titre in a young bird may simply indicate recent exposure, it is possible that high

Table 5.4 Horro model: Approach 2. Nested series of logistic regression models. At level 1, birds are assigned to being lost to natural causes or available for human uses. Level 2 models the outcomes within these categories.

Level 1	Binomial regression			Number of birds					
	1= Survival, Sale or consumption			260					
	0= Disease or predation			166					
Random	effects:			Variance	Std.Dev.				
			Farm	1.53	1.24				
			coefficient	se	OR	95% C.I.			P
(Intercept)			2.39	0.72	10.96	2.69	-	44.71	<0.01
Village		**H2B**	**reference**						
		H1A	**-2.34**	**0.81**	**0.10**	**0.02**	**-**	**0.47**	**<0.01**
		H1B	**-2.82**	**0.81**	**0.06**	**0.01**	**-**	**0.29**	**<0.01**
		H2A	**-2.18**	**0.81**	**0.11**	**0.02**	**-**	**0.56**	**0.01**
Season		**Dry**							
		Wet	**-2.32**	**0.77**	**0.10**	**0.02**	**-**	**0.45**	**<0.01**
Village: Season Interaction									
H1A		**Wet**	**2.04**	**0.95**	**7.68**	**1.20**	**-**	**49.26**	**0.03**
H1B		**Wet**	**1.87**	**0.94**	**6.52**	**1.03**	**-**	**41.34**	**0.05**
H2A		Wet	1.09	0.96	2.98	0.45	-	19.52	0.26
New bird bought in:		**No**							
		Yes	**0.86**	**0.32**	**2.36**	**1.25**	**-**	**4.45**	**0.01**
Age (months)			**0.04**	**0.02**	**1.04**	**1.00**	**-**	**1.08**	**0.04**
Level 2	Binomial regression			Number of birds					
	1 = Survival			182					
	0 = Sale or consumption			70					
Random	effects:			Variance	Std.Dev.				
			Farm	1.46	1.21				
			coefficient	se	OR	95% C.I.			P
(Intercept)			1.33	0.47	3.77	1.51	-	9.42	<0.01
Sex	**Female**		**reference**						
	Male		**-1.37**	**0.36**	**0.25**	**0.13**	**-**	**0.51**	**<0.01**
Scaly leg	**No signs or mild**		**reference**						
	Severe hyperkeratosis		**-0.97**	**0.42**	**0.38**	**0.16**	**-**	**0.87**	**0.02**
Age (months)			**0.08**	**0.03**	**1.08**	**1.01**	**-**	**1.15**	**0.02**
***Pasteurella* OD**			**1.40**	**0.55**	**4.07**	**1.38**	**-**	**12.07**	**0.01**
***Pasteurella*:Age interaction**			**-0.09**	**0.04**	**0.91**	**0.84**	**-**	**0.98**	**0.02**
Level 2	Binomial regression			Number of birds					
	1= Predation / Accident			38					
	0= Disease			146					
Random	effects:			Variance	Std.Dev.				
			Farm	**46.77**	**6.834**				
			coefficient	se	OR	95% C.I.			P
(Intercept)			3.70	1.75	40.45	1.32	-	1237	0.16
Village	**H2B**		**reference**						
	H1A		**-6.77**	**2.59**	**0.0011**	**0.00**	**-**	**0.18**	**0.01**
	H1B		**-5.73**	**1.83**	**0.0032**	**0.00**	**-**	**0.12**	**<0.01**
	H2A		**-7.70**	**3.22**	**0.0005**	**0.00**	**-**	**0.25**	**0.02**

Table 5.5 Jarso model: Approach 2. Nested series of logistic regression models. At level 1, birds are assigned to being lost to natural causes or available for human uses. Level 2 models the outcomes within these categories.

Level 1	Binomial regression		Number of birds					
	1= Survival, Sale or consumption		268					
	0= Disease or predation		93					
Random	effects:							
	Groups	Name	Variance	Std.Dev.	Corr			
	Farm	Season Dry	0.04	0.19				
		Season Wet	2.08	1.44	-0.11			
		AdjMDV	0.08	0.28	0.02	-1.00		
		coefficient	se	OR	95% C.I.			P
(Intercept)		1.92	0.50	6.82	2.56	-	27.80	<0.01
Village	J1A	reference						
	J1B	0.30	0.49	1.35	0.52	-	3.51	0.54
	J2A	-0.01	0.45	0.99	0.41	-	2.40	0.99
	J2B	-0.85	0.45	0.43	0.18	-	1.04	0.06
Season	**Dry**	**reference**						
	Wet	**0.95**	**0.33**	**2.58**	**1.34**	**-**	**4.97**	**<0.01**
Production	In lay	reference						
status	rearing chicks	-1.18	0.68	0.31	0.08	-	1.16	0.08
	incubating eggs	**-2.24**	**0.86**	**0.11**	**0.02**	**-**	**0.57**	**0.01**
	not in production	-0.47	0.50	0.62	0.24	-	1.65	0.34
	Male	**-1.00**	**0.46**	**0.37**	**0.15**	**-**	**0.91**	**0.03**
MDV OD		**-2.47**	**0.83**	**0.08**	**0.02**	**-**	**0.43**	**<0.01**
***Eimeria* oocysts per 1000 (up to 3000 epg)**		**-0.73**	**0.18**	**0.48**	**0.34**	**-**	**0.68**	**<0.01**
MDV: rearing chicks interaction		3.24	1.77	25.53	0.79	-	824	0.07
MDV: incubating eggs interaction		1.49	1.66	4.43	0.17	-	114	0.37
MDV:not in production interaction		1.64	0.96	5.14	0.79	-	33.7	0.09
MDV:Male interaction		**3.32**	**1.11**	**27.54**	**3.14**	**-**	**242**	**<0.01**
Level 2	Binomial regression		Number of birds					
	1 = Survival		291					
	0 = Sale or consumption		41					
Random	effects:							
			Variance	Std.Dev.				
		Farm	0.00	0.00				
		coefficient	se	OR	95% C.I.			P
(Intercept)		4.16	0.69	63.98	16.44	-	249.05	<0.01
Village	J1A	reference						
	J1B	**-1.67**	**0.59**	**0.19**	**0.06**	**-**	**0.61**	**0.01**
	J2A	-0.04	0.60	0.96	0.30	-	3.15	0.95
	J2B	0.62	0.73	1.86	0.44	-	7.80	0.40
Season	**Dry**	**reference**						
	Wet	**1.63**	**0.45**	**5.11**	**2.10**	**-**	**12.43**	**<0.01**
Sex	**Female**	**reference**						
	Male	**-3.68**	**0.58**	**0.03**	**0.01**	**-**	**0.08**	**<0.01**
Scaly leg	**No signs**							
	Hyperkeratosis	**-0.97**	**0.44**	**0.38**	**0.16**	**-**	**0.90**	**0.03**
Level 2	Binomial regression		Number of birds					
	1= Predation / Accident		78					
	0= Disease		41					
Random	effects:							
			Variance	Std.Dev.				
		Farm	56.50	7.52				
		coefficient	se	OR	95% C.I.			P
(Intercept)		5.23	1.17	185.98	18.83	-	1837.31	**<0.01**

titres in older birds may reflect a more chronic pasteurellosis. Chronically infected birds may be showing respiratory signs, or perhaps have low productivity, and as such may be removed from the flock. Sex was a strong predictor for sale, as male birds were more likely to be sold. Hyperkeratosis due to scaly leg mite infestation also increased the odds of being sold compared to surviving, although, like the *Pasteurella*, age and sex variables, it had no impact on the odds of dying from disease or predation.

Village was the main factor in determining whether they were lost to disease or predation; among these villages in Horro, only H2B reported predation as its biggest cause of loss, whereas disease claimed higher numbers of birds in the other three villages. However, there was considerable between-household variation in the model comparing disease to predation.

5.3.2(b) Jarso

Again, the same explanatory variables contributed significantly to the outcomes as those from the first approach, with the addition of the scaly leg score. There were fewer differences between the villages in Jarso, with only village J1B being less likely to sell birds.

It was found that for the Level 1 model, comparing “good” to “bad” outcomes, the fit was significantly improved by allowing the coefficient for season to vary between households. This suggested that the model explained most of the variation in the probability that birds would have a good outcome in the dry season, and that in this season, there was very little difference between households. However, there was far more variation between households in the rainy seasons as to whether birds would have a good outcome (Figure 5.5). Comparing the spatial distribution of the household residuals for the wet seasons to a randomly generated spatial distribution suggested that there was no spatial correlation between households with a higher probability of good outcomes, or those where birds

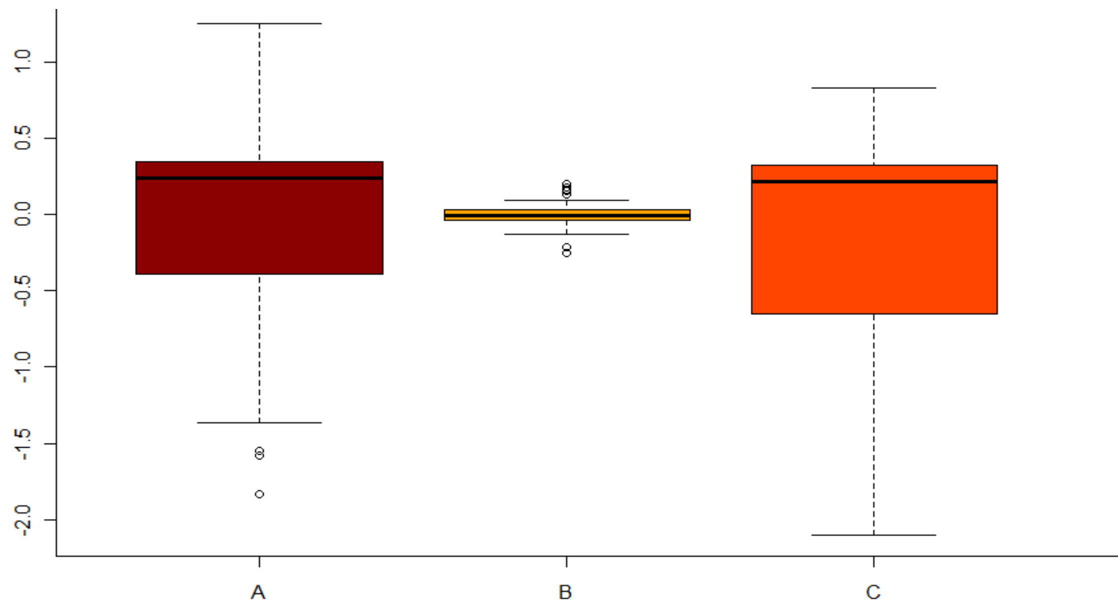

Figure 5.5 Household residuals plotted by farmer cohort, showing farms followed over the wet seasons (A and C) show far more variation as to whether their birds die from disease /predation than farms followed over the dry season (B).

were more likely to die (Appendix 5.2). This would suggest that some other, unmeasured, household variable was contributing significantly to the survival risk in the wet season.

After controlling for village and season, other variables were also important in determining the birds' fates. As in Horro, male birds were more likely to be sold, which reduced their odds of survival, but the production status of a female also appeared to influence her 6-month outcome; females which were incubating eggs at the first visit had significantly lower odds of a good outcome compared to birds in lay. Also, as in Horro, signs of hyperkeratosis increased the odds of a bird being sold or consumed compared to surviving.

Two other disease measurements significantly improved fits in this second series of models: Marek's disease antibody levels and *Eimeria* oocyst counts (up to 3000 oocysts per gram of faeces). Increasing measures of both of these diseases decreased the odds of a good outcome. MDV also demonstrated an interaction with the sex of a bird, such that increasing MDV titres decreased the odds of a good outcome in females, but not in males.

There were no variables which, given the bird died, were associated with whether it was more likely to die of disease or predation. There was, however, considerable variation between households. This may suggest unmeasured household risk factors which may alter disease risk, including management, environmental or spatial factors. It was also found that the fit of the model predicting a "good" outcome was significantly improved by fitting an interaction between household and the MDV antibody levels. This would suggest that the effect of MDV on the outcome also varies between households.

5.3.3 Approach 3

The third approach was a multinomial Poisson regression model, to compare all four outcomes simultaneously. For Horro, all variables previously mentioned were found to significantly improve the fit of the model, but some, such as the age-*Pasteurella* interaction, although approaching a critical probability of 0.05, were no longer found to significantly alter the odds of any one outcome (Table 5.6). This model reinforced many of the main findings from the previous models, namely that older birds had enhanced odds of survival; birds in households where there had been a new bird introduced within the previous 12 months were at lower risk of dying of disease, and males and birds with hyperkeratosis were most likely to be sold. There were no risk factors which were associated with an increased risk of predation among the villages in Horro (but note that predation was a relatively uncommon outcome in Horro). Village and season predominantly affected the odds of disease mortality.

In Jarso, only village, season, sex and *Eimeria* counts were important factors in the multinomial model. Hyperkeratosis and MDV were no longer found to significantly improve the fit. Males were less likely to survive, but much more likely to be sold. Birds in village J2B were less likely to be sold, and in all villages birds were much more likely to be sold in the

dry season. Increasing *Eimeria* counts (up to 3000 epg) put a bird at increased risk of subsequent death from disease (Table 5.7).

Table 5.6. Horro model: Approach 3. Four-way multinomial Poisson regression

Multinomial Poisson regression		Number of birds						
Survival		175						
Disease		119						
Sale/Consumption		67						
Predation / Accident		33						
Random effects:			**Variance**	**Std.Dev.**	**Corr**			
	Survival		0.00	0.00				
	Disease		0.29	0.54	0.00			
	Sale/Consumption		0.62	0.79	0.00		-0.82	
	Predation / Accident		0.85	0.92	0.00		-0.39	-0.213
		coefficient	**se**	**OR**	**95% C.I.**			**P**
Survival (intercept)		-0.74	0.22	0.48	0.31	-	0.73	<0.01
Village	H2B	reference						
	H1A	-0.13	0.20	0.88	0.59	-	1.29	0.50
	H1B	**-0.52**	**0.22**	**0.59**	**0.38**	**-**	**0.92**	**0.02**
	H2A	-0.19	0.21	0.82	0.55	-	1.24	0.36
Season	Dry							
	Wet	-0.29	0.16	0.75	0.55	-	1.03	0.07
Scaly leg	No signs							
	Hyperkeratosis	0.02	0.15	1.02	0.75	-	1.38	0.90
New bird bought in:	No							
	Yes	0.20	0.17	1.22	0.87	-	1.71	0.25
Sex	Female							
	Male	-0.29	0.16	0.75	0.54	-	1.03	0.07
Age (months)		**0.03**	**0.01**	**1.03**	**1.01**	**-**	**1.05**	**0.01**
Pasteurella OD		0.44	0.23	1.55	0.99	-	2.43	0.06
Pasteurella:Age interaction		-0.03	0.02	0.97	0.94	-	1.00	0.08
Disease (Intercept)		-2.43	0.41	0.09	0.04	-	0.20	<0.01
Village	**H2B**	**reference**						
	H1A	**1.09**	**0.38**	**2.97**	**1.42**	**-**	**6.22**	**<0.01**
	H1B	**1.43**	**0.37**	**4.20**	**2.02**	**-**	**8.71**	**<0.01**
	H2A	**1.45**	**0.38**	**4.27**	**2.03**	**-**	**9.00**	**<0.01**
Season	**Dry**							
	Wet	**0.62**	**0.23**	**1.85**	**1.18**	**-**	**2.92**	**0.01**
Scaly leg	No signs							
	Hyperkeratosis	-0.24	0.21	0.78	0.52	-	1.18	0.25
New bird bought in:	**No**							
	Yes	**-0.58**	**0.26**	**0.56**	**0.34**	**-**	**0.94**	**0.03**
Sex	Female							
	Male	0.07	0.20	1.08	0.72	-	1.60	0.72
Age (months)		-0.02	0.02	0.98	0.96	-	1.01	0.30
Pasteurella OD		-0.38	0.36	0.68	0.33	-	1.39	0.29
Pasteurella:Age interaction		0.02	0.03	1.02	0.97	-	1.07	0.42

		coefficient	se	OR	95% C.I.			P
Sale / consumption	(Intercept)	-1.99	0.46	0.14	0.06	-	0.33	<0.01
Village	H2B	reference						
	H1A	-0.29	0.39	0.75	0.35	-	1.60	0.45
	H1B	-0.21	0.40	0.81	0.37	-	1.76	0.59
	H2A	-0.83	0.47	0.43	0.17	-	1.10	0.08
Season	Dry							
	Wet	-0.41	0.32	0.66	0.35	-	1.25	0.20
Scaly leg	**No signs**							
	Hyperkeratosis	**0.58**	**0.30**	**1.79**	**0.99**	**-**	**3.23**	**0.05**
New bird bought in:	No							
	Yes	0.16	0.35	1.17	0.60	-	2.32	0.64
Sex	**Female**							
	Male	**0.73**	**0.29**	**2.08**	**1.17**	**-**	**3.70**	**0.01**
Age (months)		-0.03	0.03	0.97	0.92	-	1.02	0.21
Pasteurella OD		-0.57	0.44	0.56	0.24	-	1.35	0.20
Pasteurella:Age interaction		0.04	0.03	1.04	0.98	-	1.11	0.18
Predation / Accident								
(Intercept)		-2.07	0.66	0.13	0.03	-	0.46	<0.01
Village	H2B	reference						
	H1A	-0.88	0.60	0.41	0.13	-	1.34	0.14
	H1B	-0.67	0.58	0.51	0.17	-	1.59	0.25
	H2A	-1.16	0.69	0.31	0.08	-	1.22	0.09
Season	Dry							
	Wet	-0.08	0.47	0.93	0.37	-	2.31	0.87
Scaly leg	No signs							
	Hyperkeratosis	-0.23	0.43	0.80	0.34	-	1.86	0.60
New bird bought in:	No							
	Yes	0.80	0.46	2.22	0.90	-	5.51	0.09
Sex	Female							
	Male	-0.29	0.45	0.75	0.31	-	1.79	0.51
Age (months)		-0.03	0.04	0.97	0.90	-	1.05	0.49
Pasteurella OD		-0.24	0.64	0.79	0.23	-	2.74	0.71
Pasteurella:Age interaction		0.03	0.05	1.03	0.94	-	1.13	0.50

Table 5.7. Jarso model: Approach 3. Four-way multinomial Poisson regression

Single level poisson regression		Number of birds						
	Survival	220						
	Disease	30						
	Sale/Consumption	30						
	Predation / Accident	60						
Random	effects:							
	Name		Variance	Std.Dev.	Corr			
	Survival		0.00	0.00				
	Disease		2.37	1.54	0			
	Sale/Consumption		0.03	0.16	0	-1		
	Predation / Accident		0.22	0.47	0	-1		1
		coefficient	se	OR	95% C.I.			P
Survival (intercept)		-0.51	0.18	0.60	0.42	-	0.85	<0.01
Village	J1A							
	J1B	0.17	0.19	1.19	0.82	-	1.73	0.37
	J2A	0.10	0.19	1.10	0.76	-	1.59	0.61
	J2B	0.12	0.20	1.13	0.77	-	1.67	0.53
Season	Dry	reference						
	Wet	0.27	0.15	1.31	0.98	-	1.75	0.07
Sex	**Female**							
	Male	**-0.47**	**0.16**	**0.63**	**0.46**	**-**	**0.86**	**<0.01**
Eimeria oocysts per 1000 (up to 3000 epg)		-0.18	0.10	0.83	0.69	-	1.01	0.07
Disease (intercept)		-3.82	0.70	0.02	0.01	-	0.09	<0.01
Village	J1A							
	J1B	0.26	0.76	1.30	0.30	-	5.71	0.73
	J2A	-0.38	0.81	0.68	0.14	-	3.32	0.64
	J2B	-0.48	0.85	0.62	0.12	-	3.23	0.57
Season	Dry							
	Wet	-0.06	0.60	0.94	0.29	-	3.01	0.91
Sex	Female							
	Male	0.50	0.49	1.65	0.63	-	4.33	0.31
***Eimeria* oocysts per 1000 (up to 3000 epg)**		**0.56**	**0.24**	**1.74**	**1.10**	**-**	**2.77**	**0.02**
Sale/Consumption (Intercept)		-2.87	0.57	0.06	0.02	-	0.17	<0.01
Village	J1A							
	J1B	-0.88	0.52	0.41	0.15	-	1.15	0.09
	J2A	-0.66	0.44	0.52	0.22	-	1.23	0.13
	J2B	**-2.48**	**1.04**	**0.08**	**0.01**	**-**	**0.64**	**0.02**
Season	**Dry**							
	Wet	**-0.85**	**0.38**	**0.43**	**0.20**	**-**	**0.89**	**0.02**
Sex	**Female**							
	Male	**2.53**	**0.54**	**12.55**	**4.33**	**-**	**36.41**	**<0.01**
Eimeria oocysts per 1000 (up to 3000 epg)		-0.16	0.28	0.85	0.49	-	1.46	0.56
Predation / Accident (Intercept)		-2.00	0.37	0.14	0.07	-	0.28	0.00
Village	J1A							
	J1B	-0.27	0.49	0.76	0.29	-	1.99	0.58
	J2A	0.40	0.40	1.49	0.68	-	3.24	0.32
	J2B	0.65	0.39	1.92	0.90	-	4.08	0.09
Season	Dry							
	Wet	-0.39	0.29	0.68	0.39	-	1.18	0.17
Sex	Female							
	Male	0.13	0.29	1.14	0.65	-	2.00	0.65
Eimeria oocysts per 1000 (up to 3000 epg)		0.19	0.14	1.21	0.91	-	1.60	0.18

5.4 Discussion

The nature of the extensive scavenging production system under which village chickens in Ethiopia and elsewhere are kept exposes them to a variety of potential risks which impact on their chances of survival. Whilst our primary interest in this study lay in determining how exposure to different pathogens may alter the risk of dying from disease, it is clear that infections may also subtly alter the risk of other outcomes. Although a production animal, and subject to human decisions, village chickens are in some respects more akin to a wild population, and subject to a degree of natural selection, including predation and competition for feed resources. The balance of these human and environmental pressures may vary widely between farms, and modelling this mathematically has proved challenging.

The benefit of using the three different nested model structures was found to be in the interpretation of some of the more subtle effects, as the relatively small numbers of birds was likely to make the multinomial models, in particular, prone to type II error. When an explanatory variable, such as sex in Jarso, was strongly associated with two outcomes, in that males were more likely to be sold and therefore less likely to survive, the multinomial model was able to clearly demonstrate both these associations. However, some other variables did not show such clear relationships. For example, whilst increasing age in Horro was associated with improved survival, it appeared that this was because young birds were more likely to be sold *and* be predated *and* die of disease.

These relationships became more apparent when single outcomes were compared to each other. However, the conventional nested approach, where survivors were first separated from non-survivors, could have led to incorrect assumptions. It would be easy to assume that *Pasteurella* reduced the odds of survival because it would make birds more likely to die from disease, when in fact it was not shown to increase the odds of this outcome, but

rather to alter the odds of sale or consumption in birds which did not die from natural causes. This difference was highlighted only through the use of the three different model structures.

Overall, the models showed some interesting similarities and differences between the two regions. The tendency in both areas was to sell or consume males, and birds showing hyperkeratosis were also more likely to be sold in both areas. In Horro, the models also suggested that the older birds with high *Pasteurella* antibodies were also selected for sale or consumption. This might suggest that farmers are purposely selecting older birds which are showing mild signs of ill-health to remove from the flock if they wish to make a sale, allowing them to keep the younger and healthier older birds for breeding. One of the most notable differences was the difference in the effect of season between the two regions. Whereas in Horro birds were at significantly greater odds of dying of disease during the rainy season, no such effect was seen in Jarso. This contrasts with the findings reported in Chapter 4, where farmers in both regions were more likely to report that they had experienced disease outbreaks during the rainy season. However, it was also noted from the farmer reports that fewer birds per farm were lost over the rainy season, which may account for this discrepancy between the risk to individuals and the risk of disease at farm-level.

No single infection stood out as a good predictor of subsequent death from disease, but the models suggested that different diseases are of greater importance in each area, at least in the time period of this study. Whereas *Pasteurella* was only significant in Horro, *Eimeria* and MDV were of greater importance in Jarso. Previous research in Ethiopia has suggested that chicks of the Jarso ecotype raised under confined conditions were more susceptible to MDV mortalities than ecotypes from other areas of Ethiopia, including Horro (Duguma et al., 2005). *Eimeria* in this study was not differentiated into the different species, although a subset of samples from both regions was speciated by PCR (Luu et al.,

2013). Although there were no differences between the regions in the isolation of the species regarded as highly pathogenic (*Eimeria tenella, necatrix and brunetti*), Jarso did have significantly greater levels of the moderately pathogenic *Eimeria maxima,* whereas the predominant species in Horro was *E. praecox,* which is generally regarded as only mildly or non-pathogenic (McDougald and Fitz-Coy, 2008). Therefore both pathogen and host-resistance factors are likely to be contributing to regional differences in disease mortalities. There may also be differences in pathogen exposure between the regions and villages.

The MDV-sex interaction, which was included in Approach 2 in Jarso, did not significantly improve the fit of any of the other model structures. This would suggest that, whilst males were less likely to survive overall, because they were more likely to be sold, having a higher MDV titre did not greatly alter the odds of whether they are available to sell or keep within a six month period. As MDV in a village situation is likely to be of low virulence, and take the form of a chronic disease, males may be tolerating MDV infections over extended periods of time, and so still be available for sale. Conversely in females, those with higher MDV levels were found to be more likely to die from either disease or predation within the 6-month follow-up period. Research has previously shown that males are less likely to develop tumours following MDV infection (Payne and Venugopal, 2000); thus it may be that females in the clinical phase of an MDV infection, where they can be both immunosuppressed and suffering from neuropathologies, are indeed at risk of predation, or other infections, or go on to die of MDV. It may also be that farmers do not always observe the exact fate of their birds, and tend to attribute most deaths to predators, especially if they do not see signs of an obvious disease.

The possibility that farmers are not aware of the exact fate of their birds and tend to attribute unobserved deaths to a particular cause should be borne in mind. This may contribute to the strong regional difference in reported fates, where Horro birds were

more likely to be reported to have died of disease, whereas Jarso birds were more likely to be reported to have died of predation. If this was indeed a cultural effect, it may be that farmers in Jarso were underestimating the impact of diseases in their flocks, which may have implications for the uptake of any future disease-control interventions in this area. However, it may also be a genuine finding, and measures to prevent predation may be of far greater benefit to these farmers than disease control programmes. Conversely, it may be that Horro farmers overestimate disease mortality; although we have assumed they report death from disease because they find a sick or dead bird, we did not specifically elicit this information during the questionnaire interviews. Without confirming the fate of every bird in the study, it will not be possible to disentangle a regional cultural difference from a regional disease mortality difference. However, the fact that both MDV and *Eimeria* decreased the odds of a bird being available to sell or keep in Jarso suggests that diseases are still important in this region. As both of these infections may have more subtle, subclinical effects, such as reducing scavenging behaviour or feed conversion efficiency, farmers may be less aware of their impact. If interventions such as confining birds in shelters to protect them from predators are put in place, such diseases may become more important as their epidemiology may alter with a change in management.

5.5 Conclusions

Our original hypothesis was that a number of infections may be contributing to the annual increase in reported mortalities, normally put down to “fengele”, which occur during the rainy season. In order to do this it was necessary to understand both the human decision-making processes and the biological processes which go together to determine a bird’s fate. Modelling the fate of birds has proved challenging, and the use of several different approaches was found to be beneficial. Whilst no single infection was strongly associated

with subsequent disease mortality, they may still be relevant as causes of indirect loss, for example by influencing farmer decisions to cull, or by increasing vulnerability to predators. It is possible that infections did not appear associated with disease mortality as most birds we detected as infected, especially using serology, would be in the recovery stages, and therefore we may have failed to measure any which died during the acute stages of infection. Another possibility is that whilst single infections alone may not impact mortality, multiple infections may be of much greater consequence. The high variation between households in the risk of disease mortality, and especially in Jarso the variation between households in the effect of season and MDV infection on mortality, would suggest that some households may already have practices which are effectively mitigating or exacerbating these risks. Whilst, unfortunately, we have been unable to identify these practices during this study, it may be possible to elicit and promote the exchange of such information between villagers, by encouraging the formation of groups to discuss “best practice”, and a more qualitative evaluation of strategies which have been found to work well in situ. The diversity observed between the two regions would reinforce our belief that no single intervention is likely to be uniformly beneficial to reduce disease mortality.

Appendix 5.1

Table 5.8 List of explanatory variables which were tested for inclusion as fixed effects in each multivariate regression model. Farm was included as a random effect in each of the models.

Variable	Description	Comments
Household-level variables		
Region	2 levels, Horro and Jarso	
Village	4 levels within each of the two regions	Models cannot include both region and village as fixed effects
Season	"Wet" describes birds which were followed over the rainy season, i.e. from around May to October. "Dry" describes birds which were followed only through the dry season, i.e. around October to May	Cohorts A and C are "Wet" Cohort B is "Dry"
New bird	Whether or not a new birds has been bought into the flock within the last 12 month period	
Waste	Use of bird excreta as fertilizer, or disposal by other means	
Dead disposal	Method of disposal of dead birds, including throwing away near or far from the house, burying, burning or feeding to dogs	
Contact	Contact time with other flocks during the wet or dry season	
Feed	Provision of feed in the rainy compared to the dry season, can take the values "More", "the Same", "Less" or "None"	
Bird-level variables		
Age	Bird age in months	
Sex	Bird sex	
Production status	Females are subdivided into "In lay", "Rearing chicks", "Incubating eggs" or "Not in production". Males are a single category	
Source	Birds may have been home bred or bought into the flock from either the market, a private household in the same village or a private household in a different village	
BIOP	Length of time in owner's possession (months). Grouped into less than 3, 3-6, 6-12 or more than 12 months	Highly correlated with Age

Scaly leg	Degree of hyperkeratosis; “None”, “Mild” or “Severe”	
Lice total	The total count of lice of any species found on all areas of the bird	
Eimeria	1000 Oocysts per gram of faeces	Increases over 3000 do not alter the risk
Ascarid	Eggs per gram of faeces	
IBDV	The sample:positive ratio against IBDV	*
Pasteurella	The sample:positive ratio against *Pasteurella*	*
Salmonella	The sample:positive ratio against *Salmonella* O9 serotypes	*
MDV	The sample:positive ratio against MDV, after subtraction of the optical density of the sample against cell lysate. See Chapter 2 for full details	*

*These values have been adjusted for the differences between plates, as described in Chapter 2

Appendix 5.2

Figures 5.6 to 5.9 show (a) Circle plots and (b) semivariograms of household residuals from Jarso villages in the wet season (Approach 2, comparing "good" to "bad" outcomes) showing that residuals were not observed to be more spatially correlated than those generated by a random spatial distribution.

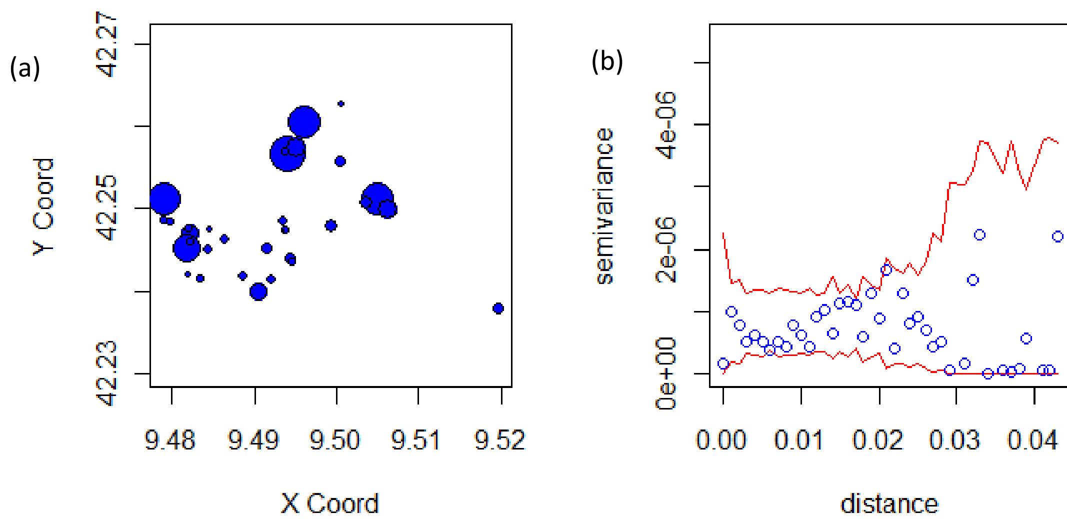

Figure 5.6 J1A wet season, p=0.52

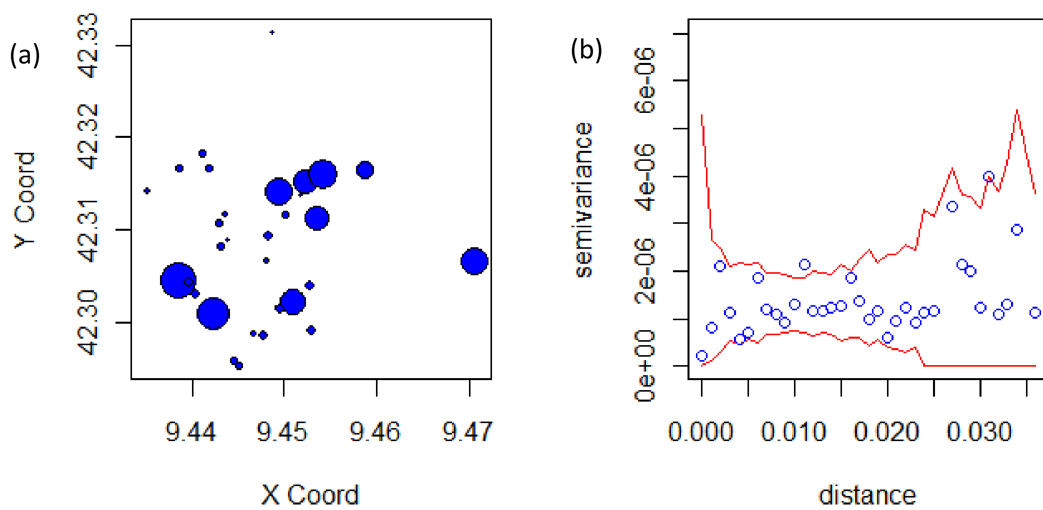

Figure 5.7 J1B wet season, p=0.34

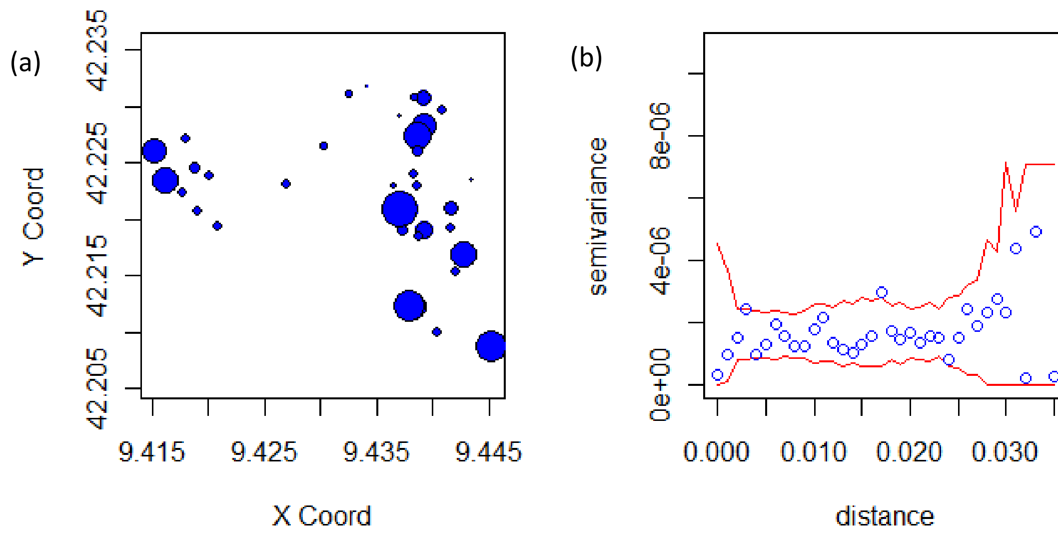

Figure 5.8 J2A wet season, p=0.53

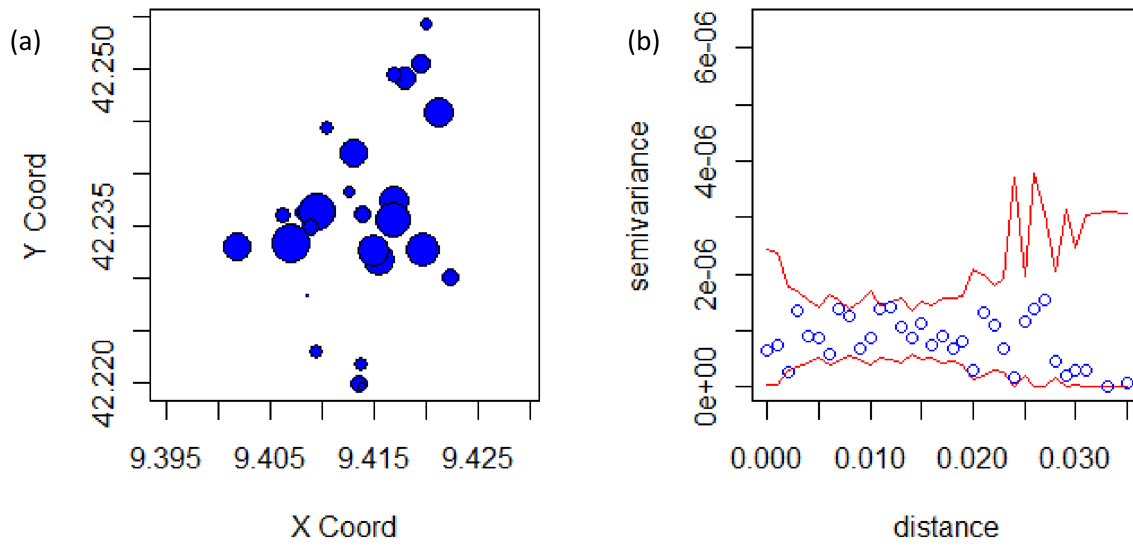

Figure 5.9 J2B wet season, p=0.54

Chapter 6

Infection-interactions in Ethiopian village chickens

Material from this chapter has been accepted for publication (article in press)

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6.1 Introduction

Infectious disease is recognised as one of the major constraints to developing village poultry production (Guèye, 1998; Mack et al., 2005). Control of Newcastle disease virus (NDV) has been identified as the most critical intervention in numerous studies throughout Africa, and other interventions such as improving feed, housing or controlling parasites are only effective if used in conjunction with vaccination (Dwinger and Unger, 2006). A number of seroprevalence surveys have demonstrated the presence of NDV in several areas of Ethiopia (Tadesse et al., 2005; Mazengia et al., 2010; Chaka et al., 2012), although little attention has been paid as yet to control strategies. However, NDV is not the only problem; chickens under a village production system are exposed concurrently or consecutively to a number of different pathogens. In Ethiopia seroprevalence surveys in village chickens have identified the presence of infectious bursal disease (Mazengia et al., 2010; Chaka et al., 2012; Jenbreie et al., 2012), salmonellosis (Berhe et al., 2012; Alebachew and Mekonnen, 2013), pasteurellosis and mycoplasma infection (Chaka et al., 2012). Marek's disease has also been identified in village chickens kept under intensive management (Duguma et al., 2005). Parasitic diseases, including coccidiosis (Ashenafi et al., 2004b; Luu et al., 2013), helminths (Tolossa et al., 2009; Molla et al., 2012) and ectoparasites (Belihu et al., 2009; Tolossa et al., 2009) have also been demonstrated to be highly prevalent in the country.

Host survival may be severely affected by multiple, coincident infections (Jolles et al., 2008) and there is evidence from studies of wild rodents that existing infections may pose greater risks for further infections than environmental variables (Telfer et al., 2010). Associations between diseases may be the result of direct interactions between the pathogens themselves or indirectly, via the bird's immune system, such that their impact on the host may be altered. Pre-infection with Marek's disease has been observed to alter the clearance of certain *Eimeria* species (Biggs et al., 1968), while both *Pasteurella multocida*

and *Salmonella enterica* serovar Enteritidis infections have been shown to be more severe in the presence of pre-infection with the nematode *Ascaridia galli* (Dahl et al., 2002; Eigaard et al., 2006). Many of these interactions are thought to be mediated through the altered differentiation of T-lymphocytes; whereas protective cell-mediated responses to intracellular microbial pathogens are primarily driven by T helper 1 (Th1) cells, protection to extracellular infections, including macroparasites, usually requires antibody and primes the immune system towards a Th2-type response. Hosts in a natural environment may have limited ability to effectively mount both types of immune response simultaneously, particularly where they are constrained by limited resources (Jolles et al., 2008). The helminth *Ascaridia galli* has also been shown to reduce the antibody response to vaccination with NDV (Horning, 2003), therefore determining pathogen interactions may have implications for disease control programmes. Indeed, the health consequences of multiple low-grade infections may become more significant as interventions reduce the impact of single diseases (Pullan and Brooker, 2008).

However, apparent interactions between infections may arise simply because diseases have other risk factors in common, including host factors, such as sex, age, socio-economic status or behaviours, or similar transmission routes or temporal patterns of exposure (Hellard et al., 2012). In Ethiopia, mortalities from infectious diseases are reportedly associated with the rainy season (Dessie and Ogle, 2001; Halima et al., 2007), when seasonal factors, such as cold or changes in the available nutrition may impact on a bird's susceptibility to new infections, and the outcome of that infection. However, a number of pathogens survive better in cool, moist environments, including the influenza and NDV viruses and the protozoan parasite, *Eimeria* (Martin, 1991; McDougald, 2003; Swayne and Halvorson, 2008). For parasitic diseases, the long dry season can impede the development of nematode larvae (Weaver et al., 2010) and seasonal fluctuations in rainfall and temperature may alter the abundance or behaviour of invertebrate intermediate hosts.

It might, therefore, be predicted that the observed tendency for a seasonal increase in chicken mortality associated with the rainy season in Ethiopia is associated with increased exposure to one or more environmental pathogens, not only NDV, as is frequently assumed. One of the objectives in undertaking this study was to investigate seasonal changes in infection prevalence with a variety of pathogens, by carrying out repeated cross-sectional surveys before and after the rainy season in two consecutive years. The aim was to explore seasonal variation both within and between years. However, if indeed other pathogens, which may also show seasonal variation, are also risk factors for infection, then this will clearly confound the analysis. The use of generalised linear mixed models, as proposed by Fenton et al. (2010) for the analysis of macroparasite data, can account for this, but assume a directional relationship between pathogens. For serological surveys which measure the host's adaptive immune response rather than the pathogen itself, a positive result does not necessarily represent a current infection. As the order of infection is important in determining the immune response to each single infection, and it is thought that susceptibility to new co-infections may return to normal soon after the clearance of the first infection (Telfer et al., 2010), models which assume a directional relationship are unsuited to cross-sectional serological data (Hellard et al., 2012).

In order to explore possible interactions between infections, we sought a method that did not assume a directional relationship and allowed us to combine the different data (continuous, count and ordinal) measurements for the range of infections we studied. Ordination methods are commonly used in ecology for community analysis: Here they have been applied to this community of pathogens, where each bird may be thought of as a "site", which has the potential to be exploited by any of the micro- and macroparasite species. Two main techniques were used to explore this data set. Principal component analysis (PCA) is an ordination technique which seeks to extract the main trends in the data set such that it may be explained by a few linearly uncorrelated principal components,

which will be less than or equal to the number of original variables. The main structures in the data may then be displayed in a graph(s) constructed from the reduced set of orthogonal axes. Graphs, known as biplots, can be scaled according either to the distances between sites or the correlations between variables (Zuur et al., 2007). Redundancy analysis (RDA) is a canonical ordination technique which combines regression with principal component analysis, and thus tests the relationships of a set of explanatory variables to the multivariate response data.

This chapter presents the results of the ordination analyses, whilst Chapter 7 presents a more detailed look at the individual distributions of infection measurements and incorporates some multivariable regression modelling to test some of the findings from the ordination. The work in the two chapters was undertaken as an iterative process, but is presented separately, to highlight different aspects of the methods and findings.

6.2 Methods

The study used data from four cross-sectional surveys carried out between May 2011 and November 2012 in two geographically distinct woredas (administrative districts), Horro and Jarso, within the Oromia region of Ethiopia. Eight kebeles (small administrative districts, roughly equivalent to a ward) were visited on four occasions; in May/June and October/November in each year of the study. These visits were timed for before and after the main rainy season, which occurs between June and September. Different households were selected on each occasion and two chickens in each household were sampled. Households which did not own at least two indigenous breed chickens of over 6 months of age were excluded from the study. Full details of the selection process can be found in Chapter 2.

Information on management and disease occurrence over the previous 12 months was collected in a questionnaire interview in the local language with an adult member of the household. In addition, two chickens of at least 6 months of age were randomly selected from the household flock and each underwent clinical examination, was condition scored using a 0-3 grading scale (Gregory and Robins, 1998), and was scored for lice using a timed count of 3 areas of the body – one side of the keel, the back and the rump - and a total count of lice at the base of the flight feathers of one wing and the tail feathers (Clayton and Drown, 2001). Birds were also scored for the degree of hyperkeratosis of the legs and feet (none, mild or severe), and scrapings were taken to confirm the presence of *Cnemidocoptes mutans* (scaly leg mite). Data on the chickens' age, origin and current status of production was collected from the owner. Blood samples were drawn from the brachial vein of the selected birds into sodium citrate and faecal samples were collected from individual birds wherever possible; environmental faecal samples were taken to represent the household, if individual samples were not forthcoming. All samples were kept chilled and were transported to the laboratory for testing.

Serological testing was carried out for bacterial and viral infections by enzyme-linked immunosorbent assays (ELISA). Infectious bursal disease (IBD) virus antibodies were tested for using a commercial kit (Flockscreen, x-OvO, Dunfermline, UK), while antibody ELISAs were developed in-house for *Pasteurella multocida* (PM) and *Salmonella* O9 serotypes, using *S.* Gallinarum (SG) antigen, according to the protocols by Beal et al. (2004); and to Marek's disease virus (MDV) according to the protocol described by Zelnik et al. (2004). A full description of all the laboratory methods can be found in Chapter 2. Optical densities for all ELISA samples were converted into a ratio to the positive control (s:p ratio) to allow comparison of samples tested on different plates, using Equation 6.1.

$$\text{s:p ratio} = \frac{\text{Mean sample OD - Negative control OD}}{\text{Positive control OD – Negative control OD}} \qquad (6.1)$$

Mixed-effects linear regression was used to control for the variation between plates and the bird-level residuals were taken as an estimation of the antibody response.

Faecal samples were examined for *Eimeria* spp. oocysts and nematode eggs using a modified version of the concentration McMaster technique and identified to genus level where possible using published keys (Lapage 1956, Soulsby 1982, Permin and Hansen 1998). Only the most common egg types, which could be attributed to nematodes of the Ascaridida order *Ascaridia galli* or *Heterakis gallinarum*, were counted. These eggs were counted as a single group, as it was not possible to accurately differentiate the two species. Although other types of nematode eggs were seen in low numbers, there were no other frequently occurring species. Cestode eggs were also counted, but were not speciated.

Analyses were performed using R software (R Development Core Team, 2008) using the vegan package (Oksanen et al., 2011), and according to the methods described by Borcard et al. (2011). A matrix of response variables was constructed from the ELISA s:p ratios for the viral and bacterial pathogens (IBD, PM, SG and MDV); counts of the three parasites (Ascaridida, *Eimeria* and lice) and the hyperkeratosis grading. In order to make all response variables dimensionally homogeneous, each variable was scaled by centring values on the mean and dividing by the standard deviation. This ensured additional weights were not given to the parasites with a larger range of values and fulfils one of the basic requirements for application of PCA. In order to test whether the results were features of the data (different data types, presence of outliers), data were re-analysed following a number of prior transformations, including log transformations of the ELISA s:p ratios, square root transformations of the individual parasite count data, and Hellinger transformations of the parasite count matrix. In addition, data were re-analysed following dichotomisation. The IBD ELISA was dichotomised using the manufacturer's recommended cut-off (0.306). The range of s:p ratios to the ELISA tests developed in-house did not demonstrate clear cut-off

points, and sufficient negative sera were not available to confidently determine positive cut-offs for these tests. A small panel of sera from birds with known exposure to vaccines or experimental infection were used to determine a conservative cut off value for each test which would still detect all known positives. Cut-off values chosen were 0.34, 0.61 and 0.29 for SG, PM and MDV, respectively. The analyses were also run with preselected cut-offs of both 0.3 and 0.5 for these three ELISAs. Presence / absence was used to dichotomise the parasite counts and hyperkeratosis.

Initially, an unconstrained PCA was carried out on the matrix to identify correlations between parasites; a second matrix of explanatory variables, including household data and bird signalment, was then incorporated using RDA. A category “production status” was used to combine data on sex and the current state of production (in lay, not in lay, brooding eggs, rearing chicks or male). Each centred response variable was regressed on the explanatory variables and the fitted values combined in a matrix. PCA of the fitted values produced canonical eigenvalues and a matrix of canonical eigenvectors, used to calculate the coordinates of the sites (chickens), either in the space of the original response variables, or in the space of the explanatory variables (i.e. on the fitted response variables). The residual values from the multiple regressions were also submitted to a PCA to obtain an unconstrained ordination of the residuals.

The adjusted R^2 value was used to test whether the inclusion of explanatory variables was a significantly better fit than the null model, and a forward selection process was used to select those significant variables which explained the greatest proportion of the variance in the response data (Borcard, 2011). Permutation tests were used to test how many RDA axes explained a significant proportion of the variation. The Kaiser-Guttman criterion, which compares each axis to the mean of all eigenvalues, was applied to unconstrained axes, to determine those which explained variation of interest. Biplots and triplots were

produced, scaled according either to the distances between observations (scaling 1 in the vegan package) or the correlations between variables (scaling 2).

6.3 Results

A total of 1056 birds, 532 from the Horro region and 524 from the Jarso region, were included in the analysis. All birds were found to be in reasonable health at the time of sampling, with only a very few displaying any clinical signs which may have been attributable to the diseases of interest. As only 9 birds over all four seasons of testing were found to be serologically positive for NDV antibodies (HAI titre of 16 or greater) this disease was not included in the ordination analyses. Cestode counts were not included in the PCA analysis, as there was some concern that the test sensitivity may have altered over time. All other infections were present in each village and at each sampling season with the exception of IBD, where no positive birds were found in one Jarso village at any time point, and seropositive birds were detected in only three villages in Horro during the second year of sampling

Using the manufacturer's cut-off of 0.306, 3.6% of birds tested positive to IBD. Using the chosen cut-off values of 0.34, 0.61 and 0.29 for *Salmonella, Pasteurella* and MDV, respectively gave corresponding estimated seroprevalence of around 86%, 69% and 32% for the study samples, with only minor fluctuations between different villages and seasons. However, some birds demonstrated OD values far in excess of the positive controls. The parasitic diseases were also present in all villages at each time of sampling, with overall detection prevalence of 17%, 56% and 34% for Ascaridida, *Eimeria* spp. and lice respectively. Birds showed considerable variation in the intensity of infection. Signs of hyperkeratosis were seen in 41% of birds; its identification was strongly associated with a positive identification of scaly leg mites from a skin scrape (McNemar's test, $p<0.01$).

Correlations between the infections were examined using scatterplots and correlation coefficients (Figure 6.1). On the whole, there was very low correlation between any of the pairs of variables. *Salmonella* and *Pasteurella* shared the highest coefficient of correlation (r=0.3), but the majority of birds did not display high antibody titres / parasite counts to more than one pathogen. There were no negative correlations identified.

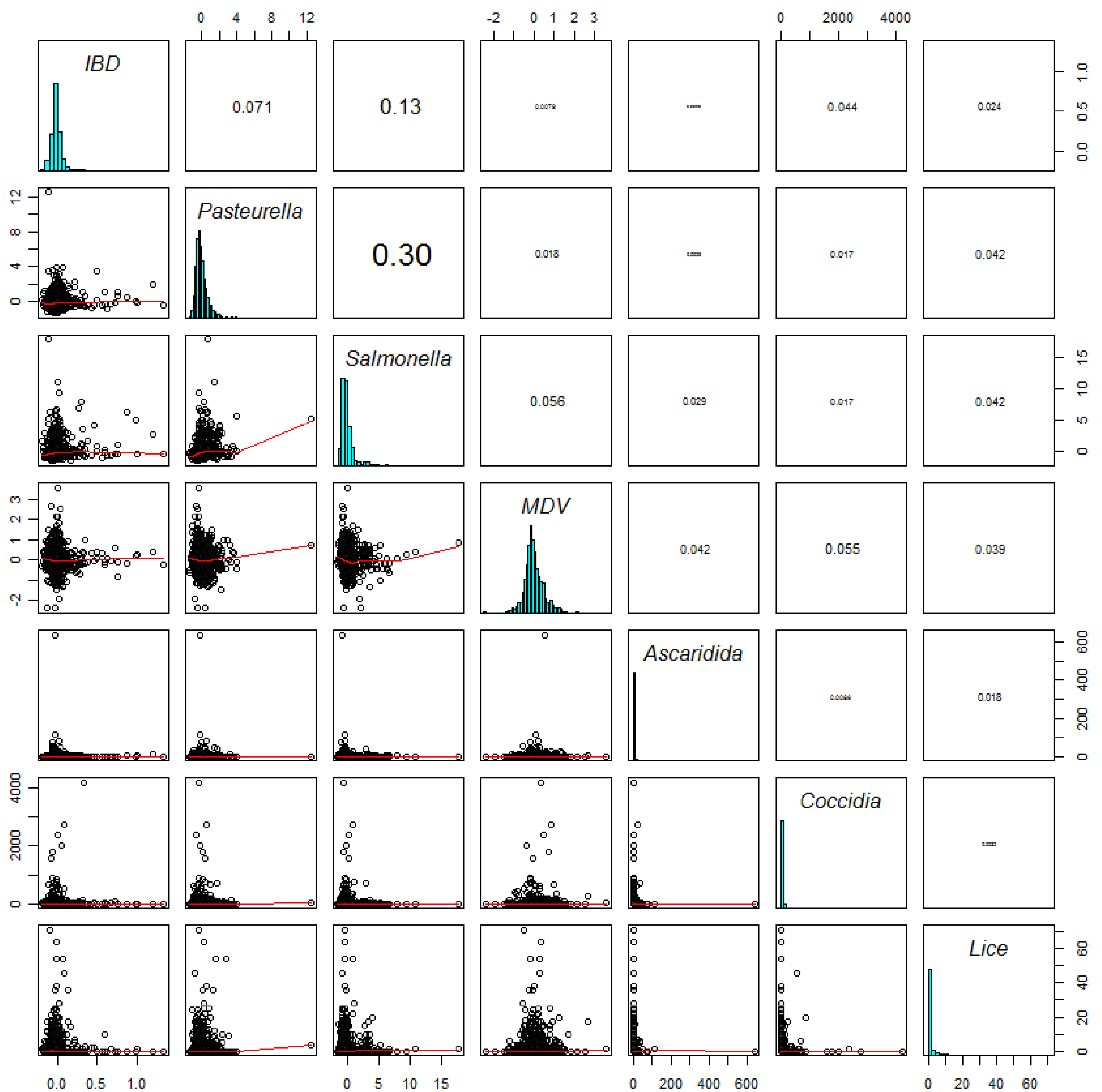

Figure 6.1 Correlations between disease variables

6.3.1 Principal component analysis

After constructing the correlation matrix of responses to each infection, principal component analysis was used to explore the variation in the scaled dataset (shown in Appendix 6.1). Regardless of the prior data transformation used, this suggested that three axes represented interesting variation in the data (using the Kaiser-Guttman criterion), and the other axes displayed essentially random variation. However, the first three axes represented less than 50% of the variation, accounting for 17.0%, 14.7% and 13.2 % (total, 44.9%) respectively on the untransformed data (Figure 6.2). Figure 6.3 shows that the majority of the data points are intermediate in all variables, with a relatively small number of birds spread out by the three axes.

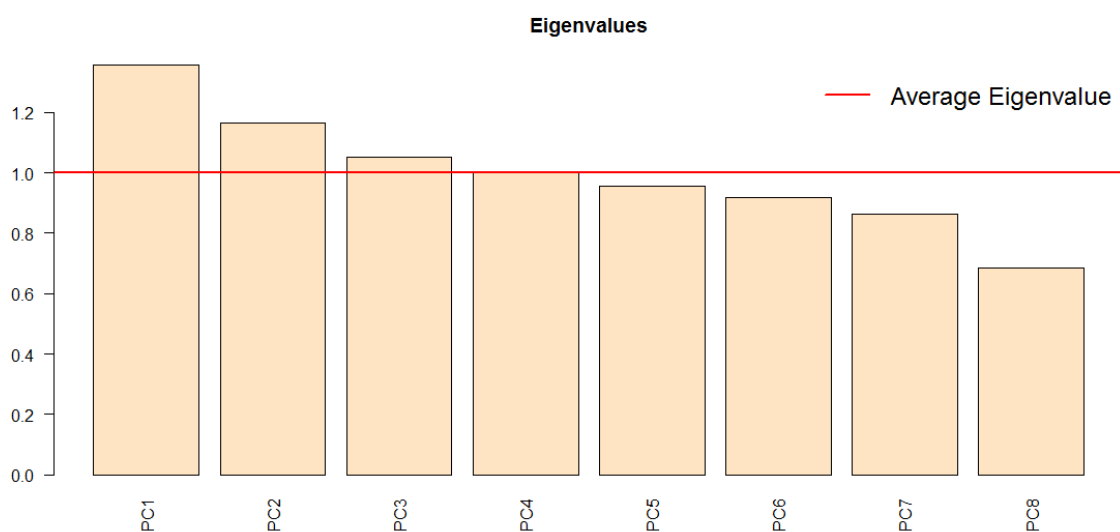

Figure 6.2 Variation in the dataset represented by the PCA axes

The first axis principally represents the variation contributed by the *Salmonella* and *Pasteurella* titres, which, as shown in the biplots, make up one group of correlated variables (Figure.6.3). The majority of the second axis consists of the variation added by scaly leg mite, MDV, *Eimeria* and, to a lesser extent, lice. This second group of correlated variables is approximately orthogonal to the first group, indicating almost no correlation between these groups. Lice counts and IBD titre are negatively correlated and form the principal part of the variation represented by the third axis; lice have a low positive correlation with scaly leg and *Pasteurella*. Ascaridida principally contribute to the fourth (non-significant) axis, and has some correlation with *Eimeria* and IBD.

Analyses following other data transformations showed similar overall groupings, although binarisation emphasised negative correlations between scaly leg and both IBD and *Eimeria* (Figure 6.4); whilst Hellinger transformations gave more weight to the distances between the three transformed parasite counts and highlighted negative correlations between *Eimeria* and lice intensity, and between Ascaridida and scaly leg (Figure 6.5). All PCA's illustrated a strong correlation between *Salmonella* and *Pasteurella* which consistently contributed the 1st principal component and therefore the greatest variation in the data.

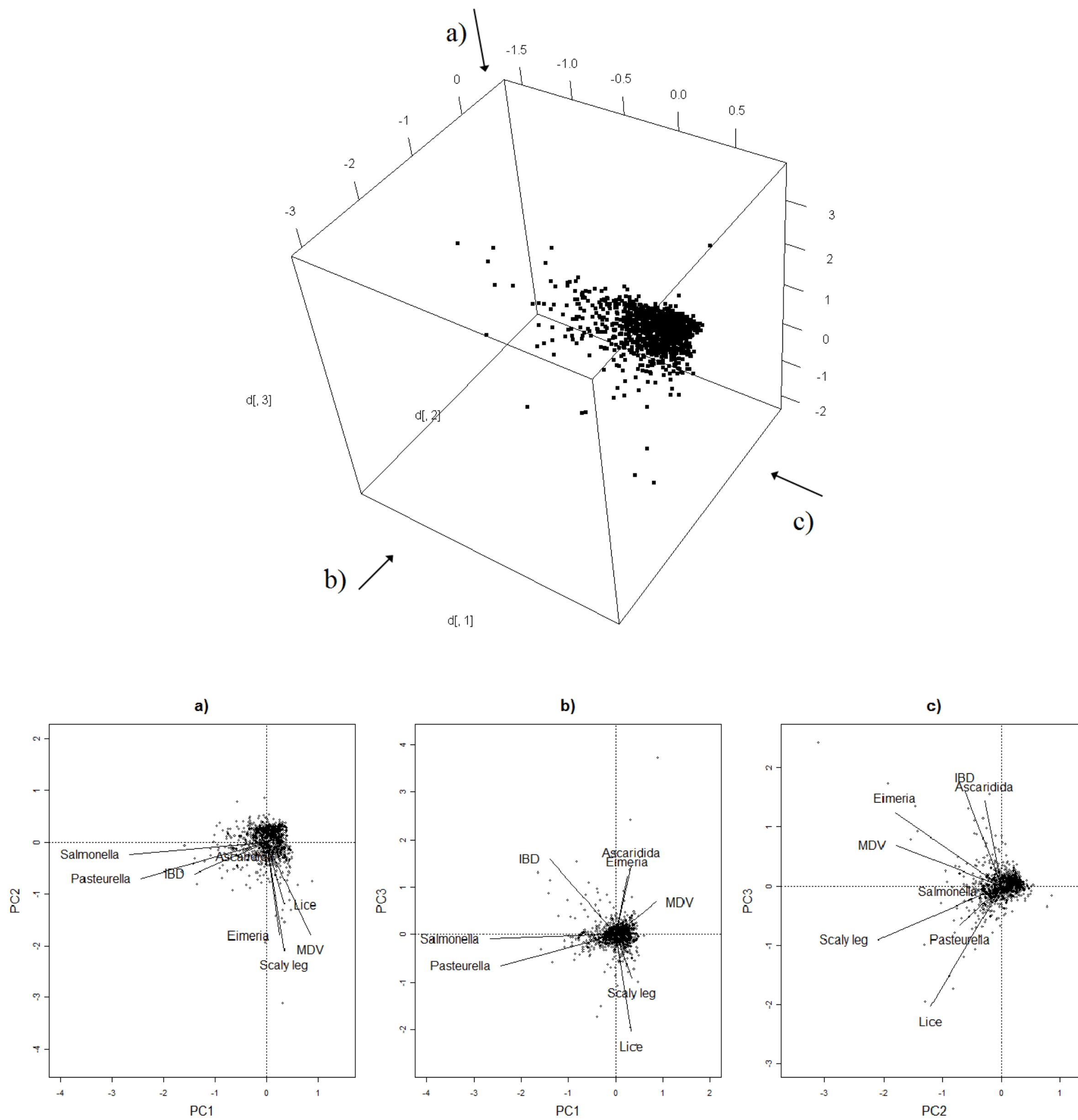

Figure 6.3 Biplots of principal components (scaling 2) showing correlations between variables. Note that correlation is indicated by the angles of the vectors, rather than the apices of the arrows. Labels a-c) correspond to the 3-dimensional views shown above.

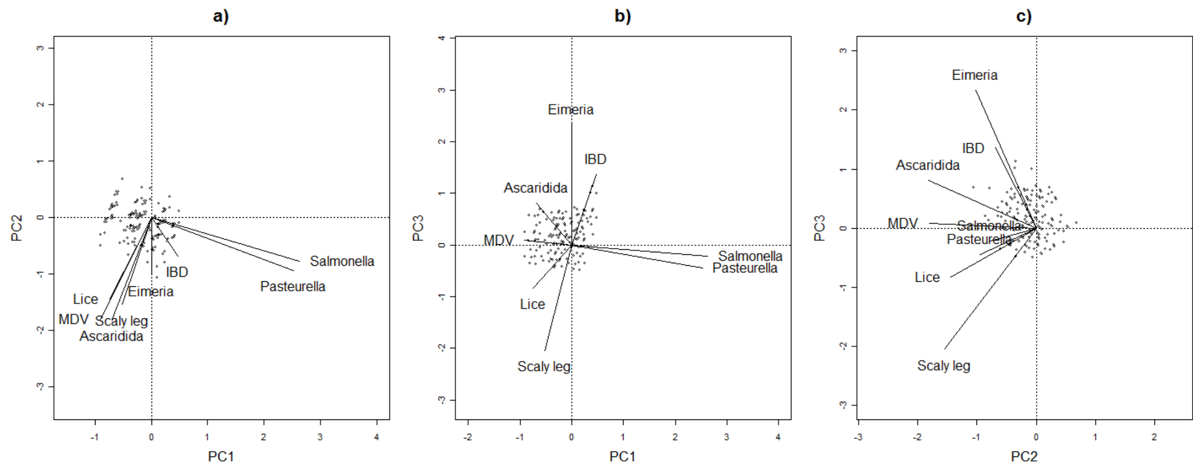

Figure 6.4 Biplots after dichotomisation of the response data

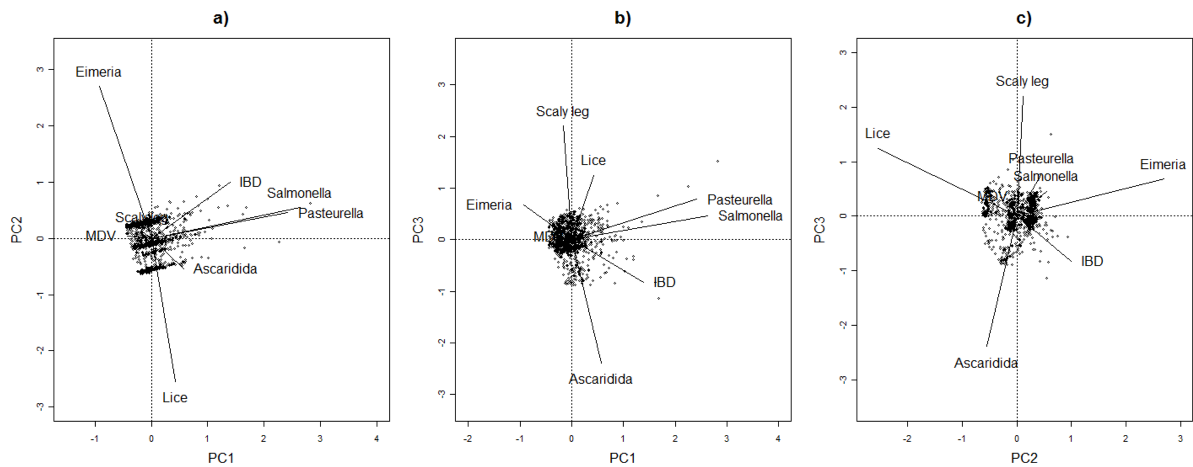

Figure 6.5 Biplots after Hellinger transformation of the parasite data

6.3.2 Constrained ordination

A forward selection process identified the following explanatory variables for inclusion in the RDA model (shown in Appendix 6.2): bird production status and weight; and at the household level, the use of chemical sprays for parasite control and whether the household had had any recent outbreaks of disease in their chicks or growing birds. Variables which were tested but not deemed to significantly improve model fit included the season in which

the household was sampled, the village or region, body condition, age of the bird, and where the bird had come from.

Although significant, the explanatory variables accounted for only a very small proportion of the total variation in the dataset (adjusted R-squared value 0.030), and a permutation test of these axes suggested that 3 of them described significant variation. However, when the household-level variation was partialled out from the model by fitting this as a fixed effect prior to fitting other explanatory variables (the permutation tests used preclude this variable from being fitted as a random effect), this explained around 59% of the variation in disease responses, and left only bird weight and production status as significant bird-level explanatory variables, and only two significant RDA axes (Figure 6.6). Appendix 6.3 shows the full RDA results.

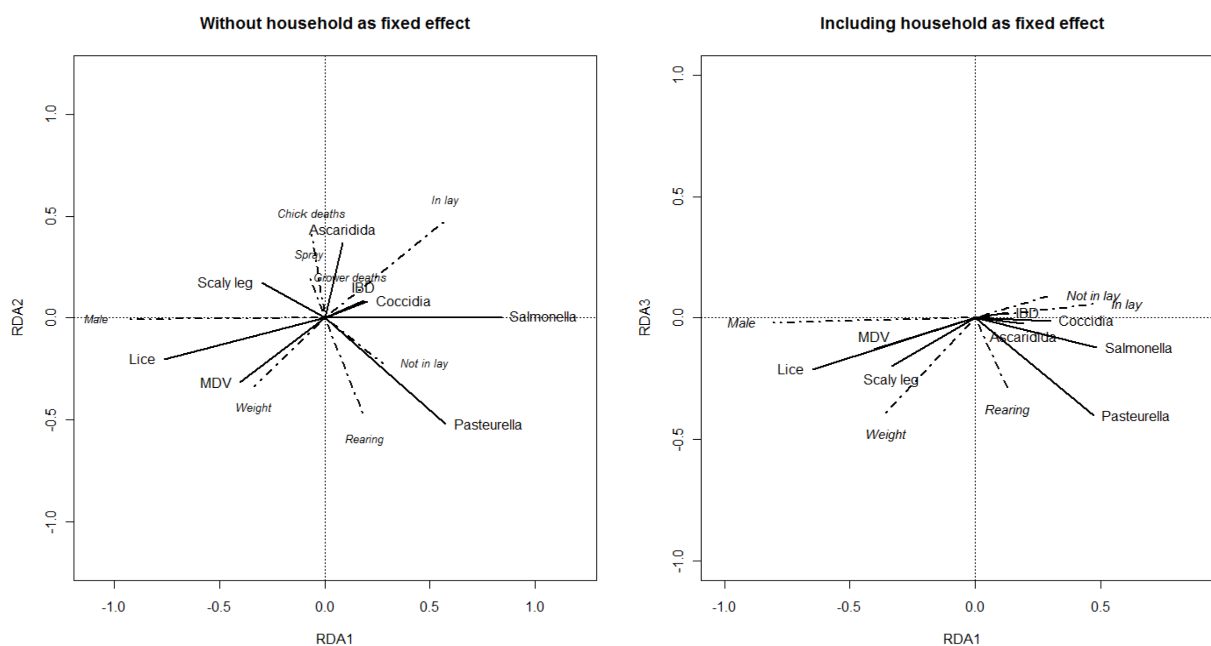

Figure 6.6 Triplots showing the relationships between response and explanatory variables between the first two canonical axes. Angles between variables reflect their correlations. Solid lines represent pathogens (outcomes); dashed lines represent the explanatory variables.

Some relationships were evident between variables: Male birds were positively correlated with lice infestation but negatively with *Salmonella*, and MDV was also correlated more with males and/or birds with heavier weights. Higher *Pasteurella* titres were observed in females which were either rearing chicks or not in production. Households reporting recent deaths in chicks appeared to have birds with greater Ascaridida shedding, but also this variable appeared correlated with the use of household sprays to control parasites. Scaly leg was also associated with households which were using sprays in the housing to treat parasite infestations. Neither the season, nor the sampling cohort explained a significant proportion of variation in the infection responses, and nor did the village or region.

The residuals from the multiple regressions were also subjected to an unconstrained PCA. The Kaiser-Guttman criterion suggested 4 axes may be required to represent the residual variation of interest in the data, and the groupings of the disease variables remain much as they were before. *Salmonella* and *Pasteurella* still maintained a positive correlation, even after accounting for the measured explanatory variables they have in common. Lice, MDV and scaly leg also maintained their previous cluster, although *Eimeria* became more distant from this group.

6.4 Discussion

Multiple micro- and macroparasites were circulating within the apparently healthy adult village chicken population in Ethiopia. However, identifying potential interactions between parasites and testing the strength of these associations, especially when the order of infection is unknown, remains a complex issue. The use of ordination methods was found to be a useful exploratory tool with this dataset, and appeared to be robust to the combination of different types of response data (ordinal scores, counts and serological titres).

PCA does not require normally distributed variables, but in community ecology count data are frequently transformed (e.g. Hellinger or chord transformations) in order to reduce the effect of large counts and to eliminate the apparent correlation of sites with double zeroes, (i.e. where two species may be absent, but for different reasons; Zuur et al. 2007). However, we did not find any advantage to using the Hellinger transformation with our parasite data. Because this transformation gives a Euclidean distance in the space of each site's (i.e. bird's) total parasite abundance, standardising the data gave a large weight to the difference between 0 and 1 parasite, but no difference to the difference between 1 and 60 parasites, if the other parasites were held constant. Since parasite intensity is important for bird health, it was decided that total burden should not be lost from the analysis. In addition, the test methods used for detecting parasite infection are not especially sensitive and the data is likely to contain a number of false negatives. The serological data, being already continuously distributed, did not require prior transformation to avoid the issue of double zeroes, and log transformations or dichotomisation to reduce the impact of outlying values resulted in relatively minor differences to the main components. In an analysis of river water quality variables, Cao et al. (1999) suggested transforming data to give weightings to observations which were outside the normal range of each variable, in order to give more biological relevance to the analysis. The untransformed scaled data maintained the distances between those birds whose infection measurements may be considered outliers from the general population. Since these may be of most interest, either in terms of their immune response and infection tolerance or because they are likely to be significant contributors to pathogen spread in this population, analysing and presenting the scaled untransformed data appeared to be of most value in this context.

The results of the ordinations show some interesting groupings of pathogens which may warrant further investigation. These correlations were maintained even after in-common measured risk factors were accounted for, and even after accounting for the effect of farm,

which might explain common exposures due to management. Therefore it would appear that either there are some attributes of the birds themselves which dictate the set of pathogens they harbour, or certain pathogens increase the risk of infection/exposure to others, resulting in the observed correlations. The closest correlation was seen between *Salmonella* and *Pasteurella* measurements: as both of these were measured by ELISA, this may be a reflection of individual's ability to mount an adaptive immune response. The higher antibody titres were correlated with being female, and *Pasteurella* titres showed a particular correlation with females which were rearing chicks. *Salmonella* Pullorum infections have been shown to recrudesce at the onset of lay in females, with the increase in bacterial numbers triggering a subsequent increase in antibody titres (Wigley et al., 2005). However, this increase in bacterial numbers does not occur in carrier males at the onset of sexual maturity. In females, sexual maturity corresponds with a decrease in T-cell proliferation (Wigley et al., 2005); therefore it is plausible that the reproductive demands placed on females may increase pathogen load, not just of *Salmonella* but of other chronic or frequently encountered infections. It is also possible that there is some other unmeasured risk factor in common, such as different male and female behaviours, which increases exposure to these pathogens, and hence antibody titres. Although all birds in our study were unconfined and scavenged freely, some cocks were observed to roam for some distance, whilst hens, especially those with chicks, tend to stay close to the homestead. This may give different rates of contact with chronically infected birds, which are thought to be the major route of transmission of both *Salmonella* and *Pasteurella* infections.

The second clustering of diseases grouped MDV loosely with *Eimeria*, and MDV also showed a positive correlation with lice counts and scaly leg scores. MDV would be expected to negatively impact the immune response through lymphocyte depletion, and previous findings have demonstrated that MDV-infected birds are less able to clear *Eimeria* infections (Biggs et al., 1968). The antibody titre to MDV is indicative of exposure, but it is

unclear as to how it relates to the extent of disease, as it is primarily a cellular immune response which regulates the latent phase of this viral infection. Birds with the highest titres tended to have low to moderate oocyst counts, and birds with very high oocyst shedding tended to have moderate MDV antibody titres (results not shown). However, a cellular immune response is also important in controlling *Eimeria*, and it may be that birds tolerating MDV infections are also able to moderate *Eimeria* infections to a certain extent, but not necessarily clear them as effectively as an MDV-uninfected bird. This grouping of diseases was most closely associated with male birds and/or heavier birds in the RDA. Males infected with MDV are less susceptible to the development of tumours (Payne and Venugopal, 2000), and in other avian species larger body sizes have been shown to correlate with greater abundances of lice (Møller and Rózsa, 2005); therefore it may be that males are simply tolerating these infections better, whilst females are more likely to succumb.

There was a large group of birds which were not spread out by the ordination analyses. *Salmonella* and *Pasteurella* antibody distributions were positively-skewed, with the cut-off value falling well below the mean (this will be further explored in Chapter 7). High antibody titres against *Salmonella* O9 serotypes are thought to be in response to *S.* Pullorum, allowing establishment of a carrier state (Chappell et al., 2009); this may be the reason behind the occurrence of a low number of birds with antibody levels far in excess of our positive control. The more moderate levels of antibody titres may represent exposure to *S.* Enteritidis. *Pasteurella multocida* is also capable of establishing a carrier state, although the mechanisms and the immune response to this bacterium remain largely unstudied in poultry. This clustering of birds, which were intermediate in all antibody measurements, may therefore suggest that most of these birds have been exposed to *Salmonella* and *Pasteurella,* but are probably not chronically infected, and are negative for IBD and MDV and all parasites. This group of birds is challenging to interpret; low antibody titres may

mean that birds have not been exposed, were infected too long ago to detect the response or simply have a poor immune response, possibly as a result of another immunosuppressive or immune-modifying infection. For the parasite species that we measured more directly, a negative count may again mean a bird has not been exposed, or has been previously infected and cleared the infection.

As illustrated by the number of axes required to explain the residual variation in disease responses, there were almost no birds which had high responses / counts to more than one pathogen group. This finding was also confirmed using the dichotomised data, which suggested that fewer birds were infected with, for example, *Salmonella* and lice than would be expected under an assumption of independence (data not shown). This may in part be due to other opposing and mutually exclusive risk factors, such as sex, in that higher lice burdens are associated with males, but females tend to have higher *Salmonella* titres. Although including household as a partial fixed effect in the RDA then prohibited the inclusion of variables measured at the household or village level, it did illustrate the significant contribution of household to many of the disease responses, suggesting that either there are some important, unmeasured, household factors or that exposure to these diseases at any single time point varies significantly even within a village. This may be due to geographical and management factors, but may also be due to the relatedness of birds in the same household giving them similar immune characteristics. The scarcity of co-positive birds may be due to a lack of synchronicity between infections. It is also possible that birds recently infected with two pathogens in a short time period, where we may expect both a high antibody titre and a high parasite burden, frequently do not survive to be measured, hence the lack of these in this dataset. In order to investigate many of these hypotheses, longitudinal studies are required; however, the ordination analyses has proved useful in the generation of these hypotheses, and in illustrating some potential

associations, both positive and negative, between different types of pathogens, even with very different types of measurement used.

The very low proportion of variation for which the explanatory variables accounted might suggest that other factors are more important in determining birds' responses to the various diseases of interest. The fact that neither season nor sampling cohort contributed significantly to the variation may suggest that there is no strong association of any of these diseases with the rainy season, although this analysis did not allow the fitting of interactions between explanatory variables. As demonstrated in Chapter 5, there were differences between regions, villages and even households as to the outcomes for birds in the rainy season. It is probable that disease measures may similarly vary, and this will be further explored in the next chapter. However, it is clear from this analysis that there are some relationships between infections which need to be controlled for when analysing other risk factors for the infection data.

The presence of multiple interacting pathogens in these regions has implications for interventions to improve chicken health and production in Ethiopia. For example, programmes aiming for genetic improvement of indigenous stock by selective breeding (Dana et al., 2011) need to be aware of the range of infectious diseases to which village chickens are exposed, and be careful not to lose important protective immune traits from the population. Our results would suggest that certain birds are better at tolerating specific pathogen types, and so maintaining diversity at the population level is likely to be important under the current production system. The impact of these multiple infections in the village chicken population is likely to be of greatest significance within the young stock that families rear for replacements, sale or consumption, and which generally mix freely with the adult population. The observed correlation between birds with high Ascaridida counts and households which had had recent deaths in their chicks may suggest that

nematodes are of particular significance for this age group, allowing targeted interventions to be considered. However, as demonstrated in other systems (Nacher, 2011), interventions such as de-worming could, whilst controlling for one pathogen, have implications for the infection biology and epidemiology of others; therefore any intervention undertaken in this population should carefully monitor any effects on other diseases, especially those with zoonotic implications.

Appendix 6.1 Principal components analysis of variance in disease outcomes

```
Partitioning of correlations:
              Inertia Proportion
Total               8          1
Unconstrained       8          1

Eigenvalues, and their contribution to the correlations

Importance of components:
                   PC1   PC2   PC3   PC4   PC5   PC6   PC7   PC8
Eigenvalue         1.356 1.165 1.051 1.003 0.956 0.917 0.863 0.6859
Prop. Explained    0.169 0.145 0.131 0.125 0.119 0.114 0.107 0.0857
Cumulative Prop.   0.169 0.315 0.446 0.571 0.691 0.806 0.913 1.0000

Scaling 2 for species and site scores
* Species are scaled proportional to eigenvalues
* Sites are unscaled: weighted dispersion equal on all dimensions
* General scaling constant of scores:  9.783041


Species scores

              PC1      PC2      PC3      PC4     PC5     PC6
AdjIBD    1.49172 -0.18750 -1.64275  0.52879  1.8596  0.6610
AdjPM     2.47948 -0.54839  0.76631 -0.69760 -0.3597 -0.1293
AdjSal    2.72670 -0.03201  0.03846 -0.05843 -0.4438 -0.2663
AdjMDV   -0.50099 -1.91311 -0.65386 -0.81159  1.4942 -1.9715
Coccidia -0.17364 -1.79034 -1.36871  1.43362 -0.6541  1.4861
AscType  -0.29059 -0.36638 -1.36809 -2.81782 -0.8659  1.0169
LiceTot  -0.22939 -1.25094  2.17937 -0.59738  1.4402  1.7073
Cnemido  -0.02846 -2.24605  0.60680  0.46791 -1.4733 -0.6419
```

Appendix 6.2 Redundancy analysis

```
Partitioning of variance:
              Inertia Proportion
Total          8.0000    1.00000
Constrained    0.2653    0.03316
Unconstrained  7.7347    0.96684


Eigenvalues, and their contribution to the variance

Importance of components:
                    RDA1    RDA2   RDA3   RDA4   RDA5    RDA6    RDA7
Eigenvalue       0.1667 0.0446 0.0251 0.0180 0.00599 0.00347  0.0016
Prop. Explained  0.0208 0.0055 0.0031 0.0022 0.00075 0.00043  0.0002
Cumulative Prop. 0.0208 0.0263 0.0295 0.0317 0.03252 0.03296  0.0331

                    PC1   PC2   PC3   PC4   PC5   PC6   PC7     PC8
Eigenvalue        1.2833 1.145 1.023 0.987 0.919 0.896 0.813 0.6659
Prop. Explained   0.1604 0.143 0.127 0.123 0.114 0.112 0.101 0.0832
Cumulative Prop.  0.1936 0.336 0.464 0.588 0.703 0.815 0.916 1.0000


Accumulated constrained eigenvalues
Importance of components:
                   RDA1   RDA2   RDA3   RDA4    RDA5    RDA6   RDA7
Eigenvalue        0.166 0.0441 0.0251 0.0180 0.00599 0.00347 0.0016
Prop. Explained   0.628 0.1664 0.0949 0.0680 0.02260 0.01308 0.0061
Cumulative Prop.  0.628 0.7951 0.8901 0.9582 0.98081 0.99389 1.0000

Scaling 2 for species and site scores
* Species are scaled proportional to eigenvalues
* Sites are unscaled: weighted dispersion equal on all dimensions
* General scaling constant of scores:  9.5712


Species scores

                         RDA1   RDA2   RDA3    RDA4    RDA5    RDA6
Cnemido                -0.313  0.425 -0.169 -0.1437  0.1583 -0.0273
AdjIBD                  0.197  0.084 -0.174  0.1229 -0.0066  0.1053
AdjPM                   0.506 -0.321 -0.253 -0.2787 -0.0075 -0.0365
AdjSal                  0.794  0.195 -0.140  0.0784 -0.0324  0.0109
AdjMDV                 -0.440 -0.235  0.071 -0.1438  0.0496  0.1132
Coccidia                0.208 -0.014  0.241 -0.0125  0.0255 -0.0925
AscType                 0.113  0.346  0.167 -0.2558 -0.1386  0.0438
LiceTot                -0.796  0.019 -0.231  0.0281 -0.1417 -0.0559

                         RDA1   RDA2    RDA3    RDA4    RDA5    RDA6
Chicks                 0.1733 -0.346 -0.5443 -0.5408 -0.4313 -0.2297
In lay                 0.5908  0.391  0.3817  0.0970  0.0843 -0.2327
Non-productive         0.2820 -0.479  0.2712  0.0166  0.1108  0.5685
Male                  -0.9593  0.119 -0.0332  0.1635  0.0174  0.0185
Outbreak (Chicks)     -0.0511  0.495  0.1143 -0.0456 -0.7976  0.2532
Outbreak (Growers)     0.1252  0.232 -0.4662  0.4660 -0.3741  0.5704
Parasite Spray        -0.0772  0.484 -0.2203 -0.5732  0.3360  0.5111
```

Appendix 6.3 Redundancy analysis conditioned on household

```
Partitioning of variance:
              Inertia Proportion
Total          8.0000    1.00000
Conditioned    4.7084    0.58855
Constrained    0.1514    0.01893
Unconstrained  3.1402    0.39252

Eigenvalues, and their contribution to the variance
after removing the contribution of conditiniong variables

Importance of components:
                          RDA1    RDA2     RDA3     RDA4     RDA5
Eigenvalue             0.11335 0.02443 0.008658 0.003467 0.001497
Prop. Explained        0.03444 0.00742 0.002630 0.001050 0.000450
Cumulative Proportion  0.03444 0.04186 0.044490 0.045540 0.046000

                     PC1   PC2   PC3   PC4   PC5   PC6    PC7    PC8
Eigenvalue         0.513 0.494 0.479 0.433 0.355 0.316 0.2796 0.2678
Prop. Explained    0.156 0.150 0.145 0.131 0.107 0.096 0.0849 0.0813
Cumulative Prop.   0.202 0.352 0.498 0.629 0.737 0.833 0.9186 1.0000


Accumulated constrained eigenvalues
Importance of components:
                         RDA1    RDA2     RDA3     RDA4     RDA5
Eigenvalue             0.1134 0.02443 0.008658 0.003467 0.001497
Proportion Explained   0.7487 0.16138 0.057180 0.022900 0.009890
Cumulative Proportion  0.7487 0.91003 0.967210 0.990110 1.000000

Scaling 2 for species and site scores
* Species are scaled proportional to eigenvalues
* Sites are unscaled: weighted dispersion equal on all dimensions
* General scaling constant of scores:  9.5712


Species scores

                       RDA1    RDA2    RDA3     RDA4     RDA5     PC1
Cnemido              -0.333 -0.1987  0.1232 -0.11621 -0.02961 -0.1889
AdjIBD                0.129  0.0211  0.0652  0.10078  0.04032 -0.4937
AdjPM                 0.472 -0.4032 -0.1057  0.03228 -0.02084 -1.5627
AdjSal                0.485 -0.1228  0.1155 -0.00723  0.00187 -1.4222
AdjMDV               -0.402 -0.1287 -0.1655 -0.04677  0.07148  0.0251
Coccidia              0.300 -0.0127  0.0299 -0.03497  0.07831  0.1452
AscType               0.191 -0.0225  0.1204 -0.04570  0.05171  0.8157
LiceTot              -0.649 -0.2119  0.1111  0.09718  0.01673 -0.6733

                       RDA1    RDA2    RDA3     RDA4     RDA5   PC1
Chicks                0.129 -0.2909 -0.0504   0.4611  -0.2116    0
In Lay                0.476  0.0569  0.3271   0.0147   0.3524    0
Non-productive        0.296  0.0908 -0.5275  -0.2077  -0.0860    0
Male                 -0.807 -0.0186  0.0999  -0.0439   0.1050    0
Bird Weight          -0.357 -0.3905  0.0991  -0.2751   0.1178    0
```

Chapter 7

Spatial and temporal patterns of endemic infections in village poultry

7.1 Introduction

Worldwide, the significance of Newcastle disease virus (NDV) as a major constraint to village chicken production is well recognised, and the benefits of vaccination, as a cost-effective intervention, have been demonstrated in 13 different African countries (Dwinger and Unger, 2006). In Tanzania and Mozambique, effective NDV control programmes have resulted in increased chicken numbers and subsequent increases in households', and particularly women's, purchasing and decision-making powers (Alders and Pym, 2009). However, as Bell (2009) remarks, the key to effective disease control is to know which diseases are prevalent in the area before starting.

In Ethiopia, whilst control programmes for NDV in village chickens are still in their infancy, there has been a growing interest in recent years. One study by Nega et al. (2012) in Ethiopia estimated that NDV thermostable vaccines reduced chicken mortality by 82%. Although likely to be of benefit, control strategies for NDV may not have such a high impact universally. As demonstrated by the wide range of reported seroprevalence values in Ethiopia (Zeleke, 2005b; Tadesse et al., 2005; Regasa et al., 2007; Mazengia et al., 2010; Chaka et al., 2012), which range from 6 – 64%, exposure to the virus varies widely, and disease may occur both endemically and as epizootic outbreaks. This pathogen's epidemiology is complicated by circulating lentogenic and avirulent strains, seasonality and a wide range of risk factors (Martin, 1991; Awan et al., 1994). In Tanzania, Komba et al. (2012) reported continued undiagnosed disease outbreaks in chicks and growers despite achieving good vaccine coverage with a thermostable NDV vaccine, suggesting other diseases also needed to be controlled.

A number of other potential pathogens, including infectious bursal disease virus, *Salmonella enterica* serovars Gallinarum and Pullorum, *Pasteurella multocida, Mycoplasma*

gallisepticum, Eimeria, helminths and ectoparasites have all been reported in rural village chickens in Ethiopia (Ashenafi et al., 2004b; Belihu et al., 2009; Tolossa et al., 2009; Degefu et al., 2010; Berhe et al., 2012; Berihun et al., 2012; Chaka et al., 2012; Jenbreie et al., 2012; Molla et al., 2012; Zeryehun and Fekadu, 2012; Luu et al., 2013). Even in the absence of reported large outbreaks, we have found in some kebeles that mortalities attributed to disease occurred in approximately 30% of adult chickens within 6 months of recruitment (Chapters 4 and 5). Nevertheless, in Ethiopia, the majority of mortalities among village chickens tend to be attributed to "*fengele*", a syndromic description which is often mistranslated as Newcastle disease. A potential concern, therefore, is that, should any vaccination programmes for NDV be implemented in future, these may not meet local people's expectations for reducing (the multi-factorial) mortality unless it is made clear that a number of other pathogens are also likely to be of significance. Furthermore, it was also noted (Chapters 4 and 5) that there was considerable variation in disease mortality between geographic areas, so the impact of vaccination may be expected to vary in space, and possibly in time.

7.1.1 Seasonal patterns of disease

The importance of seasonal climate factors on pathogens may be direct, in that cooler, wetter conditions are likely to promote the survival and spread of many pathogens in the environment. A number of pathogens survive better in cool, moist environments, including the influenza and NDV viruses and the protozoan parasite, *Eimeria* (Martin, 1991; McDougald, 2003; Swayne and Halvorson, 2008). *A*irborne spread of respiratory viruses such as influenza on water droplets is greatly affected by ambient temperature and relative humidity in temperate climates (Fuhrmann, 2010). For parasitic diseases, the long dry season can impede the development of nematode larvae (Weaver et al., 2010) and seasonal fluctuations in rainfall and temperature may alter the abundance or behaviour of

invertebrate intermediate hosts. Indirect effects of climate on disease may also be occurring. Additional stresses may be placed on the host with the change in season, making them more susceptible to infections, or contact rates may vary seasonally. In Ethiopia, feed tends to be scarcer during the rainy season, placing birds under nutritional stress, and potentially forcing them to travel further in order to scavenge enough feed, thus increasing direct or indirect contacts with other birds. Cold or heat stress, seasonal breeding and increased movement of birds through markets associated with holiday periods are also factors which can contribute to seasonal patterns of disease (Awan et al., 1994).

Seasonality of disease is well recognised in village chickens, associated with the rainy season in Ethiopia (Dessie and Ogle, 2001; Halima et al., 2007), but often associated in other countries with hot and dry or hot and humid seasons (Awan et al., 1994). Despite this well-recognised temporal variation, most knowledge of the infectious agents present is based on cross-sectional studies to assess prevalence or seroprevalence, and little account is taken of the timing of the survey. For studies which have tried to investigate seasonal effects, only a limited number of pathogens have so far been considered. One study in Tanzania has reported increasing seroprevalence and antibody titres to Newcastle disease from June to October in village chickens, and a corresponding increase in the number of virus isolates (Yongolo et al., 2002). A small study in Kenya reported higher NDV antibody titres in the wet compared to the dry season, but only 24 birds, selected purposively, were sampled at each time point (Kemboi et al., 2013). In Ethiopia, in their survey of village birds bought in markets, Chaka et al (2012) reported that birds were seropositive to significantly more diseases in the rainy season. This appeared to be predominantly due to an increase in seropositivity to *Mycoplasma gallisepticum*, as they also reported finding no significant difference in the seroprevalence of IBD, *Pasteurella* or NDV between seasons. In a survey of household flocks, there was found to be a higher NDV seroprevalence and virus detection in the dry season (Chaka, 2013b).

The effect of climate on macroparasite distributions in village chickens has primarily been studied by making comparisons between different agro-ecological zones (e.g. high- and low-altitude), but results are conflicting (Eshetu et al., 2001; Tolossa et al., 2009; Molla et al., 2012). Similarly, infection prevalence of the protozoan parasite *Eimeria* has been compared between high and low altitude situations in Ethiopia (Ashenafi et al., 2004a) and Kenya (Kaingu et al., 2010), but with conflicting results. One study in Tanzania found that burdens of the nematode *Capillaria* increased with progression of time during a single rainy season, but did not study birds in the dry season, nor investigate whether this was consistent between years (Magwisha et al., 2002). A second study in Tanzania found some seasonal differences in both prevalence and burden of selected nematodes, but also found that these patterns varied between different climatic zones (Permin et al., 1997). It has been suggested that this may be due to differences in the optimal climatic conditions for survival for free-living stages or intermediate hosts, which vary between different parasite species.

In light of the association frequently reported between disease mortalities and the main rainy season in Ethiopia (Dessie and Ogle, 2001; Halima et al., 2007; Dinka et al., 2010), the more frequent reporting of disease during the months of the rainy season by farmers at our study sites (Chapter 4) and the higher numbers of birds lost to disease during follow-up over the rainy season at one of our sites (Chapter 5), we wished to better understand the seasonal dynamics of the pathogens in the adult chicken populations. We hypothesised that the increased mortalities in the Horro region compared to the Jarso region, and the increased mortalities in Horro in the rainy season may be associated with an increase in exposure to one or more specific pathogens, which may be detectable at the population level.

Specific questions we wished to answer were:

1. Are there geographical differences in exposure to pathogens, which may be contributing to higher disease mortality in Horro compared to Jarso, and to variation in disease mortality between the Horro kebeles?
2. Are there seasonal differences in exposure to pathogens, which may contribute to the observed higher mortality, particularly in Horro in the rainy season?
3. Are there other seasonal household risk factors such as changes in feed, contacts, or housing which alter the risk of infection?

The pathogens to be investigated were chosen based on knowledge of what was likely to be important in backyard flocks, and to represent a range of pathogen types. Naturally it is not a complete list of all the diseases which may be affecting the population, but the pathogens chosen interact with a variety of immune system components, and in addition are some of those which are thought to be the most likely to cause mortality in village chickens.

7.2 Methods

The study used data from four cross-sectional surveys carried out between May 2011 and November 2012 in two geographically distinct woredas (administrative districts), Horro and Jarso, within the Oromia region of Ethiopia. Eight kebeles (small administrative districts, roughly equivalent to a ward) were visited on four occasions; in May/June and October/November in each year of the study. These visits were timed for before and after the main rainy season, which occurs between June and September. Different households were selected on each occasion and two chickens in each household were sampled. Households which did not own at least two indigenous breed chickens of over 6 months of

age were excluded from the study. Full details of the selection process can be found in Chapter 2.

Information on management and disease occurrence over the previous 12 months was collected in a questionnaire interview with an adult member of the household, normally either the household head or the main carer for the chickens. In addition, two chickens of at least 6 months of age were randomly selected from the household flock and each underwent clinical examination, was condition scored using a 0-3 grading scale (Gregory and Robins, 1998), and was scored for lice using a timed count of 3 areas of the body – one side of the keel, the back and the rump - and a total count of lice at the base of the flight feathers of one wing and the tail feathers (Clayton and Drown, 2001). Birds were also scored for the degree of hyperkeratosis of the legs and feet (none, mild or severe), and scrapings were taken to confirm the presence of *Cnemidocoptes mutans* (scaly leg mite). Data on the chickens' age, origin and current status of production was collected from the owner. Blood samples were drawn from the brachial vein of the selected birds into sodium citrate and faecal samples were collected from individual birds wherever possible; environmental faecal samples were taken to represent the household, if individual samples were not forthcoming. All samples were kept chilled and were transported to the laboratory for testing.

Serological testing was carried out for bacterial and viral infections by enzyme-linked immunosorbent assays (ELISA). Testing for Infectious bursal disease virus antibodies was performed using a commercial kit (Flockscreen, x-OvO, Dunfermline, UK), while antibody ELISAs to *Salmonella* O9 serotypes, *Pasteurella multocida* and Marek's disease virus were developed in-house. Full details of all these tests can be found in Chapter 2. Samples were tested in triplicate wherever possible, or in duplicate when reagents became limited.

Samples with high variation between replicates were retested. Positive and negative controls were run on each plate.

The test for Marek's disease virus required that samples be tested against lysates of both virus-infected and uninfected chicken kidney cells; the optical density (OD_{405}) against the uninfected cell lysate was then subtracted from the OD_{405} against the infected lysate to give a net value which was used in further calculations. Samples with a net value greater than 3x the standard deviation of the net values (after accounting for the effect of the different test plates) of were deemed to be cross-reacting to the cells in which the virus was grown and were discarded from further analyses.

Optical densities for all of the ELISA samples are converted into a ratio to the positive control (s:p ratio), using the formula;

$$\frac{\text{Mean sample OD-Negative control OD}}{\text{Positive control OD – Negative control OD}} \qquad \textit{Formula 7.1}$$

We have elected to work with the continuous data when trying to make comparisons in disease patterns, as the dichotomisation of the data may result in the loss of potentially valuable information. For the serological measurements, we have no clear cut-off value for the laboratory-developed tests to discriminate positive and negative samples, because we had no "gold standard" test or enough samples with a known infection status with which to compare our results. The range of s:p ratios to the ELISA tests developed in-house did not demonstrate clear cut-off points, and sufficient negative sera were not available to confidently determine positive cut-offs for these tests. However, in order to produce some estimates of prevalence, it was desirable to have some approximate cut-off values. A small panel of sera from birds with known exposure to vaccines or experimental infection were used to determine a conservative cut off value for each test which would still detect all known positives. Cut-off values chosen were 0.34, 0.61 and 0.29 for *Salmonella, Pasteurella*

and MDV, respectively. In addition, data were analysed with a pre-selected s:p ratio cut-off of 0.3 or 0.5, to give a generous and a conservative estimate of the prevalence, respectively. The exception to this is IBD, which, as a commercial kit, had a recommended cut-off of 0.306. However, due to issues with the running of the test (described in more detail in Chapter 2), and dictated by the data, it was decided that the cut-off needed to be lowered to 0.285.

We also chose to use the parasite count data, as the number of macroparasites in a host is highly relevant to any disease process, and of more value than simply defining their presence or absence. Definitions of the terms specific to the parasite data are given in Appendix 7.1.

7.2.1 Modelling

All models and graphical summaries were performed using R (R Development Core Team, 2008). Intercept-only random effect regression models were used to estimate the variance partitioning for the serological data, to estimate the relative contributions of each level of the hierarchical data (Bird/Household/Kebele/Market shed/Region) and the contribution of the ELISA test plate to the variation in the continuous responses. The variance partitioning was also estimated after dichotomisation of the serological responses using the latent variable method (Goldstein et al., 2002). The values chosen for dichotomisation were s:p ratios of 0.5 for the *Salmonella* and *Pasteurella* data, 0.3 for the MDV data and 0.285 for the IBD data.

The s:p ratio is designed to allow comparison of samples tested on different plates; however, there was found to be considerable variation in the data which was attributable to the plate on which the sample had been tested (which we have called “plate effect”). The cause of this was wide variation in the control values between different test plates, due to practical difficulties in standardising the laboratory conditions, such as ambient

temperature, water quality and operator accuracy. To control for this variation, where regression models have been used to model the optical density, Plate has been included as a random effect. Where optical densities are used as explanatory variables for other outcomes, values have first been corrected for the variation between plates by using a regression model to adjust for the plate effect and taking the bird-level residuals as an estimation of the antibody response.

Generalised linear mixed-effects models were used to estimate the effects of different explanatory variables on the infection measurements, with the primary interest being whether seasonal or regional variation contributed significantly, whilst controlling for the effect of other explanatory variables. Gaussian models were used for the serological data, logistic regression for parasite positivity and Poisson regression for the parasite count data for birds detected positive. The relationships between individual explanatory and responses variables were initially explored using tabulation and graphical summaries, univariable models and Generalised Additive Models. Continuous explanatory variables with a non-linear relationship were fitted piece-wise. Multivariable models were then constructed for each infection outcome, by fitting *e*xplanatory variables stepwise for each model, such that only those which significantly improved the fit of the model ($p<0.05$) were retained.

In some cases where models estimated considerable variation between households, or household residuals were non-normally distributed, attempts were made to investigate spatial autocorrelation by plotting household residuals according to their geographical co-ordinates using the RGoogleMaps package in R (Loecher, 2011) to make a visual assessment of the locations of outlying household residuals. A spatial test statistic (Eagle, unpublished) was used to assess whether the variation in households over a range of

distances was less than might be expected compared to a random distribution of the data points, which might suggest spatial clustering of the data.

A linear quantile mixed modelling approach was also investigated for some of the serological data, where the distribution of the response data did not appear to be adequately modelled by the classical linear modelling approach. Quantile regression extends regression based on the mean to the entire conditional distribution of the outcome variable, and so allows for the fact that individuals who rank differently according to the outcome variable may be affected to a different extent, or even in opposite ways, by the explanatory variables (Koenker and Hallock, 2001; Geraci, 2014). Linear quantile mixed models were fitted to each 10th percentile of the distribution using the lqmm package in R (Geraci, 2014), and plots of the parameter estimates over the distribution were produced. The bootstrapped standard errors of the parameters were used to estimate over which quantiles of the distribution the explanatory variables significantly affect the serological responses.

7.3 Results

7.3.1 Serological data

Seroprevalence estimates suggested that exposure to the bacterial pathogens was common, and that between 70-90% of birds had antibodies to *Salmonella* and *Pasteurella* (Table 7.1). Exposure to the viral pathogens was lower, with an estimated 3.6% of adult birds demonstrating antibodies to IBDV, and 20-30% of birds positive for Marek's disease antibody. There was a large range of antibody measurements to the three ELISAs developed in-house (*Salmonella, Pasteurella* and MDV), with a number of birds demonstrating values more than three times that of positive controls. Distributions of s:p ratios for each test are shown in Figure 7.1.

Intraclass correlation coefficients (ICCs), estimated on both continuous and binarised data for each disease (Fig 7.2) suggested that plate contributed significant variation to all the serological measurements, with the exception of the IBD when values were dichotomised. All measurements showed moderate household level clustering, with region, market shed and kebele contributing relatively little to the overall variance for any of the diseases.

Table 7.1 Estimates of seroprevalence for bacterial and viral diseases tested by ELISA

	Pasteurella	*Salmonella*	IBD	MDV
Valid test results (n)	1225	1244	1216	1208
Cut-off value selected based on data	0.61	0.34	0.285	0.29
Prevalence (95% CI) based on selected cut-off	69.5% (66.8 – 72.0)	85.5% (83.5 – 87.4)	3.6%* (2.7 – 4.8)	31.3% (28.7 – 34.0)
Prevalence (95% CI) cut-off value=0.3	91.5% (89.8 – 92.9)	88.3% (86.4 – 89.9)	n/a	30.6% (28.1 – 33.3)
Prevalence (95% CI) cut-off value=0.5	79.0% (76.7 – 81.2)	74.4% (71.9 – 76.7)	n/a	19.6% (17.5 – 22.0)
Number (%) of positives with s:p ratio >3	28 (2.3%)	86 (6.9%)	0	2 (0.17%)

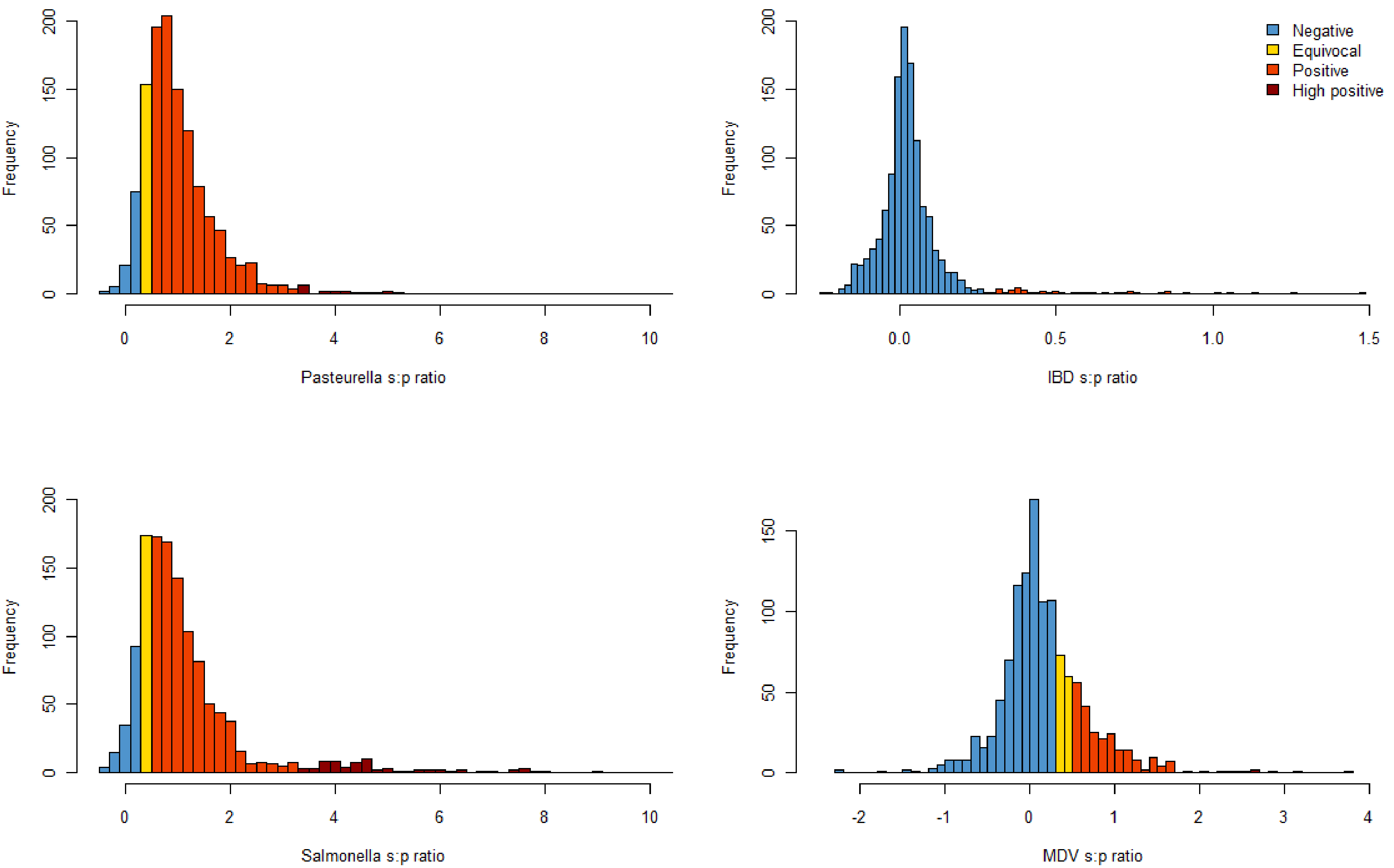

Figure 7.1 Distributions of sample:positive ratios for four ELISA tests. Values have been adjusted to control for between-plate variation.

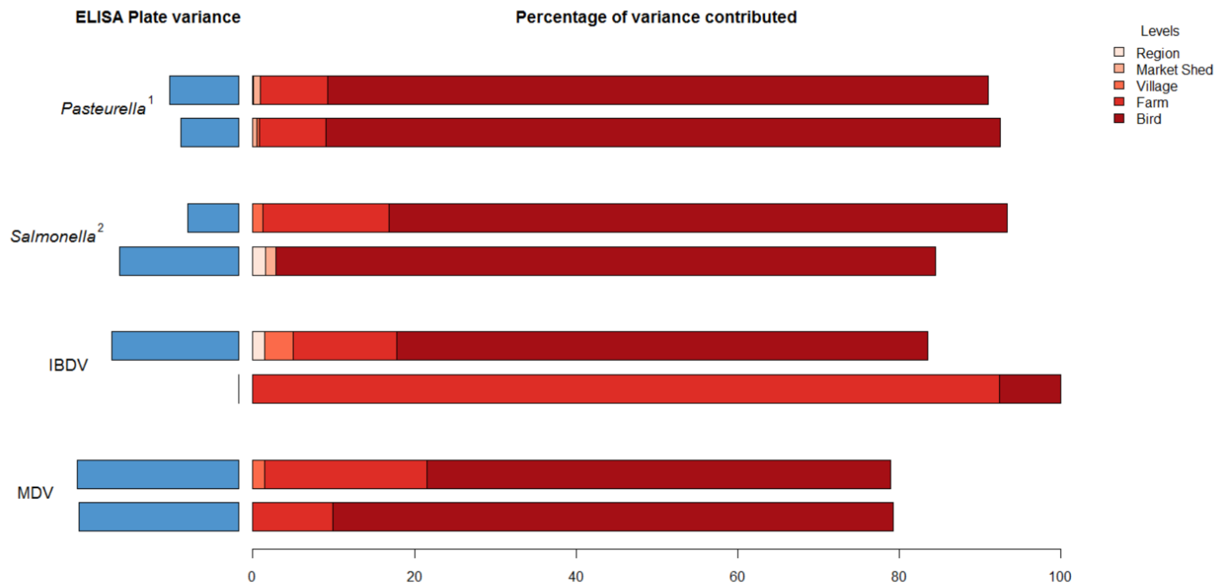

Figure 7.2 Estimates of intraclass correlation coefficients for serological data. For each pair of bars, the top bar shows the estimates using the continuous data, whilst the bottom bar shows the estimates derived from binomial data using the latent variable method. The left (blue) bars indicate the variance attributable to the ELISA plate, the right (red) bars indicate the variance due to the clustering of birds within households, villages, market sheds and regions.

1. *One outlying bird with an s:p ratio >10 has been excluded*
2. *Three outlying birds with an s:p ratio >10 have been excluded*

7.3.1(a) Pasteurella

An intercept-only random-effect model fitted to the log value of the *Pasteurella* s:p ratios suggested that at least 80% of the variation was at the bird level, even when the outlying bird was excluded, with less than 10% accounted for at the household level (Fig 7.2). When fitted as fixed effects, season, region, production status, recent outbreaks, feed provision and *Salmonella* titres all significantly improved model fit (Table 7.2).

The results suggested that antibody titres were almost, but not quite, significantly lower in Jarso, compared to Horro, but that in both regions there was an increase in the antibody levels in both sampling years across the population in October, i.e. following the rainy season. Households in Horro which had reported an outbreak in the last 12 months tended to have slightly lower antibody titres, although those in Jarso did not. Birds in households

which did not provide supplementary feed during the rainy season tended to have lower antibody titres. At the bird level, hens which were rearing chicks had higher levels of antibody, compared to those in lay, but male birds had lower titres. Antibody titres tended to increase with weight in birds in Horro, but not in Jarso. The relationship between *Salmonella* and *Pasteurella* was found to be non-linear, and so the *Salmonella* variable was fit as a piecewise term. As birds' antibody levels to *Salmonella* increased up to an s:p ratio of 2, there appeared to be a corresponding increase in *Pasteurella* antibody measurement, but further increases in the *Salmonella* titre above 2 had no effect.

Table 7.2   Model coefficients for *Pasteurella* s:p ratio

Random effects	Groups	Name	Variance	Std.Dev.
	Household	(Intercept)	0.007	0.085
	Plate	(Intercept)	0.006	0.080
	Residual		0.070	0.264

		coefficient	se	95% C.I.	P
(Intercept)		0.63	0.08	0.48 - 0.78	<0.01
Categorical variables					
Region	Horro	reference			
	Jarso	*0.16*	*0.08*	*0.00 - 0.32*	*0.06*
Season	May 2011	reference			
	Oct 2011	**0.08**	**0.03**	**0.01 - 0.14**	**0.02**
	May 2012	0.03	0.04	-0.05 - 0.11	0.46
	Oct 2012	**0.19**	**0.04**	**0.11 - 0.27**	**<0.01**
Production status	In lay	reference			
	Rearing chicks	**0.11**	**0.03**	**0.05 - 0.17**	**<0.01**
	Incubating eggs	0.03	0.04	-0.05 - 0.11	0.48
	Not in production	0.04	0.02	-0.01 - 0.09	0.13
	Male	**-0.05**	**0.02**	**-0.10 - -0.01**	**0.03**
Outbreak in last 12 months	No	reference			
	Yes	**-0.06**	**0.03**	**-0.11 - -0.01**	**0.02**
Feed in rainy season	Some	reference			
	None	**-0.20**	**0.09**	**-0.37 - -0.02**	**0.03**
Recent outbreak: Jarso interaction		-0.08	0.04	-0.16 - 0.01	0.07
Continuous variables					
Salmonella titre (≥2)		0.01	0.01	-0.01 - 0.04	0.28
Salmonella titre (<2) (Intercept)		**-0.11**	**0.05**	**-0.21 - -0.02**	**0.02**
Salmonella titre (<2)		**0.18**	**0.02**	**0.13 - 0.22**	**<0.01**
Weight (kg)		**0.12**	**0.04**	**0.05 - 0.19**	**<0.01**
Weight: Jarso interaction		**-0.13**	**0.05**	**-0.24 - -0.03**	**0.01**

7.3.1 (b) Salmonella

An intercept-only random-effect model fitted to the log values of the *Salmonella* s:p ratios suggested that around 76% of the variation was at bird level, and around 11% at household level, with no variation contributed by regions or market sheds. Both season and kebele significantly improved models fits when fitted as fixed effects, although as other variables were fitted to the model, it became apparent that the season effect was only through interactions with some of the other variables. These interactions suggested that whilst antibody levels to *Salmonella* rose with increasing *Pasteurella* titres, this effect was more pronounced in birds measured at the end of the wet season (October) compared to birds measured at the end of the dry season (May). However, the opposite effect was observed for the cell lysate, in that increasing measurements of this variable corresponded with a greater increase in *Salmonella* titre for birds measured at the end of the dry than the wet season (Figure 7.3). The model also suggested that the MDV s:p ratio was negatively correlated with the *Salmonella* s:p ratio, and male birds had significantly lower titres than females (Table 7.3).

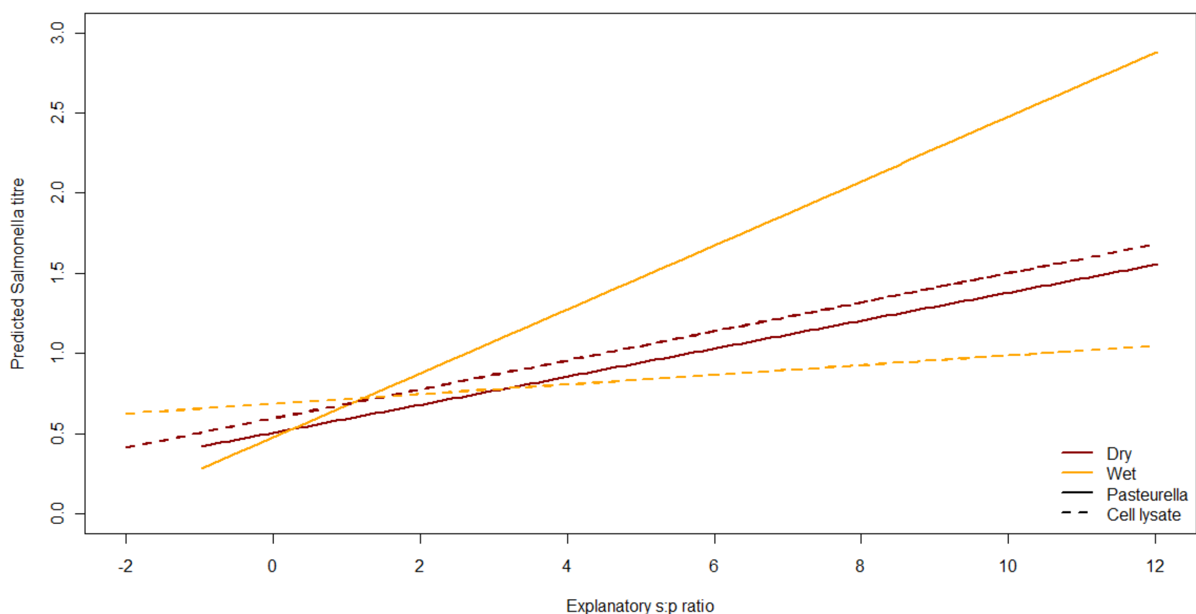

Figure 7.3 Effect of interactions between season and the s:p ratios for Pasteurella and cell lysate on predicted Salmonella s:p ratios

Table 7.3 Model coefficients for *Salmonella* s:p ratio

Random effects							
	Groups	Name		Variance	Std.Dev.		
	Household	(Intercept)		0.014	0.117		
	Plate	(Intercept)		0.008	0.089		
	Residual			0.088	0.296		
		coefficient	se	95% C.I.			P
(Intercept)		0.60	0.05	0.50	-	0.69	<0.01
Categorical variables							
Kebele	J2B	reference					
	H1A	**0.16**	**0.06**	**0.06**	**-**	**0.27**	**<0.01**
	H1B	0.02	0.06	-0.09	-	0.13	0.68
	H2A	0.07	0.06	-0.04	-	0.18	0.24
	H2B	0.00	0.05	-0.10	-	0.11	0.94
	J1A	0.09	0.06	-0.03	-	0.20	0.13
	J1B	**0.15**	**0.05**	**0.05**	**-**	**0.25**	**<0.01**
	J2A	**0.09**	**0.05**	**0.00**	**-**	**0.19**	**0.05**
Season	Dry	reference					
	Wet	-0.01	0.05	-0.10	-	0.07	0.75
Sex	Female						
	Male	**-0.16**	**0.02**	**-0.20**	**-**	**-0.12**	**<0.01**
Continuous variables							
IBD s:p ratio		**0.31**	**0.08**	**0.15**	**-**	**0.46**	**<0.01**
MDV s:p ratio		**-0.04**	**0.02**	**-0.08**	**-**	**-0.00**	**0.03**
***Pasteurella* s:p ratio**		**0.09**	**0.01**	**0.06**	**-**	**0.11**	**<0.01**
Wet season: *Pasteurella* interaction		**0.10**	**0.02**	**0.06**	**-**	**0.14**	**<0.01**
CKCL s:p ratio		**0.09**	**0.01**	**0.06**	**-**	**0.12**	**<0.01**
Wet season: CKCL interaction		**-0.06**	**0.02**	**-0.09**	**-**	**-0.03**	**<0.01**

However, examination of the model residuals suggested that there was an increase in the variance of the errors as the fitted values increased, and that the distribution of residuals was right-skewed, despite having log-transformed the response variable. Examination of the household residuals did not suggest any spatial correlation, and indeed the variance on the household errors was small, and they were reasonably normally distributed. This suggested that the model only poorly explained the *Salmonella* antibody responses in birds with measurements at the upper end of the distribution, and that these high responses were unlikely to be attributable to a localised outbreak.

One possible interpretation of the higher *Salmonella* titres is that these come from birds which were chronically infected with *S.* Pullorum, whereas more moderate levels of antibody titres may represent exposure to *S.* Enteritidis or *S.* Gallinarum. Tabulating the numbers of birds with s:p ratios greater than 2 demonstrated that there was a considerable differences in the proportion of these birds between kebeles, and that those kebeles with the highest proportions tended to be the ones which the model had suggested were significantly different from the reference kebele (Table 7.4). This raised the question of how to interpret the results of the model, as it suggested that the mean s:p ratio for birds in kebele H1A was 1.2 times greater than birds in kebele J2B. However, this may be due to the greater proportion of birds with very high titres, rather than because all birds in kebele H1A had been more recently exposed to *Salmonella.* It was therefore decided to use a quantile mixed effects regression model to look at the effects of the explanatory variables over the entire distribution of *Salmonella* measurements.

Quantile regression suggested that the effect of different kebeles was not consistent across all quantiles of the *Salmonella* distribution (Figure 7.4). Below the 40th percentile, there were no significant differences between kebeles. Whilst kebele J1B appeared to be a significant risk factor for increase in titres across the whole distribution above the 40th percentile, resulting in a relatively flat curve, kebele H1A was only a significant risk factor at the upper quantiles of the distribution, above the 70th percentile. If our interpretation of the antibody responses is correct, then this may represent more widespread recent exposure in kebele J1B, but higher numbers of persistent carrier birds in kebele H1A.

Table 7.4 Proportion of birds with high *Salmonella* s:p ratios in each kebele

Kebele	H1A	H1B	H2A	H2B	J1A	J1B	J2A	J2B
Proportion of birds with *Salmonella* s:p ratio >2	0.18	0.07	0.12	0.09	0.13	0.15	0.09	0.03

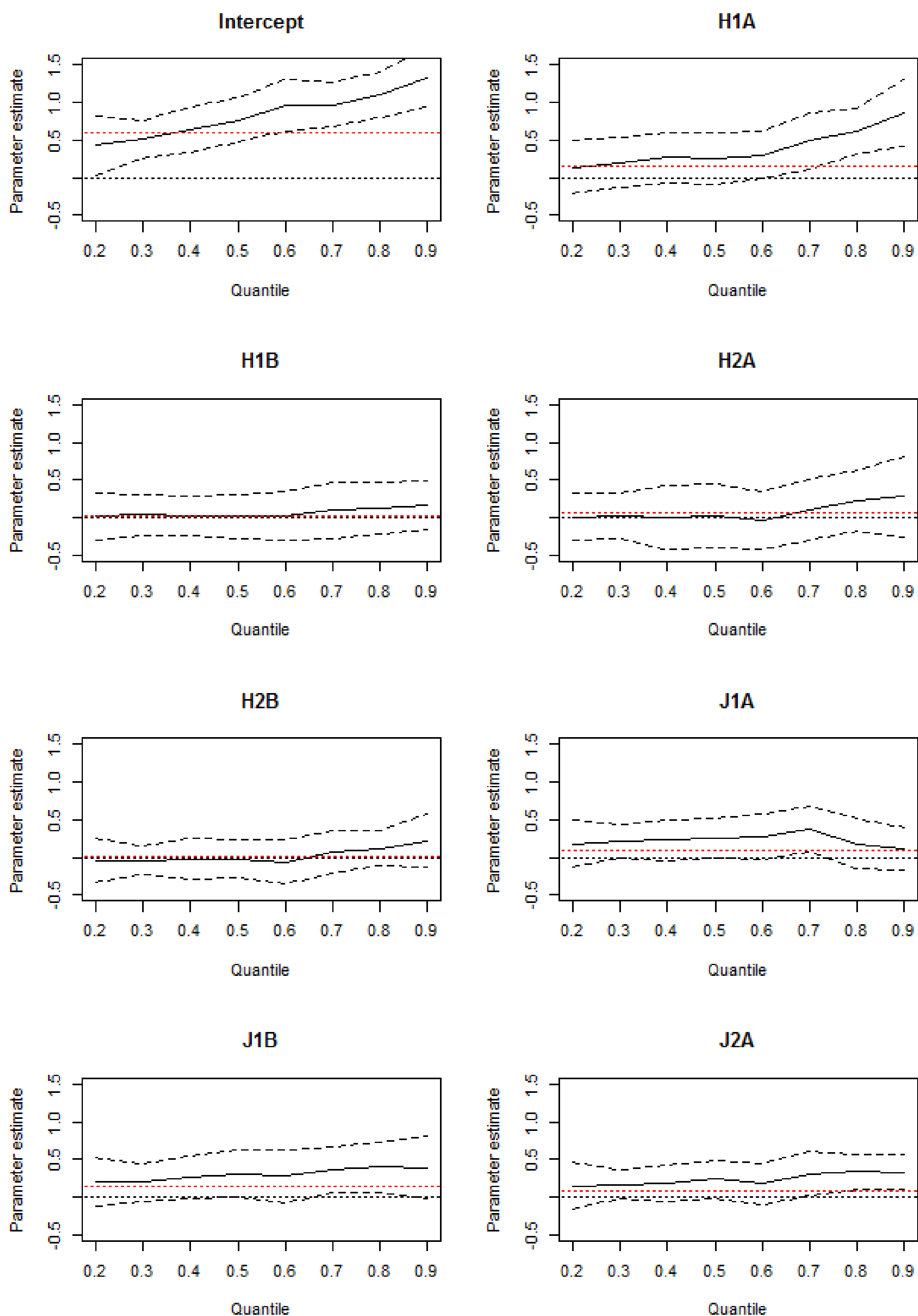

Figure 7.4 Plots of parameter estimates at different quantiles for the Salmonella quantile regression models. Solid lines show estimates, dashed lines show bootstrapped 95% confidence intervals. The red dotted lines show estimates based on mean regression. Black dotted lines indicate the reference kebele J2B.

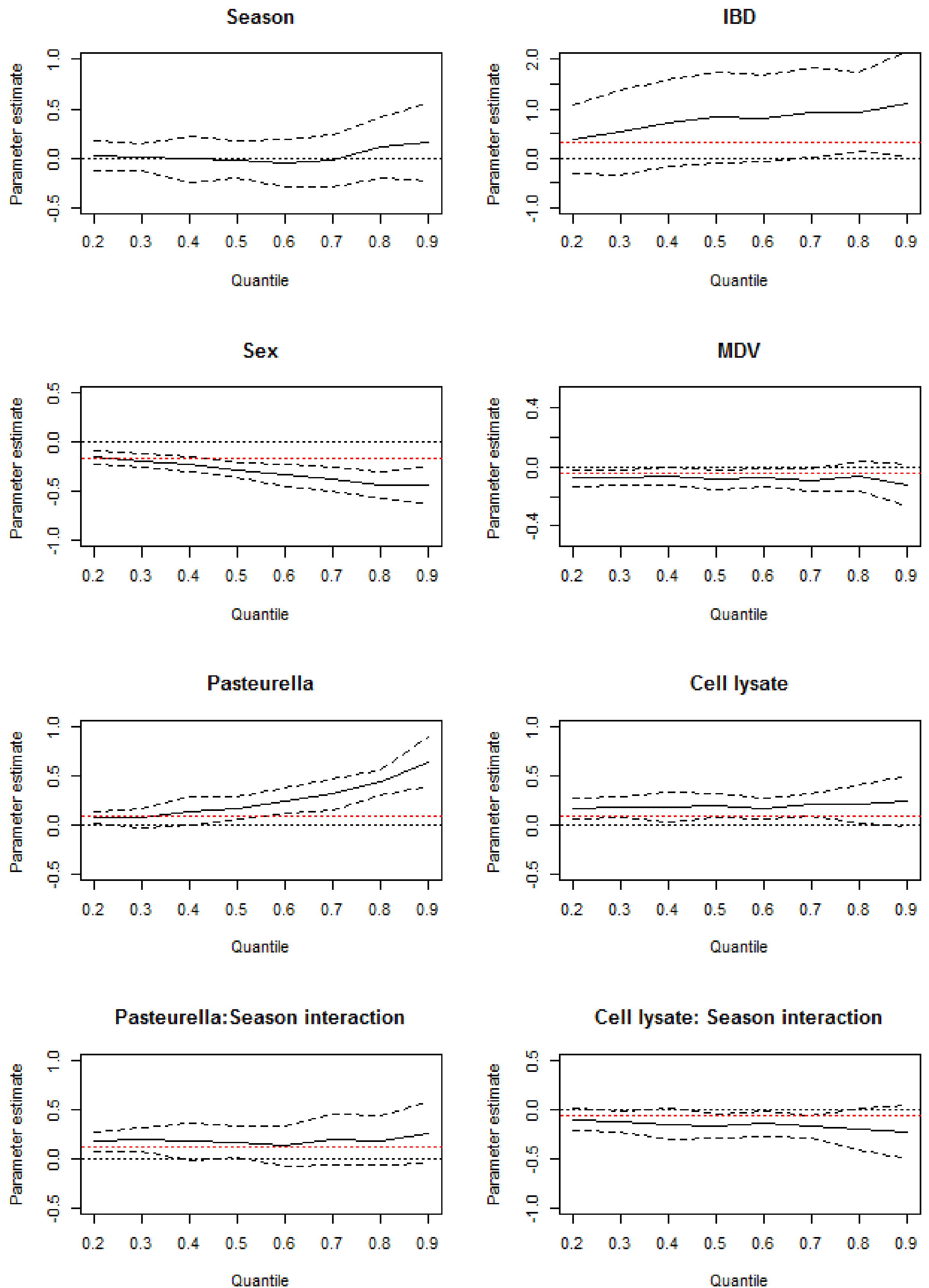

Figure 7.5 Plots of parameter estimates at different quantiles for the Salmonella quantile regression models. Solid lines show estimates, dashed lines show bootstrapped 95% confidence intervals. The red dotted lines show estimates based on mean regression. Black dotted lines indicate a female reference bird from kebele J2B measured in the dry season with average IBD, Pasteurella, MDV and cell lysate values.

Similarly, other risk factors also showed differences in the effect across different quantiles of the distribution. Whilst both sex and *Pasteurella* measurements appeared to be significant risk factors across all quantiles, they both exerted a much greater effect on *Salmonella* measurements at the higher quantiles of the distribution. IBD appeared to only significantly affect *Salmonella* at the upper quantiles, but the interaction between season and *Pasteurella* was only significant for the lower third of the distribution.

7.3.1(c) Infectious Bursal Disease

An intercept-only model suggested that households contributed around 13% of the variation in IBD s:p ratios, although when the outcome was binarised it was estimated that household contributed over 90% of the variation (using the latent variable method). Risk factors identified to significantly improve the fit of a multivariable model were kebele, season, *Salmonella* measurement and if the household had reported an outbreak of disease in their growing birds within the last 12 months (Table 7.6).

Only one kebele appeared to have significantly higher antibody levels (H1A), and the inclusion of each of the other kebeles as separate risk factors did not significantly improve the fit of the model (judged on both likelihood ratio tests and the Akaike information criterion). Therefore kebele was fitted as a two-level risk factor, comparing kebele H1A to all other kebeles. Mean s:p ratios in all kebeles were slightly higher in Season B compared to other sampling seasons. Birds from households which had reported recent outbreaks of disease in growing birds also tended to have slightly higher levels of antibody, but outbreaks affecting other age groups were not found to have a significant effect. For kebele H1A there appeared to be some spatial autocorrelation of households over very short distances with high residuals in Seasons B and D (p=0.048, Figure 7.6), although this was not evident in other kebeles or in all seasons.

Table 7.5 Model coefficients for IBD s:p ratio

Random effects							
	Groups	Name		Variance	Std.Dev.		
	Household	(Intercept)		0.002	0.017		
	Plate	(Intercept)		0.004	0.061		
	Residual			0.013	0.113		
		coefficient	se	95% C.I.			P
(Intercept)		0.00	0.02	-0.03	-	0.03	0.98
Categorical variables							
Kebele	All other kebeles	reference					
	H1A	0.07	0.02	0.03	-	0.12	>0.01
Season	A	reference					
	B	**0.04**	**0.02**	**0.01**	**-**	**0.08**	**0.02**
	C	0.03	0.03	-0.02		0.08	0.23
	D	0.04	0.03	-0.02	-	0.09	0.17
Outbreak in growers	No	reference					
	Yes	**0.03**	**0.01**	**0.00**	**-**	**0.06**	**0.05**
Continuous variables							
***Salmonella* s:p ratio**		**0.13**	**0.06**	**0.02**	**-**	**0.25**	**0.02**

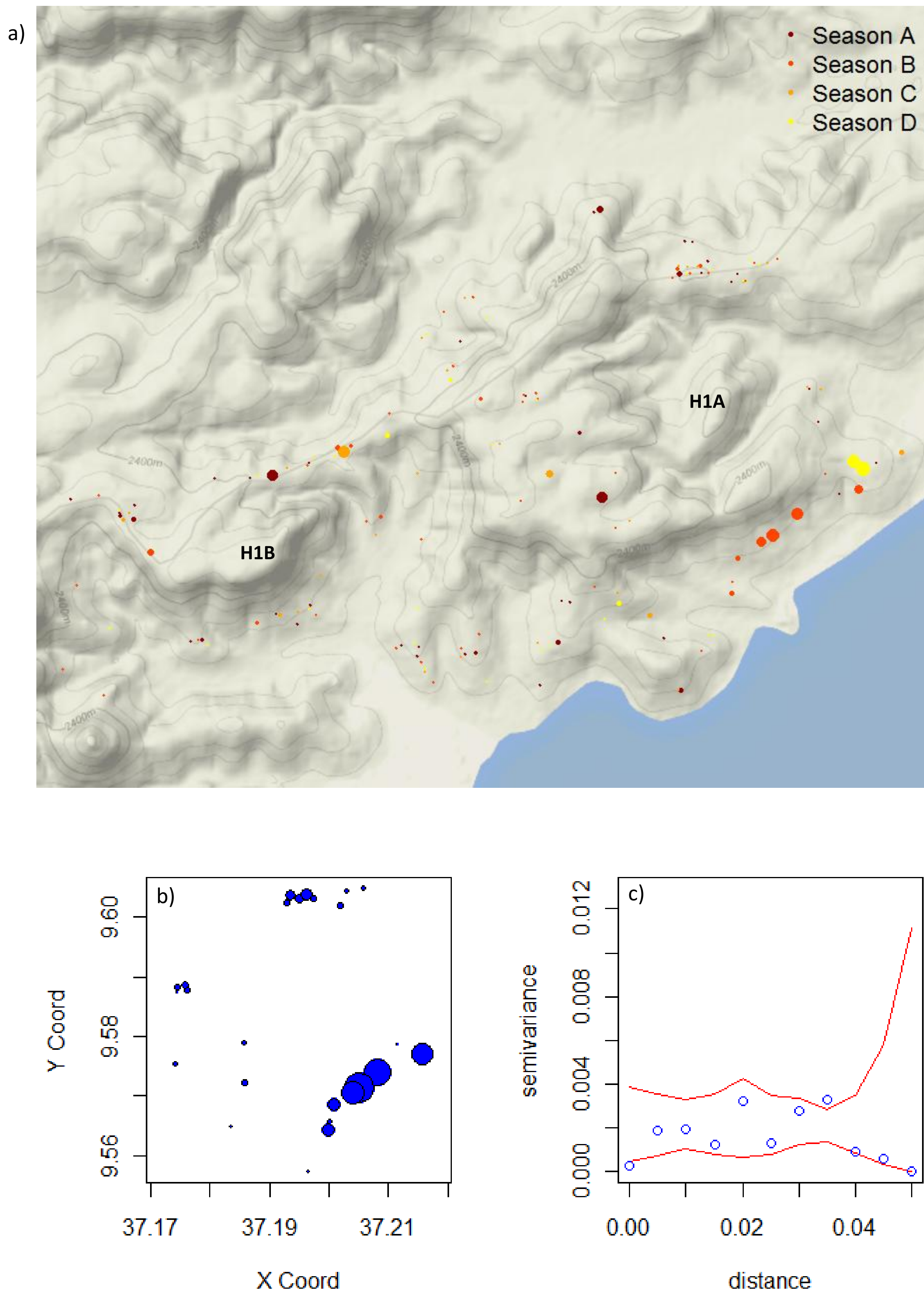

Figure 7.6 a) Map of household-level residuals for one Horro market shed for a mixed-effects model of IBD s:p ratios. b) Plot of model residuals and c) semivariogram for model residuals for kebele H1A in Season B showing spatial autocorrelation over short distances.

7.3.1 (d) Marek's Disease Virus

A mixed effects model of the MDV s:p ratios suggested that both season and kebele contributed significantly to variation in antibody measurements, with an interaction between them. In order to simplify model interpretation, a combined kebele/season variable was created, such that each kebele/season combination was compared to kebele J1B in season D, which had the lowest mean s:p ratio. This suggested that s:p ratios in villages H2B and J2A were significantly greater in three sampling seasons, and other villages had occasional seasons where s:p ratios rose. However, there did not appear to be any consistent pattern across seasons or villages in the same region. Figure 7.7 shows the proportion of birds with MDV s:p ratios over 0.5 in each kebele and sampling season, which ranged from 8 to 52%.

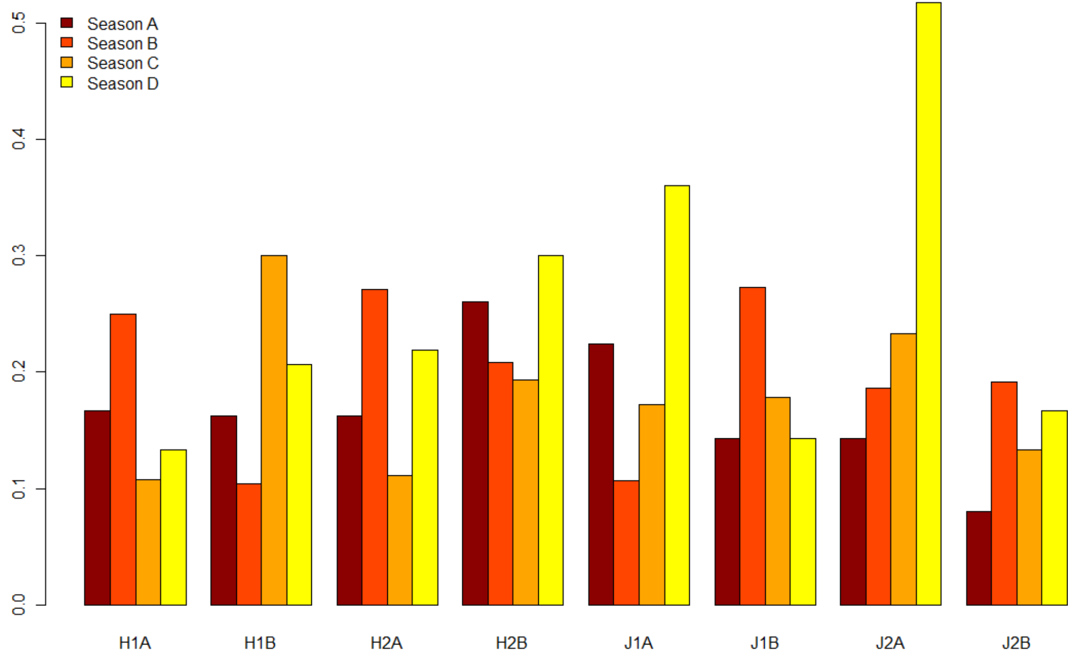

Figure 7.7 Proportion of birds with MDV sample:positive >0.5 in each kebele during each season of sampling

Table 7.6 Model coefficients for MDV s:p ratio

Random effects	Groups	Name	Variance	Std.Dev.
	Household	(Intercept)	0.065	0.254
	Plate	(Intercept)	0.104	0.322
	Residual		0.196	0.443

			coefficient	se	95% C.I.			P
(Intercept)			-0.39	0.22	-0.83	-	0.04	0.08
Categorical variables			coefficient	se	95% C.I.			P
	Village	**Season**						
	J1B	**D**	**Reference category**					
	H1A	**A**	**0.50**	**0.24**	**0.02**	-	**0.97**	**0.04**
	H1A	B	0.38	0.28	-0.17	-	0.93	0.18
	H1A	C	0.36	0.27	-0.18	-	0.89	0.19
	H1A	D	0.55	0.30	-0.04	-	1.14	0.07
	H1B	**A**	**0.55**	**0.26**	**0.04**	-	**1.06**	**0.03**
	H1B	B	0.34	0.24	-0.14	-	0.82	0.17
	H1B	**C**	**0.50**	**0.25**	**0.01**	-	**1.00**	**0.05**
	H1B	D	0.28	0.30	-0.31	-	0.86	0.35
	H2A	A	0.26	0.26	-0.25	-	0.77	0.31
	H2A	**B**	**0.54**	**0.25**	**0.05**	-	**1.03**	**0.03**
	H2A	C	0.11	0.27	-0.43	-	0.64	0.69
	H2A	D	0.38	0.28	-0.18	-	0.93	0.19
	H2B	A	0.42	0.25	-0.06	-	0.91	0.09
	H2B	**B**	**0.62**	**0.23**	**0.16**	-	**1.08**	**0.01**
	H2B	**C**	**0.53**	**0.27**	**0.00**	-	**1.06**	**0.05**
	H2B	**D**	**0.87**	**0.29**	**0.31**	-	**1.44**	**<0.01**
	J1A	**A**	**0.70**	**0.24**	**0.23**	-	**1.17**	**<0.01**
	J1A	B	0.36	0.26	-0.14	-	0.86	0.16
	J1A	**C**	**0.61**	**0.27**	**0.07**	-	**1.15**	**0.03**
	J1A	D	0.33	0.18	-0.03	-	0.69	0.07
	J1B	A	0.18	0.24	-0.30	-	0.66	0.46
	J1B	**B**	**0.68**	**0.26**	**0.16**	-	**1.19**	**0.01**
	J1B	C	0.47	0.27	-0.05	-	0.99	0.08
	J2A	A	0.36	0.25	-0.13	-	0.85	0.15
	J2A	**B**	**0.53**	**0.26**	**0.03**	-	**1.03**	**0.04**
	J2A	**C**	**0.53**	**0.26**	**0.02**	-	**1.04**	**0.04**
	J2A	**D**	**1.19**	**0.19**	**0.83**	**-**	**1.56**	**<0.01**
	J2B	A	0.32	0.25	-0.18	-	0.81	0.21
	J2B	B	0.24	0.28	-0.30	-	0.78	0.38
	J2B	C	0.45	0.26	-0.06	-	0.95	0.08
	J2B	**D**	**0.69**	**0.29**	**0.12**	**-**	**1.25**	**0.02**
Production status		Male	reference					
		Rearing Chicks	-0.05	0.05	-0.15	-	0.06	0.37
		Incubating Eggs	**-0.26**	**0.08**	**-0.41**	-	**-0.11**	**<0.01**
		In lay	**-0.16**	**0.04**	**-0.24**	-	**-0.08**	**<0.01**
		Non-productive	-0.01	0.04	-0.09	-	0.07	0.78
Continuous variables								
***Salmonella* titre (≥1.7)**			**0.04**	**0.02**	**0.01**	**-**	**0.08**	**0.02**
***Salmonella* titre (<1.7) (Intercept)**			**0.16**	**0.06**	**0.05**	-	**0.27**	**0.01**
***Salmonella* titre (<1.7)**			**-0.12**	**0.05**	**-0.22**	-	**-0.03**	**0.01**

The multivariable model also suggested that female birds which were either in lay or incubating eggs had significantly lower antibody levels compared to male birds, and that MDV titres were also correlated with *Salmonella* titres. *Salmonella* and MDV showed a non-linear relationship, so this variable was fit as a piece-wise term. There was a negative correlation up to a *Salmonella* s:p ratio of 1.7, but a positive correlation above 1.7. However, a positive correlation (r=0.25) was also noted between the *Salmonella* s:p ratio and the s:p ratio to the cell lysate, which contributes to the final MDV measurement. This was particularly noticeable for test results at the lower end of the s:p ratio; below 2 for the *Salmonella* and 4 for the cell lysate.

7.3.2 Parasitic infections

Faecal samples were processed for 1239 birds. However, only 1039 samples were known to come directly from the randomly selected birds used in the survey. Sixty two samples processed were recorded as coming from the environment around the household and the source of another 138 was not recorded, but would have either come from the individual bird or the household environment. These latter samples can therefore be attributed to the household, but not necessarily to the individuals. Figure 7.8 shows the estimates of intraclass correlation co-efficients for the parasite data (calculated on presence/absence) using the latent variable method (Goldstein et al., 2002). Estimates were also calculated using the binary linearization method, but were within 5% of the latent variable method estimates in all cases (data not shown).

7.3.2 (a) Ascarids

Worm eggs of the order Ascaridida (likely to belong either to *Ascaridia galli* or *Heterakis gallinarum*) were identified in 207 out of 1239 samples (16.7%). The median intensity was 60 epg (range 20 to 12800 epg). This was not different from the prevalence in the samples

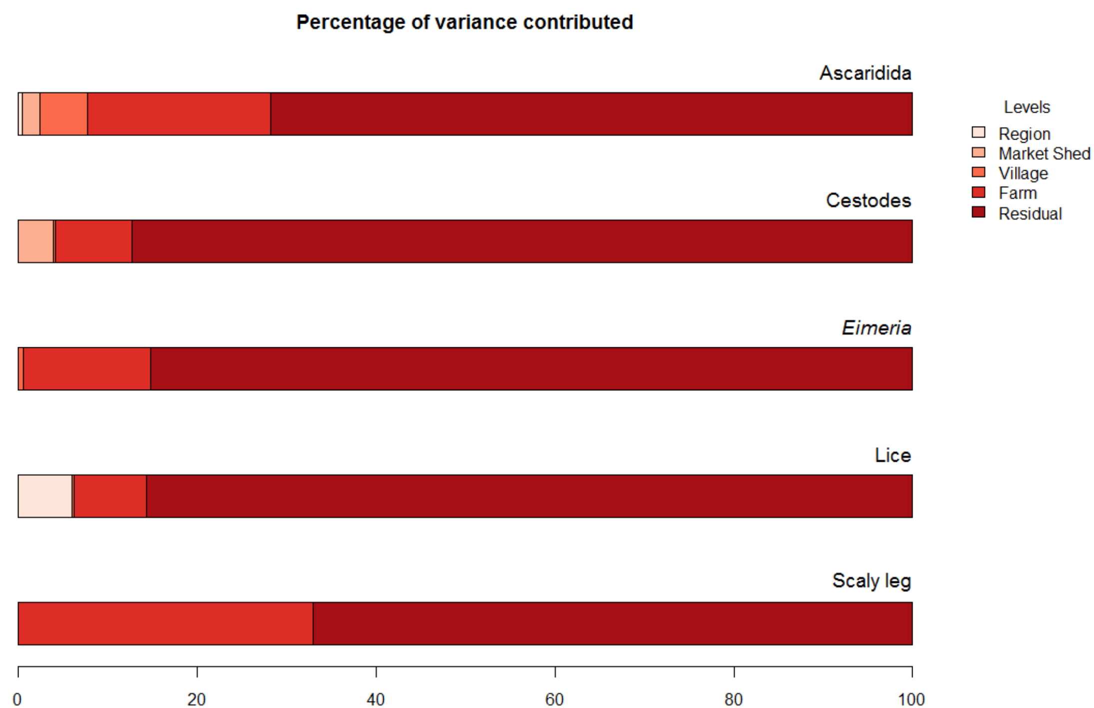

Figure 7.8   Intraclass correlation coefficients for the parasitic diseases (latent variable method)

known to have come from a randomly sampled bird, at 16.2% with the median intensity still 60 epg.

Parasite burdens (measured by the number of eggs shed in faeces) were highly aggregated, as measured by the corrected moment estimate of k (0.008) and the index of discrepancy (0.96). A Kolmogorov-Smirnov test indicated that distributions of parasites were not the same between the two regions (p value <0.01), although no seasonal differences were detected in either region. The infection prevalence in Horro was 21.8% (95% C.I. 18.7 – 25.3%), and the median number of eggs shed by an infected bird was 60 epg. In Jarso, the prevalence of infected birds was only 11.3% (95% C.I. 9.0 – 14.2%) but infected birds had a median number of 80 epg of faeces, and overall, far fewer parasite eggs were counted in the Jarso population (see Figure 7.9.a).

Table 7.7 Logistic regression model coefficients for Ascaridida positivity

Random effects						
	Groups	Name		Variance	Std.Dev.	
	Household	(Intercept)		2.45	1.57	
		OR	**se**	**95% C.I.**		**P**
(Intercept)		0.31	0.34	0.16 - 0.60		<0.01
Categorical variables						
Kebele	H2B	reference				
	H1A	**0.29**	**0.44**	**0.12 - 0.70**		**0.01**
	H1B	0.80	0.41	0.36 - 1.79		0.59
	H2A	**0.26**	**0.47**	**0.10 - 0.66**		**<0.01**
	J1A	**0.38**	**0.44**	**0.16 - 0.89**		**0.03**
	J1B	**0.25**	**0.47**	**0.10 - 0.64**		**<0.01**
	J2A	**0.07**	**0.65**	**0.02 - 0.27**		**<0.01**
	J2B	**0.10**	**0.56**	**0.03 - 0.30**		**<0.01**
Sex	Female					
	Male	**0.57**	**0.23**	**0.37 - 0.90**		**0.02**
Hyperkeratosis	Negative	reference				
	Positive	**0.57**	**0.24**	**0.35 - 0.92**		**0.02**
Eimeria	Negative	reference				
	Positive	**1.80**	**0.24**	**1.43 - 2.87**		**0.75**
Continuous variables						
MDV s:p ratio		**3.39**	**0.34**	**1.43 - 8.04**		**0.01**
MDV s:p ratio: village interaction						
	H1A	0.29	0.81	0.06 - 1.40		0.12
	H1B	0.32	0.81	0.07 - 1.56		0.16
	H2A	1.62	0.79	0.34 - 7.64		0.54
	J1A	**0.18**	**0.83**	**0.04 - 0.92**		**0.04**
	J1B	0.94	0.76	0.21 - 4.21		0.94
	J2A	**0.09**	**1.05**	**0.01 - 0.68**		**0.02**
	J2B	0.64	1.17	0.06 - 6.31		0.70

A multivariable logistic regression model suggested that the odds of positivity for Ascaridida varied significantly between the kebeles, with kebeles H2B and H1B having the highest risk. However, kebele was not a significant risk factor for the infection intensity (number of epg of faeces, given that a bird was positive). There appeared to be little seasonal variation either in infection prevalence or intensity, once other risk factors were

Table 7.8 Poisson regression model coefficients for Ascaridida count, given positivity

Random effects						
	Groups	Name		Variance	Std.Dev.	
	Household	(Intercept)		1.402	1.184	
		OR	se	95% C.I.		P
(Intercept)		6.42	0.16	4.67 - 8.82		<0.01
Categorical variables						
Region	Horro	reference				
	Jarso	0.71	0.29	0.40 - 1.26		0.24
Sex	Female					
	Male	**0.49**	**0.12**	**0.39 - 0.62**		**<0.01**
Eimeria	Negative	reference				
	Positive	**0.56**	**0.17**	**0.40 - 0.78**		**<0.01**
Sex:Region Interaction	*Male:Jarso*	*1.47*	*0.21*	*0.97 - 2.21*		*0.07*
Eimeria:Region interaction	**Positive:Jarso**	**2.00**	**0.28**	**1.15 - 3.48**		**0.01**
Continuous variables						
***Salmonella* s:p ratio**		**0.70**	**0.06**	**0.62 - 0.79**		**<0.01**

taken into account. Male birds appeared to be at significantly lower risk of being positive for Ascarids, and to have significantly lower epg of faeces if they were positive in Horro. However, in Jarso, the difference in infection intensity between the sexes appeared less, reflected in the almost, but not quite significant interaction between the region and sex variables in the Poisson regression model.

Other infections also were found to significantly affect the risk of positivity and/or intensity. Higher MDV s:p ratios significantly increased the odds of being positive in some, but not all villages, but appeared to have no effect on infection intensity. *Salmonella,* on the other hand did not appear to alter the odds of being positive, but higher *Salmonella* s:p ratios were associated with lower faecal egg counts in positive birds. *Eimeria* appeared to affect both the odds of being positive and the infection intensity, but in opposite directions: *Eimeria*-positive birds were at greater odds of being positive for Ascaridida, but

Ascaridida-infected birds in Horro tended to have a lower intensity of infection if they were positive for *Eimeria.* This did not, however, appear to be significant in Jarso (Tables 7.7 and 7.8).

7.3.2 (b) Cestodes

Cestode eggs were found in 284 samples, giving an overall prevalence of 22.9% (95% C.I. 20.6 – 25.4%). Again, excluding samples not taken directly from the randomly selected birds made no difference to the estimate of prevalence. The median intensity was 80epg, with a range of 20 to 12300epg. Overall, 37% of households sampled had one or more infected birds present.

No eggs were detected in the Horro samples from season A: However, these were the first set of samples tested and the lack of positive samples is likely to be due to a problem with the identification guides available, which meant if any cestode eggs were seen in these samples, they were probably ignored by the readers. This could potentially also affect some of the samples from Jarso in season A, but as the order in which samples were tested was not recorded, it is not possible to tell which ones are truly negative and which may simply have been tested earlier. This alteration in test sensitivity over time is likely to bias any further analysis looking for regional or seasonal differences, and so Season A samples have been excluded from further analyses.

A Kolmogorov-Smirnov test did not indicate a significant difference existed between the regions (p value = 0.12) (Figure 7.8.b). However, a logistic regression model indicated that birds sampled in Horro in October 2012 were at considerably greater odds of being positive for cestodes. The only other variable which significantly affected infection was *Pasteurella*, higher titres of which reduced the odds of cestode infection (Table 7.9). Attempts to model cestode intensity in positive birds suggested several variables were significant, but many showed significant interactions with the region, and some also with the sex of the bird.

Table 7.9 Logistic regression model coefficients for cestode positivity

Random effects	Groups	Name	Variance	Std.Dev.
	Household	(Intercept)	0.376	0.613

		OR	se	95% C.I.			P
(Intercept)		0.34	0.23	0.22	-	0.53	<0.01
Categorical variables							
Region	Jarso	reference					
	Horro	0.87	0.33	0.46	-	1.65	0.67
Season	May 2012						
	October 2011	1.11	0.29	0.62	-	1.97	0.72
	October 2012	0.64	0.35	0.32	-	1.27	0.21
Region:Season interaction							
	Horro: October 2011	1.34	0.42	0.60	-	3.04	0.48
	Horro: October 2012	**6.20**	**0.47**	**2.46**	**-**	**15.60**	**<0.01**
Continuous variables							
***Pasteurella* s:p ratio**		**0.77**	**0.11**	**0.62**	**-**	**0.96**	**0.02**

Table 7.10 Poisson regression model coefficients for cestode egg count, given positivity (Jarso)

Random effects	Groups	Name	Variance	Std.Dev.
	Household	(Intercept)	2.45	1.57

		OR	se	95% C.I.			P
(Intercept)		0.33	0.96	0.05	-	2.19	0.25
Categorical variables							
Sex	Male	reference					
	Female	16.25	1.05	2.09	-	126.6	0.01
Hyperkeratosis	Negative	reference					
	Positive	**0.51**	**0.25**	**0.31**	**-**	**0.84**	**0.01**
Eimeria	Negative	reference					
	Positive	**8.33**	**0.63**	**2.41**	**-**	**28.81**	**<0.01**
Sex:*Eimeria* interaction							
	Female:Positive	**0.13**	**0.68**	**0.04**	**-**	**0.50**	**<0.01**
Continuous variables							
Body condition score		**3.48**	**0.44**	**1.46**	**-**	**8.29**	**<0.01**
BCS: sex interaction							
	Female	**0.28**	**0.47**	**0.11**	**-**	**0.70**	**0.01**
MDV s:p ratio		**0.26**	**0.12**	**0.20**	**-**	**0.33**	**<0.01**

Table 7.11 Poisson regression model coefficients for cestode egg count, given positivity (Horro)

Random effects				
	Groups	Name	Variance	Std.Dev.
	Household	(Intercept)	1.31	1.14

		OR	se	95% C.I.	P
(Intercept)		1.31	0.60	0.40 - 4.26	0.65
Categorical variables					
Sex	Male	reference			
	Female	1.59	0.62	0.47 - 5.34	0.45
Season	May 2012				
	October 2011	**6.82**	**0.43**	**2.96 - 15.73**	**<0.01**
	October 2012	**6.24**	**0.42**	**2.77 - 14.08**	**<0.01**
Sex:Season interaction					
	Female: October 2011	**0.20**	**0.41**	**0.09 - 0.44**	**<0.01**
	Female: October 2012	**0.42**	**0.41**	**0.19 - 0.94**	**0.03**
Hyperkeratosis	Negative	reference			
	Positive	**1.95**	**0.15**	**1.44 - 2.64**	**<0.01**
Eimeria	Negative	reference			
	Positive	**2.06**	**0.24**	**1.29 - 3.30**	**<0.01**
Sex:*Eimeria* interaction					
	Female:Positive	**0.39**	**0.30**	**0.21 - 0.69**	**<0.01**
Continuous variables					
Body condition score		**0.57**	**0.24**	**0.36 - 0.90**	**0.02**
BCS: sex interaction					
	Female	**2.11**	**0.28**	**1.20 - 3.68**	**0.01**
MDV s:p ratio		**0.22**	**0.35**	**0.11 - 0.43**	**<0.01**
MDV s:p ratio: sex interaction					
	Female	**3.75**	**0.39**	**1.73 - 8.14**	**<0.01**
***Pasteurella* s:p ratio**		**0.76**	**0.09**	**0.64 - 0.89**	**<0.01**

Therefore it was decided to model the regions separately. In both regions higher MDV s:p ratios were associated with reduced numbers of eggs, but in Jarso this was seen in birds of both sexes, whilst in Horro it was only seen in male birds. *Eimeria* showed a similar sex interaction, such that in Jarso, birds positive for *Eimeria* had significantly higher cestode counts, but in Horro this was again only seen in males.

Although hyperkeratosis, associated with scaly-leg mite, significantly affected cestode counts in both regions, it showed opposite effects, being associated with higher counts in Horro, but lower counts in Jarso. Body condition scores in males, but not females, also affected counts in both regions, but again in opposite directions; higher cestode numbers were associated with birds of low body condition in Horro, but higher body condition in Jarso. *Pasteurella* and sampling season were only significant risk factors in Horro, where raised *Pasteurella* measurements were associated with decreased egg counts. In Horro, higher egg counts were found in males (but not females) in the October sampling season in both years, compared to those birds sampled in May 2012. As previously mentioned, the first sampling season was excluded from the analysis.

7.3.2 (c) Eimeria

Eimeria oocysts were detected in 689 samples, giving a prevalence of 55.6%. The median intensity was 200 eggs per gram (epg) of faeces, with a range of 20 to 84,120 epg. If only the samples known to be attributable to a sampled bird are considered, prevalence was 56.2%, and a median intensity of 220 epg.

Parasites were highly aggregated, as measured by the corrected moment estimate of k (0.035) and the index of discrepancy (0.91). The Kolmogorov-Smirnov test indicated that distributions of parasites were not significantly different between the two regions (p value = 0.96) (Figure 7.8.c). However, there were found to be significant differences in the probability of being positive between different villages, with birds significantly more likely to be positive in kebeles H1A, H2A, H2B and J1A, compared to H1B. Intensity of infection also varied between kebeles and regions. Seasonal variation was observed in the probability, but not the intensity of infection, with birds being at significantly greater risk of infection in October 2011 and May 2012. Multivariable models also suggested that

Salmonella, Ascarids, cestodes and scaly leg mite also significantly affected *Eimeria* infections, and that sex and age were also important risk factors.

Table 7.12 Binomial regression model coefficients for *Eimeria* positivity

Random effects							
	Groups	Name		Variance	Std.Dev.		
	Household	(Intercept)		0.552	0.743		
		OR	se	95% C.I.			P
(Intercept)		0.24	0.33	0.13	-	0.47	<0.01
Categorical variables							
Village	H1B	reference					
	H1A	**2.87**	**0.31**	**1.58**	**-**	**5.23**	**<0.01**
	H2A	**2.35**	**0.30**	**1.31**		**4.22**	**<0.01**
	H2B	**2.29**	**0.30**	**1.27**	**-**	**4.10**	**0.01**
	J1A	**2.11**	**0.30**	**1.17**	**-**	**3.79**	**0.01**
	J1B	1.82	0.32	0.98	-	3.38	0.06
	J2A	1.38	0.30	0.77	-	2.46	0.28
	J2B	1.83	0.31	0.99	-	3.38	0.06
Season	May 2011	reference					
	October 2011	**4.15**	**0.35**	**2.07**	**-**	**8.32**	**<0.01**
	May 2012	**2.44**	**0.45**	**1.00**	**-**	**5.92**	**0.05**
	October 2012	2.15	0.42	0.94	-	4.92	0.07
Cestodes	Negative	reference					
	Positive	**2.64**	**0.19**	**1.80**	**-**	**3.87**	**<0.01**
Continuous variables							
Age		**1.04**	**0.02**	**1.01**	**-**	**1.08**	**0.02**
Age: season interaction							
	October 2011	**0.95**	**0.02**	**0.91**	**-**	**0.99**	**0.01**
	May 2012	**0.95**	**0.03**	**0.90**	**-**	**1.00**	**0.05**
	October 2012	0.98	0.02	0.93	-	1.02	0.33
***Salmonella* s:p ratio**		**0.89**	**0.05**	**0.81**	**-**	**0.99**	**0.04**

Table 7.13 Poisson regression model coefficients for *Eimeria* count, given positivity

Random effects							
	Groups	Name		Variance	Std.Dev.		
	Household	(Intercept)		4.04	2.01		
		OR	se	95% C.I.			P
(Intercept)		24.55	0.24	15.30	-	39.4	<0.01
Categorical variables							
Region	Horro	reference					
	Jarso	1.23	0.20	0.82	-	1.84	0.31
Sex	Female	reference					
	Male	**0.43**	**0.02**	**0.41**	**-**	**0.45**	**<0.01**
Cestodes	Negative	reference					
	Positive	**2.01**	**0.04**	**1.88**	**-**	**2.16**	**<0.01**
Scaly leg	None/mild	reference					
	Severe	**1.69**	**0.03**	**1.58**	**-**	**1.80**	**<0.01**
Continuous variables							
Age (≤9 months)		**0.85**	**0.03**	**0.81**	**-**	**0.90**	**<0.01**
Age (>9 months – Intercept)		0.31	0.20	0.21	-	0.46	**<0.01**
Age (>9 months)		**1.17**	**0.03**	**1.11**	**-**	**1.23**	**<0.01**
***Salmonella* s:p ratio**		**0.46**	**0.02**	**0.44**	**-**	**0.48**	**<0.01**
***Salmonella* s:p ratio squared**		**1.39**	**0.01**	**1.36**	**-**	**1.43**	**<0.01**
Ascarid count		**0.98**	**<0.01**	**0.97**	**-**	**0.99**	**<0.01**
Ascarid: Jarso region interaction		**1.23**	**0.01**	**1.21**	**-**	**1.25**	**<0.01**

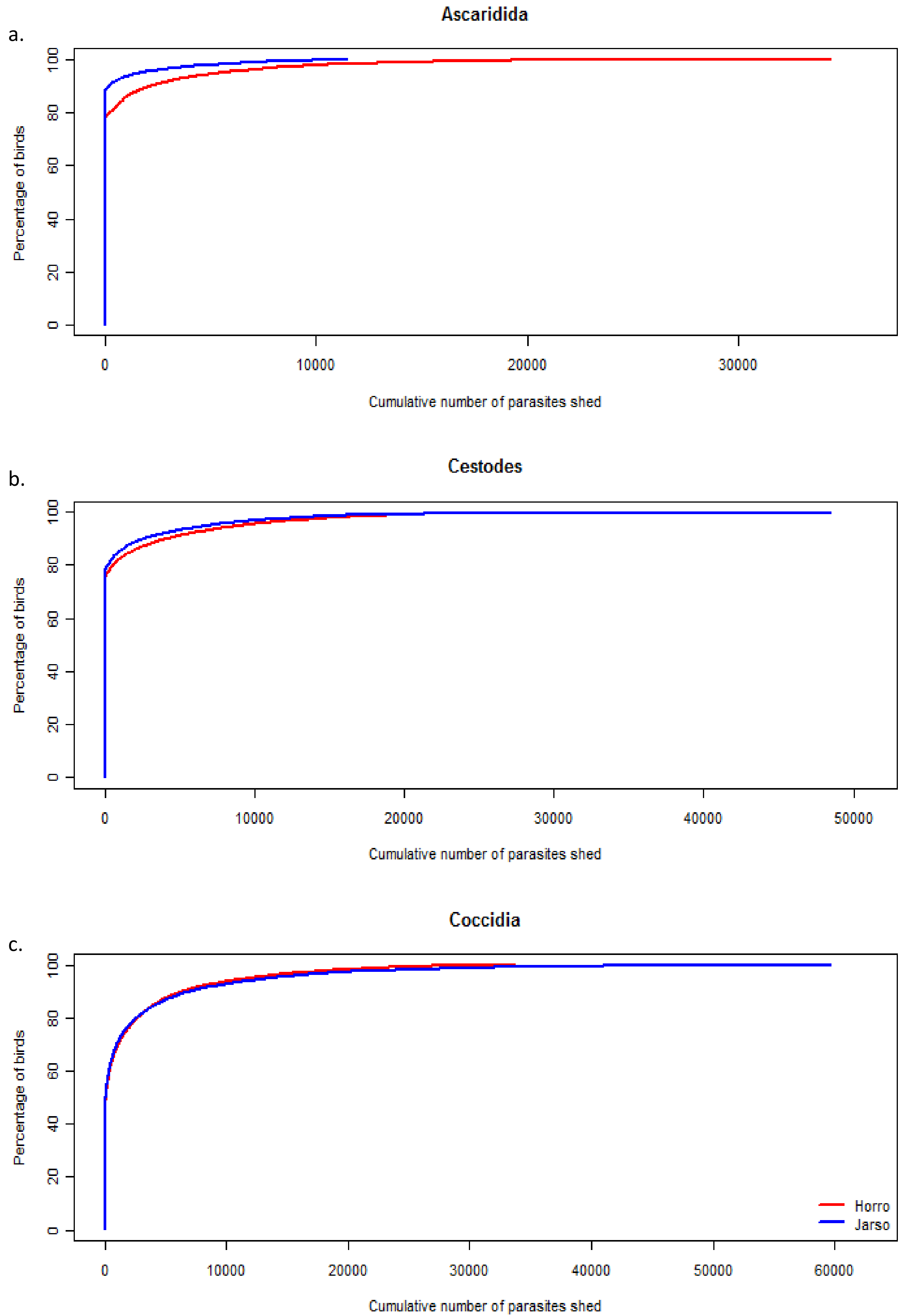

Figure 7.9 Cumulative faecal parasite densities among chickens in each region

7.3.2 (d) Lice

The prevalence of lice infestation was 33.2% (95% C.I. 30.6 – 35.9%). However, there were significant differences in lice distributions between the two regions (Kolmogorov-Smirnov test, D=0.1996, p<0.001). Although in both regions the median parasite intensity was 3, in Jarso, only 23.3% of birds were infested (95% C.I. 20.1 -26.8%), and parasites were highly aggregated, with a corrected moment estimate of k =0.05 and the index of discrepancy =0.91. However, in Horro, prevalence was much higher, and 43% of birds were observed to be carrying lice (95%C.I. 39.3 – 47.2%), but lice burdens were less aggregated between birds (corrected moment estimate of k =0.21, index of discrepancy =0.81, Figure 7.10).

Within each region, there were fewer positive birds in Season C, but there was found to be no significant difference in the intensity of infection between seasons after other explanatory variables were controlled for (Table 7.14 and 7.15). Sex was an important risk factor both for being found positive and the number of lice found, but interacted with the weight and body condition for this latter outcome. This suggested that hens were not only

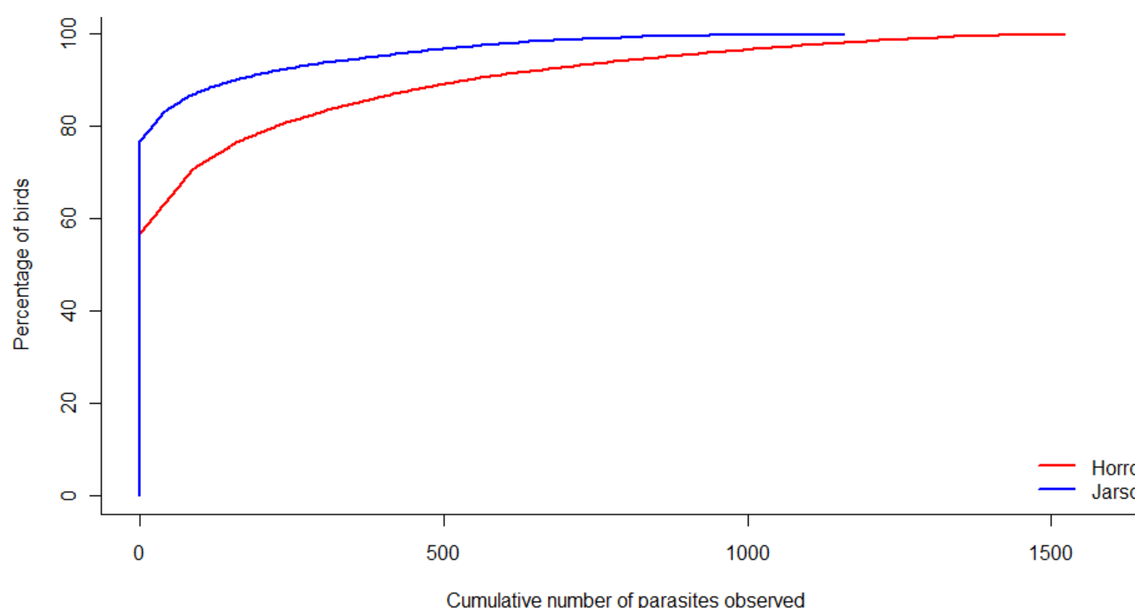

Figure 7.10 Cumulative louse densities among chickens in each region

Table 7.14 Binomial regression model coefficients for lice positivity

Random effects							
	Groups	Name		Variance	Std.Dev.		
	Household	(Intercept)		0.71	0.84		
		OR	se	95% C.I.			P
(Intercept)		0.49	0.16	0.36	-	0.67	<0.00
Categorical variables							
Region	Horro	reference					
	Jarso	**0.36**	**0.15**	**0.27**	**-**	**0.49**	**<0.01**
Season	May 2011	reference					
	Oct 2011	0.70	0.19	0.48	-	1.02	0.06
	May 2012	**0.59**	**0.23**	**0.37**	**-**	**0.93**	**0.02**
	Oct 2012	1.15	0.22	0.75	-	1.76	0.51
Sex	Female	reference					
	Male	**3.72**	**0.14**	**2.81**	**-**	**4.91**	**<0.01**
Fumigate housing	No	reference					
	Yes	**1.61**	**0.22**	**1.05**	**-**	**2.46**	**0.03**

Table 7.15 Poisson regression model coefficients for lice intensity

Random effects							
	Groups	Name		Variance	Std.Dev.		
	Household	(Intercept)		0.852	0.923		
		OR	se	95% C.I.			P
(Intercept)		4.12	0.91	0.69	-	24.46	0.12
Categorical variables							
Sex	Female						
	Male	**0.05**	**1.06**	**0.01**	**-**	**0.38**	**<0.01**
Use of parasite control	Yes						
	No	**1.44**	**0.13**	**1.12**	**-**	**1.84**	**<0.01**
Continuous variables							
Age (≥24 months)		**1.10**	**0.02**	**1.07**	**-**	**1.13**	**<0.01**
Age (<24 months - Intercept)		6.65	0.48	2.62	-	16.9	<0.01
Age (<24 months)		0.94	0.02	0.90	-	0.98	<0.01
Weight		0.10	0.68	0.03	-	0.39	<0.01
Weight: male interaction		16.64	0.83	3.29	-	84.1	<0.01
Weight: body condition interaction		**2.59**	**0.35**	**1.31**	**-**	**5.11**	**0.01**
Body condition score		0.27	0.41	0.12	-	0.60	<0.01
Body condition: male interaction		**6.17**	**0.54**	**2.14**	**-**	**17.81**	**<0.01**
Weight:body condition:male interaction		0.29	0.41	0.13	-	0.62	<0.01

less likely to be infected, but also tended to have fewer lice as their weight and body condition increased; however, increases in these variables in cocks predicted greater louse burdens. The region variable became non-significant once these variables were added to the model, suggesting that the observed regional variation in louse densities may be largely explained by the larger body sizes of chickens in the Horro region. Age was fitted as a piecewise variable, as louse intensity tended to decrease up to the age of 24 months, but increase thereafter. Increasing *Pasteurella* s:p ratio was also associated with a significant increase in the louse intensity, but no other infections contributed significant variation. Birds in households which did not use any form of parasite control tended to have higher louse burdens, but birds in households which fumigated the housing were more likely to be positive.

7.3.2 (e) Cnemidocoptes mutans (Scaly leg mite)

Scaly leg mite infestation prevalence, judged by the number of birds with hyperkeratosis, was estimated at 41.2% (95% C.I. 38.5-44.0%). Of those birds showing signs of hyperkeratosis, 48% were graded as “severe”. Identification of hyperkeratosis was strongly associated with a positive identification of scaly leg mites from a skin scrape (McNemar’s test, $p<0.01$).

In Jarso there was an almost, but not quite, significantly lower prevalence of observed hyperkeratosis in the first sampling season (May 2011), compared to the final one (October 2012); whereas in Horro the highest prevalence was observed in the first sampling season, and indeed all other seasons had significantly higher prevalence compared to the final season. In both regions, the odds of an infested bird having severe hyperkeratosis were greater in the middle two seasons. Males were both more likely to be infested and to have severe hyperkeratosis, if infected, but females incubating eggs were also more likely to have hyperkeratosis, compared to those not in production. Fumigation of the housing

Table 7.16 Binomial regression model coefficients for hyperkeratosis

Random effects							
	Groups	Name		Variance	Std.Dev.		
	Household	(Intercept)		1.15	1.07		
		OR	se	95% C.I.			P
(Intercept)		0.52	0.36	0.25	-	1.05	0.07
Categorical variables							
Region	Jarso	reference					
	Horro	**0.31**	**0.44**	**0.13**	**-**	**0.74**	**0.01**
Season	October 2012	reference					
	May 2011	*0.50*	*0.36*	*0.25*	*-*	*1.02*	*0.06*
	October 2011	0.86	0.38	0.40	-	1.82	0.68
	May 2012	0.74	0.45	0.31	-	1.80	0.51
Season: Horro interaction							
	May 2011	**10.66**	**0.56**	**3.59**	**-**	**31.6**	**<0.01**
	October 2011	**3.04**	**0.55**	**1.04**	**-**	**8.91**	**0.04**
	May 2012	**4.17**	**0.65**	**1.16**	**-**	**14.99**	**0.03**
Disposal of dead birds	In yard	reference					
	Far from yard	0.82	0.26	0.49	-	1.36	0.44
	Bury	0.69	0.36	0.34	-	1.39	0.30
	Burn	**0.05**	**1.30**	**0.00**	**-**	**0.65**	**0.02**
	Fed to dog	0.51	0.86	0.09	-	2.78	0.44
Fumigate housing	No	reference					
	Yes	**0.55**	**0.28**	**0.32**	**-**	**0.95**	**0.03**
Eimeria	Negative	reference					
	Positive	**1.72**	**0.18**	**1.22**	**-**	**2.44**	**<0.01**
Production status	Non-productive	reference					
	In lay	1.43	0.25	0.88	-	2.35	0.15
	Incubating eggs	**2.75**	**0.42**	**1.22**	**-**	**6.22**	**0.01**
	Rearing chicks	1.26	0.33	0.67	-	2.38	0.48
	Male	**2.14**	**0.22**	**1.38**	**-**	**3.32**	**<0.01**

appeared to be protective, as did burning the carcasses of dead birds, but severe infestations were more likely in households which used chemical sprays on the birds themselves. Severe infestations were also associated with decreasing numbers of chicks in the household flock. *Eimeria* infection was also identified as significant risk factor, but a concurrent Ascarid infection appeared to reduce severity (Tables 7.16 and 7.17).

Table 7.17 Binomial regression model coefficients for severe hyperkeratosis, given presence

Random effects					
	Groups	Name		Variance	Std.Dev.
	Household	(Intercept)		0.390	0.625

		OR	se	95% C.I.	P
(Intercept)		0.55	0.23	0.35 - 0.86	0.01
Categorical variables					
Season	May 2011	reference			
	October 2011	**3.57**	**0.27**	**2.10 - 6.09**	**<0.01**
	May 2012	**2.24**	**0.30**	**1.24 - 4.04**	**0.01**
	October 2012	1.29	0.32	0.69 - 2.40	0.42
Sex	Female	reference			
	Male	**1.57**	**0.21**	**1.04 - 2.38**	**0.03**
Ascaridida	Negative	reference			
	Positive	**0.48**	**0.32**	**0.26 - 0.89**	**0.02**
Chemical spray on birds	No	reference			
	Yes	**3.53**	**0.59**	**1.12 - 11.1**	**0.03**
Continuous variables					
Chick numbers in household flock		**0.94**	**0.03**	**0.89 - 0.99**	**0.01**

7.4 Discussion

Our results have shown that a number of different infectious micro- and macroparasites are circulating in the two poultry populations under study. Exposure to *Salmonella, Pasteurella,* MDV and IBD, as measured by the distributions of antibody titres, was observed to be broadly similar between the two study regions. Whilst the antibody distributions to *Pasteurella* varied very little between kebeles, the kebele was a significant risk factor for *Salmonella*, MDV and IBD, with birds in kebele H1A in particular being observed to have higher responses to IBD. The MDV responses varied considerably within kebeles over time, such that no single kebele was at consistently greater risk, but several demonstrated at least one season where antibody responses were found to be significantly

above the average. For *Salmonella,* there were kebeles in both Horro and Jarso which had significantly higher mean antibody titres, but whereas for the kebeles in Jarso this appeared to be due to a general increase in antibody measures across the distribution, in Horro the mean s:p ratio was influenced by a large number of outlying birds with antibody measures far in excess of the positive control in the kebele in question. This may be a reflection of different *Salmonella* serotypes with differing prevalence between the two regions (for example, a higher proportion of infections in Horro being attributable to *S.*Pullorum, rather than *S.* Enteritidis), or differences in bird antibody responses in the two bird ecotypes.

Of the parasitic diseases, *Eimeria* counts were also found to have similar distributions between the two regions, although prevalence varied between kebeles, with three Horro kebeles and one Jarso kebele exhibiting significantly higher prevalence over the course of the study. The prevalence of scaly leg infestation fluctuated over the course of the study, with Horro having a higher prevalence in the first season, but a significantly lower prevalence by the final sampling 18 months later. Both regions also showed increasing numbers of severe cases of hyperkeratosis in the second and third sampling seasons. Cestode prevalence and intensity appeared broadly similar between regions, although exhibited more seasonal variation in Horro. Lice counts and Ascaridida egg counts showed in both cases that higher numbers of parasites were to be found in Horro, and that these were less aggregated between hosts, leading to a higher prevalence of infected birds in both cases.

All of these diseases were clustered to some extent within households, particularly IBD, which had a household level ICC of 0.13-0.9, depending on the method used. Previous studies have also suggested that this disease has a relatively high degree of clustering within household, with ICC reported as 0.39 by Otte and Gumm (1997). In kebele H1A, for

two of the four sampling seasons, it was observed that the household residuals for the IBD model were not spatially independent. These observations would be consistent with sporadic, localised IBD outbreaks within the kebele, with virus readily transmitted within and between neighbouring flocks. The model also suggested that slightly higher titres in the adult birds in this study were observed in households which had reported recent losses among the growing birds. This is consistent with the pathogenesis of IBD, which tends to only cause clinical signs in birds of 3-12 weeks of age (Eterradossi and Saif, 2008).

Marek's disease was also found to exhibit moderate household level clustering, whilst ICCs for the bacterial diseases were somewhat lower. In their calculations of ICCs for 20 livestock infections, Otte and Gumm (1997) also reported that the bacterial diseases leptospirosis and brucellosis had some of the lowest levels of clustering. This may be due to chronically infected individuals within households being the main route of spread of these bacterial infections, so although exposure to both *Salmonella* and *Pasteurella* was found to be widespread in our study, antibody titres of birds within households still exhibit considerable variation, as they are perhaps more strongly influenced by individual factors, including the timing of infection and individual response. Parasites in our study also exhibited considerable household-level clustering, particularly the scaly leg mite (0.33), Ascarid nematodes (0.20) and *Eimeria* (0.14). These latter two are similar to the ICCs reported for directly transmitted nematodes and *Eimeria* in cattle (Otte and Gumm, 1997).

Correlations between serological measurements, which were observed during multivariate analysis (see Chapter 6), were borne out by the regression models. *Salmonella* and *Pasteurella* were positively correlated, and each was found to be a significant risk factor for the other. *Salmonella* was also correlated positively with IBD, and negatively with MDV. However, further investigation of this latter relationship suggested that *Salmonella* may be more strongly correlated with the cell lysate response, which was subtracted from the

MDV-infected cell response to give the final MDV ratio. These correlations tended to only hold true for *Salmonella* s:p ratios up to around 2 times the positive control, whilst the model suggested that *Salmonella* titres above this were primarily influenced by some other, unknown bird-level factor. These correlations between the ELISA test results at lower optical densities may reflect variation in non-specific antibody binding between birds. Parmentier et al. (2004) observed that naive birds selected for high or low antibody responses to sheep red blood cells exhibited similar high or low antibody binding to seven other novel antigens, suggesting differences in the level of natural antibodies. These are antigen-binding antibodies, frequently polyreactive, which are present in the absence of infection or immunisation and are normally dismissed as "background" in ELISA tests (Ochsenbein et al., 1999). A large proportion may be auto-antibodies, which may explain their binding to the chicken kidney cell lysate, but they also bind with low affinity to a wide range of evolutionary conserved molecules, and so form a part of the innate immune response to viral and bacterial pathogens (Ochsenbein et al., 1999). Although dilution of sera for the ELISA tests should reduce the effect of this non-specific binding, it is possible that by choosing to use the continuous data, rather than dichotomising the ELISA results, we have incorporated a measure of these natural antibodies into our data, and therefore the correlations between the bacterial infections are not simply a result of common exposure but at least partly reflect an individual's ability to produce antibody.

In light of the fact that most previous studies have agreed that disease tends to occur during the rainy season, we anticipated that we may observe a seasonal pattern to one or more infections at the population level. In contrast to this, the majority of the infections we measured did not exhibit a clear seasonal pattern, or in some cases (such as *Salmonella*) the apparent seasonality was explained by other risk factors, particularly covariance with other infections. This emphasises the importance of controlling for co-infections when examining risk factors for individual disease. The only infection to show a consistent

increase in antibody levels after the rainy season in both years of the study was *Pasteurella,* although the increase in the mean was relatively small. Although not examined in detail, quantile regression did suggest that these seasonal effects were not consistent over the entire distribution of *Pasteurella* s:p ratios, and as such this finding may benefit from further investigation. Previous studies examining risk factors for infection have considered all birds in a village as a single epidemiological unit (e.g. Otim et al. (2007), Jenbreie et al. (2012)). From our findings, as demonstrated by the high level of household clustering, the spatial correlation of household residuals, and the finding that some risk factors only appear to influence specific quantiles of response distributions, it seems more likely that small, localised outbreaks causing elevated antibody titres in a small number of birds are primarily responsible for the observed spatial and temporal variation in our study.

Given our earlier findings (reported in Chapters 4 and 5) that farmers in Horro reported more disease mortality in their birds, especially during the wet season, it was somewhat surprising that pathogen levels appeared so similar between the two regions. However, there may be other unmeasured pathogens, including NDV, responsible for the reported mortalities. As we appeared to have conducted the study during an inter-epidemic period (see Chapter 4), we may not have detected sporadic high-mortality outbreaks. It may be that small outbreaks caused by a variety of single pathogens occasionally overlap to give the appearance of larger “epidemics” of high mortality. Other factors and stressors, such as nutrition or temperature, may also be acting synergistically with these infections to cause increases in morbidity/ mortality. Our data on these are not sufficiently detailed to be able to investigate these hypotheses, however the influence of factors such as bodyweight and body condition on both selected antibody titres and parasite burdens might suggest nutrition may be a contributing factor and worth further investigation.

Table 7.18 Summary of observed seasonal variation of infections in (a) Horro and (b) Jarso

(a)

Horro	*Pasteurella*	*Salmonella*	IBD	MDV	Ascarids	Cestodes	*Eimeria*	Lice	Scaly leg
May 2011	-	-	-	*increase (H1A & H1B)*	-	*n/a*	-	-	*increase (prevalence)*
October 2011	*increase*	-	*increase*	*increase (H2A & H2B)*	-	*increase (counts in males)*	*increase (prevalence)*	-	*increase (severity)*
May 2012	-	-	-	*increase (H1B &H2B)*	-	-	*increase (prevalence)*	*decrease (prevalence)*	*increase (severity)*
October 2012	*increase*	-	-	*increase (H2B)*	-	*increase (prevalence and counts in males)*	-	-	-

(b)

Jarso	*Pasteurella*	*Salmonella*	IBD	MDV	Ascarids	Cestodes	*Eimeria*	Lice	Scaly leg
May 2011	-	-	-	*increase (J1A)*	-	*n/a*	-	-	*decrease (prevalence)*
October 2011	*increase*	-	*increase*	*increase (J1B & J2A)*	-	-	*increase (prevalence)*	-	*increase (severity)*
May 2012	-	-	-	*increase (J1A & J2A)*	-	-	*increase (prevalence)*	*decrease (prevalence)*	*increase (severity)*
October 2012	*increase*	-	-	*increase (J2A & J2B)*	-	-	-	-	-

The infections which showed the greatest and most consistent regional differences across the study period were those which are not normally associated with mortality in adult birds; namely the parasitic Ascarid and lice infections, both of which were more abundant in Horro. Cestode infection was the single infection the prevalence of which was observed to rise only in Horro after the rainy season in both years of the study. However, these observed changes in parasite burdens do suffer from significant limitations. In the case of faecal egg counts, it is difficult to interpret these, as they do not approximate the worm burden (Permin and Hansen, 1998). They may be influenced by the number of worms, their age and fecundity, by host factors, such as the age, sex and immune status of the host and the consistency of the faeces, which is in turn influenced by diet and the freshness of the sample. *Ascaridia galli* are particularly immunogenic worms, and it has been shown that their individual fecundity decreases as worm burden increases, through the chicken's immune response to the parasite. Ascarids also have a relatively long pre-patent period (4-8 weeks) so recent infections may not be detected. The lower limit of detection for the test method we used was 20 epg of faeces. False negative tests may be expected to be a feature of these data.

Lice burdens are also affected by many factors. Although we have grouped them as one taxa, bird lice are formed of two main sub-orders; *Amblycera,* which live in direct contact with host skin and feed on skin secretions, debris and blood from developing feathers; and *Ischnocera,* which live on feather surfaces and eat keratin. Work done on the samples we collected throughout the course of this study suggested that there was greater taxonomic diversity of both these sub-orders of lice in Horro compared to Jarso (Collins, unpublished data). Although we did not quantify abundance of the lice species separately, the majority of species identified were *Amblycera.* Møller and Rózsa (2005) found that taxonomic richness in *Ischnocera* was correlated with both *Amblycera* abundance and with host body

size. This corresponds with our findings, in that Horro birds tend to have a larger body size, as well as both more lice and a greater diversity of lice species.

Louse abundance and intensity of Amblycerans has also been observed to be positively correlated with larger group sizes in the host population (Whiteman and Parker, 2004), and group size can also affect the degree of aggregation of the more mobile Amblycerans. Louse abundances have also been observed to increase following nutritional stress in birds and to show seasonal changes related to breeding and moulting (Freed et al., 2008), and *Amblycera* diversity has been observed to correlate with an increasing T-cell response in nestlings (Møller and Rózsa, 2005). As Horro farmers tend to keep larger flocks, breed more frequently and have different types of supplementary grain to feed, there may be a number of potential factors which contribute to the differences in louse populations.

Therefore it is difficult to interpret the observed differences in the parasite burdens seen in our study populations. One hypothesis is that differences in the chickens' immune response between the two regions may contribute to the observed differences, as this may cause suppression of both worm fecundity and of lice numbers. Although these parasites are generally thought to have little impact on host survival, their higher abundance in Horro may potentially be of some significance for the birds. Taken together with our findings of low co-positivity (Chapter 6), we may speculate that there is ecological interference between parasitic and bacterial/ viral infections, such that birds infected with both are less likely to survive. If this is occurring at higher rates in Horro, it may be a possible explanation for the mortality difference between the regions. It would be interesting to determine whether effective anti-parasiticides have a positive impact on chicken survival in one or both of these very different ecosystems.

It is clear from our results that there is a complex system of interactions present among the numerous pathogens present in the scavenging production system, and their interactions

with the host are affected on a variety of levels, from interactions at the cellular level to those human interventions which are the most frequently considered risk factors in most epidemiological studies. Modifying any management practices at the human level might, therefore, be expected to perturb this ecosystem, and could have implications for infections beyond those targeted for control.

Appendix 7.1

Parasite burdens are described in various ways, as the number of parasites per host is an important factor in the ecology of the species. The definition of certain terms is provided here. (Margolis et al., 1982; Rozsa et al., 2000)

Prevalence: $\frac{\text{Number of hosts infected with a particular parasite species}}{\text{Total number of hosts examined}}$

Intensity: Number of individuals of a parasite species (determined directly or indirectly) within each infected host. Often given as a range.

Mean / Median intensity: The mean / median number of individuals of a particular parasite species within the total number of infected hosts (i.e. excluding all hosts with 0 parasites)

Abundance: The total number of individuals of a particular parasite species in a sample of hosts

Mean / Median abundance: The mean / median number of individuals of a particular parasite species within the total number of sampled hosts (i.e. infected and uninfected)

Aggregation: The tendency for parasite intensities to be unevenly distributed throughout the host population, such that a small number of hosts carry large numbers of parasites

Corrected moment estimate of k: Estimate of the parameter k of the negative binomial distribution, which approximately describes the parasite distribution among hosts, adjusted for sample size (Gregory and Woolhouse, 1993)

$$k = \frac{\overline{x}^2}{s^2 - \overline{x}} \tag{7.11}$$

Index of discrepancy: A measure of aggregation, comparing the parasite distribution among sampled hosts with the hypothetical situation, where all hosts harbour the same number of parasites (Poulin, 1993). Found by the equation:

$$D = 1 - \frac{\sum_{i=1}^{N}(\sum_{j=1}^{i} x_j)}{\overline{x}N(N+1)} \tag{7.12}$$

Where x is the number of parasites in host j (hosts are ranked from least to most infected) and N is the total number of hosts in the sample.

Chapter 8

General discussion

8.1 Introduction

Village chickens are still important to millions of people in less economically developed countries, and likely to remain so for many years to come (Alders and Pym, 2009). The purpose of this research project was to inform ongoing development programmes in Ethiopia, which place heavy emphasis on genetic improvement of local stock, and use selective and cross-breeding programmes to produce "improved" birds for the Ethiopian producer (Dana et al., 2010; Moges et al., 2010b). Farmers who participate in such improvement programmes are often required to radically alter their management system, in terms of housing and feeding the birds, and buying in chicks from large distributors, rather than rearing their own replacement stock. A potential concern with these "improved" birds is that they may have lost some of their natural disease resistance, which is a trait highly valued by farmers, and thus re-introduction into field conditions may not be as successful as hoped.

Among the previous literature on village chickens there are numerous studies characterising seroprevalence of various infections in healthy birds, and many reports of disease as a constraint to poultry production. There is, however, very little literature which covers the gap between infection and disease, and addresses how birds in a village situation are tolerating the pathogens with which they come into contact. The specific objectives of this thesis were to better understand the village production system and particularly the practices which may impact infectious disease ecology; to quantify the impact of disease on village poultry production; and to describe some of the infections present in birds in two geographically distinct populations, and identify potential risk factors, including the impact of co-infections, in order to help prioritise avian diseases for control in East Africa.

Through a series of cross-sectional studies, this study has attempted to provide a detailed observation of the impacts of infectious disease on chicken keeping in two geographically distinct areas of Ethiopia. The two regions, selected based on known cultural differences, and chosen to represent two well-characterised chicken ecotypes thought to be genetically distinct, were expected, *a priori*, to have some differences which would influence the way that people interacted with their chickens, due to differences in the socio-cultural importance of chickens in the two human populations. It was also believed that the two chicken populations may have differences in their genetic susceptibility to certain diseases, or have different phenotypic adaptations to their local environments. Evidence for these differences has been found through the socio-economic and genetic analyses performed by other members of the study team. It was also anticipated that there may be differences in the epidemiology of infectious diseases between the two regions, perhaps through variation in exposure due to different management systems, differences in climate or in social factors such as marketing practices. Previous studies in Ethiopia have attributed the majority of chicken mortality to "*fengele",* generally translated as Newcastle disease (ND), although numerous other chicken pathogens have been identified in the country. We believe this is one of the first studies in village chickens to not only identify the presence of multiple infections, but to consider their impact on bird survival, by studying both infection and mortality in the populations, and to try and fit this in the context of the local agro-ecology of the regions studied. Whilst it has been largely hypothesis-generating, it can nevertheless provide some useful insights for current development programmes in Ethiopia.

8.2 Main findings

The two regions have different agro-ecologies and cultures, and data about these was gathered through rapid rural appraisals, from existing published data and from data provided by the local agricultural offices. The predominantly Christian Horro region has high agricultural potential, and produces cattle, teff and niger seed, and values chickens for their cultural importance in welcoming guests and to use in celebratory dishes at festivals. Horro farmers particularly value larger body sizes in cocks, and good mothering ability in hens, and tend to sell and eat more birds, and rear more chicks. These findings were reflected in the observations of the chicken population in Horro, where birds had larger body weights, better body condition scores, more pronounced secondary sex characteristics, and flock sizes were larger and comprised a greater proportion of young stock. The main business model may be analogous to that of a broiler-breeder flock, as the majority of eggs go into reproduction, although it should be noted that most farmers do not make regular income from their chickens, and they are consumed only infrequently. Maintaining a flock as an asset, the sale of which can be used to cover small, incidental expenses ("safety net" role) appeared to be the most common purpose of chicken production in both areas.

Jarso is a predominantly Muslim area, where chickens are eaten infrequently, and many families did not know how to prepare chicken. The area is food insecure, but is a high producer of the mild stimulant, chat (*Catha edulis*), which is sold as a cash crop. Fewer chickens are reared than in Horro, and even fewer sold. Although birds still fulfil the "safety net" role, they may be less important due to the predominance of chat, which provides cash income. The traits most valued by farmers are appearance in cocks, and egg-laying in hens. Hens are reportedly less broody than in Horro, and chicks tend to be reared only for a limited period each year, which it might be supposed would result in more of a layer flock

business model; however, in practice it would appear that eggs are sold or eaten relatively infrequently, and many may be potentially wasted.

The day-to-day management of chickens, covering such items as feed provision, waste management, housing and confinement, appeared broadly very similar between the two regions. However, differences in the breeding management were more obvious, both in the frequency and the timing of rearing broods of chicks; whereas in Horro chicks were reared from September to May, farmers in Jarso tended to rear fewer broods and mainly confined rearing to the months of June, July and August, when the additional vegetation in the main rainy season helped birds to avoid predation, which Jarso farmers cited as their biggest constraint to chicken rearing. Horro farmers reported disease as the biggest cause of loss, especially disease “outbreaks” during the rainy season. To minimise the problems associated with cold and mud during the rainy season, Horro farmers preferred to avoid rearing chicks during this period. Horro farmers were also more likely to provide additional feed during the rainy season, and around one third reported that their birds were less likely to have contact with birds from other flocks at this time of year. A higher proportion of them also used forms of parasite control in their birds, largely in the form of ethnoveterinary treatments, including cleaning and hygiene measures.

Despite the fact that many factors which might be predicted to decrease disease risk, such as lower frequency of direct contacts with other flocks, more supplementary feed provided during the rainy season and more attention to parasite control through hygiene measures were more commonly characteristics of farms in Horro than Jarso, our findings from Chapter 4 suggested that disease was actually more prevalent in Horro. This result, gathered from questionnaire surveys of farmers’ recent experiences of disease events in their flocks, agreed with the reports from the rapid rural appraisal, as did the finding that the majority of disease “outbreaks” that only affected the young stock were reported

during the months of the rainy season, justifying the practice in Horro of not setting eggs during this period. The management practices which might be expected to decrease disease especially during high-risk periods may, therefore, be responses of farmers to an existing problem, and the efficacy of these in reducing disease risk is difficult to assess.

We found that our definition of "outbreak" did not necessarily correspond with what farmers regarded as an outbreak, as a significant proportion of them described events where only a single bird suffered mortality due to disease. It was found that there was indeed a regional difference in how often outbreaks were reported, with Horro farmers both more likely to report the occurrence of disease in their chickens, and more likely to report higher numbers of deaths in any disease event than farmers in Jarso. From farmer reports, it appeared that a larger epidemic had occurred in Horro the year before the study began (summer 2010), but that no such epidemic occurred during the study period. This, combined with the very low levels of ND seropositivity (<1%) led us to believe that we had conducted the study in an inter-epidemic period for ND, and that other infections, which led to farmers describing a variety of symptoms affecting different age groups, were contributing to the seasonal increase in disease mortality, which farmers still described as "*fengele".* It appeared that most of this seasonal rise in mortality was attributable to an increased frequency of small-scale losses, including single birds being lost, whereas individual farms reporting large-scale losses occurred sporadically throughout the year. However, over time, farmers tended to overlook the small-scale losses and focus on the large outbreaks when discussing the problem of disease.

We also examined factors which may predict individual bird mortality and survival, and found a complex web of factors which may lead to a bird being more likely to be lost due to disease or predation, or available for farmers' uses –either to be sold, consumed or retained. Although overall survival rates were relatively constant, reasons for non-survival

varied considerably between villages even within a region. The odds of an individual bird dying of disease in Horro were significantly greater during the rainy season, but also varied from year to year. However, in Jarso, the individual risk of dying from disease did not show a significant seasonal fluctuation. This perhaps correlates with findings from the previous chapter in that, whilst farmers in the wet season were more likely to report a disease outbreak, they also tended to lose fewer birds overall during this period, which may result in a relatively constant risk at the individual bird level.

Factors predicting the fate of an individual varied between regions, although male birds and birds with a scaly leg mite infestation in both regions were more likely to be sold or eaten. Previous infections did appear to influence a bird's subsequent fate, although none strongly predicted death from disease. In Horro, older birds with high *Pasteurella* antibody levels were more likely to be sold or eaten, perhaps as these may represent chronically infected birds, which may show respiratory signs or show reduced productivity. In Jarso, Marek's disease and *Eimeria* infections both increased the probability that birds died from disease or predation within the subsequent 6 months, reducing their availability for farmer use. There was high variation between households in the risk of disease mortality, and in Jarso the effect of season and MDV exposure on the risk of disease varied between households, suggesting some households may be effectively mitigating these risks.

It was also noted that whilst males were less likely to survive overall, because they were more likely to be sold, having a higher MDV titre did not greatly alter the odds of whether they are available to sell or keep within a six month period. However, females in Jarso with high MDV titres were at greater risk of dying from disease or predation within the 6-month follow-up period, perhaps indicating that there was not only regional variation between birds' resistance to disease, but also perhaps some differences between the sexes, and that in females, even the production status may have some impact on birds' survival, with

females incubating eggs at the time of initial sampling having the lowest odds of surviving the subsequent 6 months.

In the final two chapters, patterns of infection and co-infection were examined by using both multivariate analyses, and regression models to look at each infection outcome individually. The temporal and spatial variation of infections was examined to see if any differences may account for the different patterns of mortality observed between the two study regions, whilst controlling for other risk factors, including co-infections. Little difference in exposure to the bacterial or viral infections was found between the two regions. It was found that several of the infections appeared to interact with each other, such that birds rarely had high levels of more than one infection, with the exception of *Pasteurella* and *Salmonella*, high antibody titres to which were observed to co-occur more frequently than expected under an assumption of independence. However, MDV and parasitic infections appeared to cluster away from these bacterial infections, and higher burdens appeared more frequent in male birds, which may be more tolerant of such chronic infections. Strong seasonal patterns of infection were not observed, but Horro demonstrated higher prevalence and lower aggregation of the macroparasitic lice and Ascarid infections, and seasonal fluctuations of cestode infections, with a rise in birds sampled at the end of the rainy season. As these infections are generally not regarded as causing mortality in adult birds, it may be that birds infected with both a parasitic and a bacterial infection are more likely to be unable to combat both simultaneously and die; hence the lack of these observations in our dataset, and potentially why more mortalities are observed in Horro, where higher parasite prevalence would make co-infection likely to occur more frequently.

However, we did not observe a current infection with any of the parasitic diseases in Horro to impact on a bird’s likelihood of dying of disease within the 6-month follow-up period. It

was observed that there were slight increases in *Pasteurella* s:p ratios after the rainy season in both regions in both years of the study, and measurements of other diseases tended to fluctuate. Thus it may be that what farmers sometimes observe as an "outbreak" in the rainy season is multiple coincident infections causing a rise in mortality, rather than a single epidemic infection, although the latter may also occur from time to time. Although exposure to these infections is widespread, the majority of birds we examined in our study showed no clinical signs, and were clearly able to tolerate a wide range of infections. Other factors may be at play during the rainy season, such as cold stress and a lack of energy-rich feed, which impact on birds' immunity and contribute to the observed mortality epidemic in Horro. Jarso may be less at risk, due to the dryer climate and the fact that more maize is grown in this area, and commonly forms a part of birds' dietary supplementation.

However, other factors may also be at play. Climate and macroparasite infections were not the only thing to differ between the two regions. There were also phenotypic differences in the birds, in that Horro birds grew larger, and had more pronounced secondary sex characteristics, such as combs and wattles, and brighter plumage colours. They also reared more chicks, influenced by the choices the farmers made as to when and how often they set eggs for hatching. Ecological trade-off theory would suggest that this investment into reproduction reduces the energy available for maintenance, including immunity. This would offer another possible explanation for the shorter lifespan, greater disease-mortality and higher intensity and prevalence of macroparasite infections observed in Horro. Variation between birds would appear to play a substantial role in the differences we observed in infection measurements. Even though there was a moderate degree of clustering within farms for all of the diseases we looked at, modelling suggested that even after this and other farm-level risk factors were controlled for, there were still substantial differences between birds on the same farm, particularly for *Salmonella* and *Pasteurella*, where bird level risk factors such as sex were also important determinants of s:p ratios.

Whether this reflects differences in bird behaviour between the sexes and consequent differences in exposure, or whether this is due to differences in the bird immune response between the sexes cannot be determined from our data. However, what is clear is that there is no simple answer to the question of which infections are having the greatest impact on bird survival, due to this complex interplay between the pathogens, the birds, their environment and the human influences in this system.

Study strengths and limitations

Perhaps the greatest limitation of this study is that direct observations were very limited, both of the pathogens and of the birds. By choosing to measure a single serological response rather than isolating pathogens, it is often difficult to know how to interpret these in the context of the questions we were trying to answer. Although we can detect a small rise in mean antibody titre over the population, this does not necessarily equate to widespread exposure to the pathogen in question, but may be due to a small number of birds with much higher titres being included in the sample. Quantile regression appeared to be a promising approach to exploring this question. Spatial analysis of household residuals was also helpful in identifying potential small localised outbreaks. However, where household clustering was lower, for example *Salmonella,* this could not explain the appearance of individual birds with very high titres within households. More data on the pathogens themselves, such as isolation and identification of the circulating *Salmonella* serovars, would be useful, in order to aid interpretation of the antibody response. Similarly, parasitic infections such as the lice and *Eimeria* have been treated in this analysis as a single species, although subsets of them were speciated by MSc students and differences in the diversity and prevailing species were shown between the two regions, which may clearly have implications for bird health as species differ in their pathogenicity.

The other problem with serological responses is that they do not indicate current infection, and therefore using them in regression models which assume a directional response is potentially misleading. We have tried to overcome this problem by using both a multivariate analysis as well as regression to explore potential correlations between infections, and controlling for infections which co-vary when trying to explore other risk factors. By combining several different methods, we have illustrated the complexity and the numerous interactions in the system, and are able to gain a more holistic picture of possible risk factors.

The other main outcome that we did not observe directly was that of the fate of the birds. Although we were able to confirm survival in most cases (with the exception of around 10% of birds which farmers presented as "survived", but were found on comparison of previous photos to be different birds), we relied entirely on farmers to tell us if birds had died of disease, predation, or some other cause. There is therefore likely to be some error, not only because farmers cannot always identify individuals accurately, but also because deaths are not necessarily witnessed. Therefore deaths attributed to predators may be due to disease morbidity/ mortality and birds being scavenged by predators. It is hard to know how much people's perception of what happened to their birds is influenced by the prevailing local beliefs as to the major constraints they are under. Where people reported birds to have died from disease, we tried to collect data on symptoms displayed, and whether other birds in the flock were also affected, but we are still relying heavily on people's ability to recall such events.

Although we have collected many data on these two regions and were therefore able to explore in detail how disease exposure and survival varied within and between these regions, some of the factors that we hypothesise may drive how birds and pathogens are interacting, such as climate, agro-ecology and human social and economic factors have

mainly been collected at the regional level, and therefore no conclusions can be drawn on possible correlations between these factors, as there are only two data points available. In addition, it should be borne in mind that we only have data for two years, and therefore observations such as the seasonal fluctuation of risk of disease mortality in one region but not the other may not be representative of variation or trends in the longer-term.

Implications of the findings and possible future work

This study was designed to inform the Ethiopian genetic improvement programme, where selective breeding of indigenous birds on station has been going on for several years to produce a dual-purpose chicken which meets local farmers' requirements. The 8th generation of these "improved" Horro chickens are now being trialled on farms in the Horro region, as well as in villages around the research centre in Debre Zeit where the selection took place. It will be interesting to see the outcome of this trial, as our results suggest that i) pathogens may impact mortality differently in different areas and ii) there is diversity amongst birds within a village, such that they tolerate different pathogens, yet fewer birds than expected are present which appear able to tolerate both a bacterial and parasitic infection simultaneously. The implications of these findings are that birds would appear to be locally adapted, and that it is important to maintain the diversity at the population level, so that the population can continue to recover from the dynamic and diverse array of pathogenic infections. Birds distributed from the research centre will have passed through a population bottleneck, and the risk is that some genetic diversity and adaptive immune traits may have been lost from this population, which may impact their survival.

The continued reporting by farmers of deaths from "*fengele*", despite our finding that almost all birds were seronegative for NDV, would suggest that "*fengele*" is probably

comprised of several of the diverse array of infections we have found in the population. This has important implications for any vaccination programme, as this discrepancy needs to be carefully explained lest farmers have unrealistic expectations for a vaccine, and lose incentive to use it. When we revisited the villages in May 2014 and held public meetings to explain our findings and provide some education about the various diseases, this was appreciated by most farmers, who confirmed that they saw all the clinical syndromes which we described, and that it made sense that multiple diseases were involved. Although it may seem very obvious from a veterinary standpoint that multiple infections will be present, the lack of veterinary input into a lot of the previous research in Ethiopia may have contributed to an over-simplification of the problem of infectious disease in village chickens, and led to the erroneous belief that ND is the only disease of importance (Dessie and Jobre, 2004; Halima et al., 2007; Dinka et al., 2010; Moges et al., 2010b). Whilst it is likely that an ND vaccination programme would bring benefits, it should perhaps be emphasised that it may not have an equal impact in all areas, as our study has demonstrated it does not circulate continuously in all regions, and large epidemics do not affect all areas every year, as previously seems to have been assumed (Dessie and Jobre, 2004; Halima et al., 2007; Moges et al., 2010a). In some areas, protection from predators, basic hygiene and biosecurity and improvements in breeding management may also bring substantial benefits, and thus extension services should be tailored to the local needs, rather than adopting a blanket approach. This applies equally whether the approach is through distribution of “improved” birds or other technologies to improve health or productivity. Different production models, such as concentrating on birds for meat or egg production may in fact suit different areas, depending on the local markets, or what markets people are able to access. Current strategies also rely on substantially altering the production environment by introducing measures such as full-time housing of birds and providing all feed. This contradicts the premise of utilising the existing semi-scavenging system, to which

the indigenous birds are highly adapted, and is likely to radically alter the epidemiology of the existing infections in these areas. Certainly infections such as MDV and *Eimeria* would be likely to become far more significant under housed conditions.

In view of the fact that the landscape of chicken production is changing rapidly in Ethiopia, with more and more money going into projects focused on breeding high-producing birds for the environment, coupled with a focus on changing production systems towards (semi-)intensification, perhaps the most important role of infectious disease research at present would be to monitor the impact such rapid changes are having on the epidemiology and ecology of infections. We have demonstrated that apparent interactions exist between, for example, *Salmonella* and Ascarid worms, but should a worming control programme be introduced, for example, it is difficult to predict whether *Salmonella* burdens would increase, due to the removal of ecological interference and survival of birds which may previously have died due to co-infections, or whether it would decrease due to birds being better able to control bacterial infections and reduction in faecal shedding. However, since this disease is clearly of zoonotic importance, it would be of interest to monitor whether such a programme would have impacts on non-target diseases, as well as improvements in survival. Although in this study we have focused on mortality as the main reason for investigating infectious diseases, gains in productivity may also be had through infection controls. The question is whether such benefits will outweigh the costs, particularly if the costs include increasing burdens of zoonotic diseases in smallholder communities which continue to manage their chickens in a traditional system.

Due to the growing pressures of food security and increasing demand for animal protein, the scavenging village chicken clearly still has a valuable role, as it can utilise food waste, weeds and insects and therefore dose not compete with humans for food, nor require labour to provide all its feed requirements. Changing to an intensive system where all feed

is provided will not necessarily bring all the hoped-for economic advantages to a small-scale producer. However, the current smallholder system appears to be very wasteful, with around 30% of adult birds lost from the system every 6 months, according to our findings, and reported rates of chick loss are even higher (Dessie and Ogle, 2001). Given one hypothesis from this study is that the bird ecotype which grew larger and reared more offspring appeared more prone to die of disease, we could ask whether breeding for larger birds and birds which lay more eggs within the current scavenging system will be economically advantageous, or whether it will in fact introduce more wastage into the system. However, it may be that small alterations, such as providing more energy-rich supplementary feed at periods of stress may have profound benefits, if indeed energy is a main limiting factor. Clearly assessments of the cost/benefit would also need to be made of any strategies, as well as their impact on the birds' health. However, it should be borne in mind that as most people currently appear to keep their birds for their "safety net" role, wastage in many flocks may be a natural consequence of farmers under-utilising their birds, and would require cultural change in order to shift from simply maintaining flocks to seeing them as a productive asset.

Our experiences with Ethiopian farmers would indicate that they are very keen to access new technologies which might allow them to reduce the burden of disease in their chicken flocks. However, most of what is known about poultry disease, even in industrialised countries, is aimed at control in intensively farmed flocks where there is heavy reliance on biosecurity measures to prevent introduction of infections. Trying to exclude infections in the backyard situation is clearly unfeasible. Yet over-reliance on vaccines to control infections has demonstrably driven the evolution of pathogen strains of increasing virulence (for examples, see Atkins et al. (2013), Gandon et al. (2001)). Therefore, as a long-term strategy, widespread vaccination against multiple endemic infections in village chickens may not be desirable either. Although the Ethiopian Government's focus is on a

drive towards (semi)-intensification of chicken production, it is interesting to contrast this with the situation in more economically developed countries such as the UK and USA, where numbers of backyard flocks are increasing. However, in the main, such small flocks are not maintained for the economic benefits of chicken production, and veterinary involvement and service provision to the backyard poultry sector is also inadequate in these countries (Garber et al., 2007; Karabozhilova et al., 2012).

Findings from the genetics part of the Chicken Health for Development project suggested that the Horro and Jarso chicken populations both had high levels of diversity, yet were clearly differentiated, which may be attributed partly to adaptation to their particular ecological niche (Desta, personal communication). Furthermore, different genetic loci associated with *Salmonella* and IBD resistance were found in the two populations (Psifidi, personal communication). Rather than focus solely on developing intensive farming systems, where animals need the provision of large quantities of energy and protein-dense food, which often has to be shipped in and could be used for human nutrition, developing countries, such as Ethiopia, might be well-placed to lead the way in devising strategies to derive the greatest benefits from smallholder poultry production by utilising their robust and locally adapted chicken ecotypes to fulfil the various roles that people demand of them in diverse production systems. It has been suggested that in order to farm sustainably, we need to optimise, rather than maximise production levels, as high-producing animals can no longer meet their metabolic demands from local produce. (van Dijk, 2014). Perhaps considering endemic infections within the ecological framework of the smallholder production system, as this study has done, will help to devise ways which allow chickens to tolerate the pathogens, yet still produce enough to have a valuable contribution to nutrition and livelihoods worldwide.

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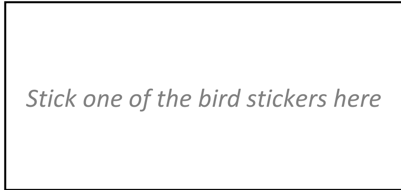
General Appendix A

Questionnaires used in the study

A.1	Health and genetics questionnaire
A.2	Socio-economic questionnaire
A.3	Bird examination recording sheet
A.4	Follow-up questionnaire
A.5	Follow-up bird examination recording sheet

Chicken Health for Development – Cross sectional study.



1. **Start by greeting the respondent in their language!**

2. **Explain the following to the respondent!**

This questionnaire is part of a research study into the health problems of village poultry in rural Ethiopia, Jarso and Horro. The study is conducted by International Livestock Research Institute, the Ethiopian Institute of Agricultural Research and the University of Liverpool in Great Britain.

Before you decide if you want to participate, we will explain to you why the research is being done and what it will involve for you. Please feel free to ask us if you would like more information or if there is anything that you do not understand.
We hope to find out which the most important diseases in Habesha poultry are and why this affects some birds more than others. By doing this, we hope we can develop ways to reduce the problems with disease that will work in your village. Many other villages in Ethiopia are also taking part.

We want to examine two of your birds; then take some blood samples, some photographs of the birds and identify them with leg rings. If you wish to sell the birds, you can remove the rings. It is possible that your chicken could develop a bruise or an infection under the wing, where we take the blood sample, but the risk of this is very small. We will keep a small amount of blood from your chickens which we will send to researchers in England, who will test it to see whether certain chickens in Ethiopia are better at resisting diseases and whether we can recognise those chickens. The rest of the tests will be done here in Ethiopia, in Debre Zeyit. If you would like us to stop the examination or the sampling of your chickens at any time, you are free to tell us to stop without giving us a reason.
We would also like to ask you some questions about the management of your birds. The questions will take about 30 minutes. We will record the answers on paper, and they will be stored in a safe place, where no-one else can see them.

We would like to stress that you do not have to accept this invitation and should take part only if you want to. If you change your mind after you have answered the questions and would not like to be included in our study, we can destroy your answers or samples from your birds so they are not used. If you are worried for any reason, or have any problems with your birds after the sampling, please tell your Development Agent, who will contact us.

Thank you in advance for your willingness to discuss with us!

If the household is happy to take part and has agreed for the chickens to be sampled, please tick this box. ☐

Have you ever used any vaccinations in your birds? Yes ☐ No ☐

If yes, can you provide any details of **when** they were vaccinated and against **which diseases**?
....
....
....

***If the household has used any vaccines in the last 2 years, unfortunately we cannot use them for this survey*.**

Date /dd/mm/yyyy/:..

Name of the enumerator:..

Poultry ownership

Poultry by Age and sex group	Hens	Cocks	Pullets	Cockerels	Chicks
Number currently owned					

Housing

1. Where do birds sleep at night?
   - Separate shelter.....................☐
   - Perches in the house..............☐
   - Perches in the kitchen............☐
   - Perches on the veranda..........☐
   - Other (please describe)..

2. If you don't have a separate house for your chickens, why not? [1]
   - Lack of knowledge (Awareness)..☐
   - Lack of importance of poultry...☐
   - Lack of construction materials (Availability and Cost)...........☐
   - Risk of predators..☐
   - Risk of theft...☐
   - Other (please describe)..

3. What do you do with bird waste products such as excreta?
   - Used as fertiliser......☐
   - Burning...................☐
   - Thrown away...........☐

4. Do you provide nest boxes for incubating/ brooding hens and chicks? Yes ☐ No ☐

Feed and water

5. Do you ever provide any supplementary feed for your birds? Yes ☐ No ☐

6. Do you provide separate supplementary feed just for chicks? Yes ☐ No ☐

7. During the rainy season, do you provide:
   - More feed.........................☐
   - The same amount of feed.☐
   - Less feed...........................☐
   - No feed.............................☐

[1] *This question was part of an MSc student's survey on the reproductive aspects of poultry keeping, and was only included during the second season of sampling*

8. What type(s) of supplementary feed do you provide? (Multiple answers possible)[2]
   Wheat.................☐ Sorghum........................☐
   Maize..................☐ Household scraps...........☐
   Barley..................☐
   Other(describe)..

9. Are there periods during the year when birds are confined within their housing eg planting, harvesting?
   Yes ☐ No ☐

   If yes, which months?...

Contact with other birds

10. For each season, can you grade the amount of time you estimate your birds spend in areas where they can mix with other peoples' birds? Only consider adult birds without chicks for this exercise.

	Not at all	Rarely	A few times a week, for less than one hour	A few times a week for more than one hour	Every day for less than one hour	Every day for more than one hour	Don't know
Dry season	0	1	2	3	4	5	99
Rainy season	0	1	2	3	4	5	99

Source of new birds

11. When was the last time you bought or were given a new bird?

    Within the last 12 months.........☐.........If yes, which month?..
    Between 1 and 2 years ago........☐
    Between 2 and 3 years ago........☐
    More than 3 years ago..............☐

    Was this bird: From someone you know in your village?.......................☐
    From someone you know in a different village?..............☐
    From the market?...☐

    From another source (please describe)..

[2] *This question was not asked in the first sampling season. It was added as part of an MSc student survey during the second season of sampling, and retained thereafter*

Poultry and egg selling places and target seasons (multiple answers possible)

12. Selling places (A) — Main seasons at which you sell them (B)

Selling places (A)	Main seasons at which you sell them (B)
Neighbours....................☐	Christmas..☐
Local collectors..............☐	Easter...☐
Near market..................☐	Ramadan...☐
Take to far markets........☐	Meskel..☐
To restaurants...............☐	New year..☐
Others..	Any time needs arise or birds available...........☐

13. Do you practice culling of birds? [3] Yes ☐ No ☐

If yes, what is your reason for culling (multiple answers are possible)

Old age..............☐	Low production.........☐	Unwanted plumage colour/pattern...☐
Illness................☐	Bad temperament.....☐	Other (specify)..................................

Loss of birds to diseases

14. In the last 12 months, how many outbreaks have you had, where you lost several of your birds to the same disease? ....

For each outbreak, can you **write how many** birds you lost in the month the outbreak occurred and **tick which age groups** were affected for each outbreak?

	Sept	Oct	Nov	Dec	Jan	Feb	Mar	April	May	Jun	Jul	Aug
Total lost in month (number)												
Chicks (√)												
Growers (√)												
Adults (√)												

Treatment of sick birds and treatment of healthy birds to prevent disease

15. If you had an outbreak in the last 12 months, did you attempt any treatment of **sick** birds?

Yes ☐ No ☐

a. If Yes, what did you try?

....

....

b. Was your treatment successful at curing the sick birds?

Completely ☐ Partly ☐ Not at all ☐

[3] *This question was not asked in the first sampling season. It was added as part of an MSc student survey during the second season of sampling, and retained thereafter*

c. Did you give the same treatment to healthy birds to prevent them from becoming sick?
Yes ☐ No ☐

d. Was your treatment successful at preventing birds from becoming sick?
Completely ☐ Partly ☐ Not at all ☐

16. Do you use any other measures to try and reduce losses from disease outbreaks?
    - Sold birds before your flock was affected by disease............... ☐
    - Sold healthy birds after you had seen disease in your flock...... ☐
    - Isolated sick birds from healthy birds...................................... ☐
    - Slaughtered birds showing signs of disease............................. ☐
    - Slaughtered healthy birds... ☐
    - Use ethno-veterinary treatment(s) to prevent disease............ ☐

    Other.(please describe) ....
    ....

17. What do you do with carcasses of birds which have died from disease?
    - Throw away in or close to your yard....... ☐ Bury.. ☐
    - Throw away far away from your yard...... ☐ Burn... ☐

    Other (please describe)..

18. Do you see any skin parasites on the birds? [4] Yes ☐ No ☐

If yes, please show pictures to farmer and ask them to identify any they have seen.

	Tick any seen on the bird	Where on the bird do you see them?	Do you remove any by hand?	Tick any seen in the housing
a. Stick-tight flea	☐	....	☐	☐
b. Soft tick	☐	....	☐	☐
c. Hard tick	☐	....	☐	☐
d. Lice	☐	....	☐	☐
e. Other (Please write name)	....	....	☐	☐

[4] *This question was part of an MSc student survey on ectoparasites, and was only included during the fourth season of sampling*

19. Do you use any treatments against parasites?
    Insecticide spray treatment of housing......☐ Insecticide spray on birds..................☐
    Treating housing by smoking.....................☐ Other treatment of birds....................☐
    Other, please describe ....

20. Do you have any birds with disease problem at the moment? Yes ☐ If Yes, how many?...........
    No ☐

21. Do you have access to advisory/technical/ support from extension workers on poultry production?
    Yes ☐ No ☐

    If yes, how often do they visit you per month? ....

Characteristics of your chickens

22. Have you observed any variation in disease resistance among your chickens? [5]

    Yes ☐ No ☐

23. How would you describe the temperament of your chickens? [6]
    Docile ☐ Moderately tractable ☐ Wild ☐

24. Do your birds take dust bath regularly? [6] Yes ☐ No ☐

Breeding and Hatching chicks

25.

Reproductive traits [6]	Hen	Cock
Age at first mating(mo)		
Age at first lay(mo)		

26. Average number of clutches per hen per year (circle) [6] 1 2 3 4 5

    Average length of inter-clutch period (wk) [6]...

    Average length of single clutch (wk) [6]...

    Average number of eggs per clutch (N) [6]...

[5] *This question was part of the genetics survey and was not included during the third round of sampling*
[6] *This question was part of an MSc student's survey on the reproductive aspects of poultry keeping, and was only included during the second season of sampling*

27. How many times a year do you incubate eggs for hatching..

28. **Underline** the months when you prefer to set eggs for hatching
    Sept Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug

29. **Underline** the months (if any) when you will **not** set eggs for hatching
    Sept Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug

    Why do you not set in these months? [7]...

30. Do you select eggs for incubation? [7] Yes ☐ No ☐

31. For how long (days) do you store eggs before incubation? [7] ....

32.

Average number of eggs set to a broody hen [7]	Average hatch rate (%) in the dry season[7]	Average hatch rate (%) in the wet season[7]	Survival rate of chicks to 8 weeks (%)[7]

33. How would you describe broodiness in your hens (circle)[7]
    Common ☐ Sometimes ☐ Rare ☐

34. Interval between two consecutive brooding period (months[7])..

35. How do you deal with unwanted broodiness behaviour? (multiple answers are possible) [7]
    Hanging hen upside-down...☐ Disturbing.....................☐ Inserting feathers through nostrils...☐
    Taking to another place........☐ Taking away brooding nest.☐ Immersing in cold water.......☐
    Other (specify) ....

36. Which two characteristics of hens are most important to you? [7]

    ....

    ....

37. Do you have your own cock [7] Yes ☐ No ☐

38. Which two characteristics of cocks are most important to you? [7]

    ....

    ....

---

[7] *This question was part of an MSc student's survey on the reproductive aspects of poultry keeping, and was only included during the second season of sampling*

Preferences for appearance of chickens

39. Plumage colour of your preference (rank, 1=most important)[8]

39.1. Hen

1. .... 2. .... 3. ....

39.2.Cock

1. .... 2. .... 3. ....

39.3. Which plumage colours do you dislike? [8]

1. .... 2. .... 3. ....

39.4. Reason for plumage colour preference [8]

1. Aesthetic value.................................. ☐ 2. High market value...................... ☐
3. Cultural and religious value................ ☐ 4. Other(Specify).................................

40. Which comb type do you prefer? [8]

1. Single ☐ 2. Double ☐ 3.Others (Specify)...............................

40.1. Reason for comb type preference [8]

1. Aesthetic value.................................. ☐ 2. High market value...................... ☐
3. Cultural and religious value................ ☐ 4. Other(Specify).................................

41. Which shank colour do you prefer? [8]

1. Yellow ☐ 2. White ☐ 3. Black/grey ☐

42. Do you prefer [8]

1. Normal feathered chicken ☐ 2. Naked neck ☐

43. Which type of chicken do you prefer? [9] Crested ☐ No crest ☐ No preference ☐

43.1. Why?

1. Aesthetic value................................☐ 2. High market value....................☐
3. Cultural and religious value.............☐ 4. Other(Specify).................................

---

[8] *This question was only included in the first two rounds of sampling as part of the genetics survey*
[9] *This question was only included during the third round of sampling, as part of the genetics survey*

44. Presence of any cultural or religious belief to rear a special type of chicken[10]

1. Yes   2. No

If yes; specify the type of cultural/religious belief to rear a special type of chicken

____

Presence of any cultural or religious belief not to eat chicken meat and eggs

1. Yes   2. No

If yes; specify the type of cultural/religious belief not to eat chicken meat and eggs

____

Presence of any cultural or religious belief not to sell chicken and eggs

1. Yes   2. No

If yes; specify the type of cultural or religious belief not sell chicken and eggs

____

Thank you very much for taking part in this survey

[10] *This question was part of an MSc student's survey on the reproductive aspects of poultry keeping, and was only included during the second season of sampling*

Chicken Health for Development cross sectional study. Introductory sheet (SE only)

Questionnaire Code number

3. **Start by greeting the respondent in their language!**

4. **Explain the following briefly to the respondent!**

This is a questionnaire is part of a research study into the economic and health problems of village poultry in rural Ethiopia, Jarso and Horro. The study is conducted by International Livestock Research Institute and the University of Liverpool. Before you decide whether to participate, we would like to explain to you why the research is being done and what it will involve. Please feel free to ask us if you would like more information or if there is anything that you do not understand.

The data to be generated will be on socioeconomic importance of poultry in your household and poultry health services. This questionnaire will take about 90 minutes but respondents have the right to stop the interview at any time. Yet, the data will only be used if the questionnaire is completed. Data generated with this questionnaire will not be transferred to a third person and will be used only for the purpose of the study.

Some households are also having their birds sampled, but we do not need to do this in every household, so we have chosen these by a lottery. We are returning to sample more birds in the village later this year and again next year, so we may ask to sample your birds in the future. We hope to find out which the most important diseases are in your village, and why this affects some birds more than others. By doing this, we hope we can develop ways to reduce the problems with disease that will work in your village.

We would like to stress that you do not have to take part today and should do so only if you want to. If you decide later you would like your data to be removed, you can tell your DA who will contact us.

Thank you in advance for your willingness to discuss with us!

If the household is happy to take part, please tick this box. ☐

Date /dd/mm/yyyy/:...

Name of the enumerator:...

Village name...

Time started..

NOTE: QUESTIONS CAN HAVE MORE THAN ONE ANSWER!

Part-A

I. Households' access to and participation in poultry extension services and knowledge of poultry diseases.

Now we would like to know about poultry extension services and health related issues.

1. How would you say the problem of poultry disease in your area?
    1. Good
    2. Poor
    3. Worst
2. Is there animal health clinic in your village? A/ yes B/ No
3. How far is animal health clinic from your home? _____km or _______walking minutes
4. Does the animal health clinic give health service for poultry? A. Yes B. No
5. Do you know that poultry can be treated in animal health clinic like other livestock?
    1. Yes 2. No
6. Do you have access to advisory/technical/ support from extension workers on poultry production? A. Yes B. No
7. If yes, how often they visit you per month?___________________________
8. If the government starts some auxiliary services to help with poultry production, are you likely to access these?
    1. Yes
    2. No
9. What type of auxiliary services would be important to you?
    1. Training on bird management
    2. Subsidising feed
    3. Providing vaccination services
    4. Providing disease resistant birds
    5. Others specify__________________

Part-B

I. Family structure

We will now start by asking some questions about your family structure, how many members, what age and who does what etc. Let us start with you/head of the household.

1. Family size, activity, and related issues

No	HH head/ relation to HH head(A)	Marital status of HH head (B)	Sex (C)	Age in years	Education (D)	Main Work (E)	Secondary work (E)
1	HH head						
2							
3							
4							
5							
6							
7							
8							
9							
10							

(A) 1= Parent; 2 = Spouse; 3= Son/daughter; 4 = Sibling; 5 = Others
(B) 1= Single, 2= married, 3= Divorced, 4= Widowed
(C) 1-female, 2-male
(D) 0= None, 1= primary school, 2= secondary school , 3= high school, 4= vocational training; 5= college; 6 = degree and above
(E) 1- farmer/family farm work, 2- family non/off farm activity, 3-student, 4- casual wage worker (farm/off-farm) 5- salaried worker , 6-unemployed, looking for a job, 7-unwilling work/ retired/not able to work, , 8-Other (specify)

II. Resource ownership and allocation

Now we would like to ask you some questions about resources related to housing, land allocation, crop production and income, livestock production and use, and other sources of livelihoods.

2. Dwelling/Housing

	Roofing (A)	No. of rooms	Exterior walls (B)	Flooring (C)	Electric supply(D)	Energy source(E)	Drinking water sources(F)
Main							
Kitchen							
Toilet							
Other							

(A) 1-grass roofed 2-Plastic; 3 - Iron sheet roofed; 4 – Combination, 5- Not roofed
(B) 1-Mud plastered, 2-Mud plastered and reinforces with cement 3-covered with straw
(C) 1-Mud/leveled earth, 2--Covered by plastics 3 Cement and stone, , 4-Others
(D) 1-yes, 2-no
(E) 1-Fire-wood, 2-dung, 3-charcoal,4-fuel/like kerosene/, 5- electricity
(F) 1- natural spring-unprotected, 2-natural spring-protected, 3- potable-supplied at water point, 4-potable-supplied at home, 5 other______________________________

3. Land size, allocation and crop income during the last cropping season:

   3.1 Total land size in hactar/olma/sanga/qarxi_________________

   3.2 Land allocation, costs and crop income in the last cropping season

Crop / Land /input/output/	Crop-1	Crop-2	Crop-3	Crop-4	Crop-5	Fallow land And/or *kalo*
Land allocated in *sanga/olma/qarxi Hectare*[*]						
Quantity of seed used/Quintal or KG/						
Value of seed used/Birr/						
Quantity of fertilizer used/Quintal/-DAP						
Value of fertilizer used/Birr/-DAP						
Quantity of fertilizer used/Quintal/-UREA						
Value of fertilizer used/Birr/-UREA						
Quantity of Pesticide used/litre/						
Value of Pesticide used/Birr/						
Capital cost/interest paid//Birr/						
Size of land rented-in						
Value of land rent-in / in Birr/						
Oxen rent /value in Birr/						
labour cost-employed in the year -crop						
labour cost-employed in the year -Value						
Labor cost for land preparation /Birr/						

Labor cost for weeding /Birr/						
Labor cost for harvesting /Birr/						
Total cost/Birr/						
Harvest in *KG/quintal/*						
Sold harvest in *KG/quintal*						
Value of sold harvest/Birr/						
Consumed from harvest/Quintal or KG/						
Value of consumed crop/Birr/						
Crop at store/Quintal or kg/						
Value of crop at store/Birr/						
Land rent/for land rented out/-value/Birr/						
Sell of *kallo/*hay//Birr/						
Total revenue						

Instruction

Please, write the name of crops in a column provided/crop-1, crop-2, crop-3, crop-4, crop-5.../!!!!!!

4. Livestock and other asset ownership, expenditure, sale and income from livestock product:

Livestock type	No. currently owned	Current market value of currently owned	No. /quantity/ sold in the last 12 months	price sold for/valve of rent /	No. purchased in the last 12 months	Purchase price
Cows						
• Income from sale of milk						
• Income from sale of yogurt						
• Income from sale of butter						
Oxen						
• Income from rent						
• Hides/skin						
Bull						
Heifer						
Calves						
Goats(young)						
Goats(adult)						
Sheep (young)						
Sheep (adult)						
Donkey(young)						
Donkey(adult)						
Horses						
Mules						
Others						
Home appliance		Year of purchase	Purchase price			
Radio						

Television						
Tape recorder						
Bicycle						
Others						

5. Livestock expenditure: have you had any of the following expenditures related to livestock during the last cropping season?

Type of expenditure	**Cash value**
Labour for herding	
Feed	
Veterinary services/medicine	
Other expenses	

6. Other livelihoods type and income from each

Livelihoods type	**Number in hh engaged**		**Income in last 12 months**	
Petty trade	Adults:	Children:	Adult:	Children:
Wage labor	Adults:	Children:	Adult:	Children:
Food for work	Adults:	Children:	Adult:	Children:
Skilled labor employment	Adults:	Children:	Adult:	Children:
Others	Adults:	Children:	Adult:	Children:

7. Other incomes and expenditures:
    1. Saving at bank/home(in cash-Birr)________
    2. Saving rate income in the year(in cash-Birr)____________
    3. Remittance (in Birr) ____________
    4. Transfers out of household (in Birr)__________________
    5. House rent paid (in Birr)______________
    6. House rent that would have been paid(in Birr)_____________

III. Poultry production and marketing practice

We would also like to learn about poultry production and marketing practice of your household.

Questions 8 to 11 to households that don't keep poultry currently. For poulty keeping households go to question 12.

8. You don't keep poultry currently, but did you ever keep poultry in the past?
    1. Yes, please specify how long back?____________ years/months ago.
    2. No
9. What is the main reason(s) for not currently keeping poultry?
    1. Busy with other activity
    2. Feed is too costly
    3. Frequent diseases outbreak
    4. Low market price
    5. Recently sold
    6. Recent massive death due to disease
    7. Others____________
10. If the government helps you with auxiliary services, are you likely to consider keeping poultry?
    1. Yes
    2. No
11. If yes, then what type of auxiliary services would be important to you?
    1. Training on bird management

2. Providing vaccination services
3. Providing disease resistant birds
4. Subsidising feed
5. Others, specify ______________________________

Questions 12 to end of section to households that keep poultry.

12. How long have you kept poultry for?___________
13. What/who are sources of hen/chicken to begin poultry production?
    1. Market
    2. Neighbour areas
    3. Office of Agriculture and Rural development
    4. Others_____________
14. What is the main reason for choosing the above source?
    1. Easily accessible
    2. Characteristics of chicken trustable
    3. Known for their good productivity
    4. Chicken health well known
    5. Others_________________________
15. If answer is not from market, why?
    1. Production performance cannot be known/trusted
    2. May be infected by disease
    3. Others____
16. Do you have exotic/improved mother birds?
    1. Yes
    2. No
17. Which breed do you prefer for reproduction?
    1. Exotic
    2. Indigenous
    3. Both
18. Why do you prefer this breed?
    1. Good in brooding and hatchability
    2. Produce more eggs
    3. Resist local conditions, like low inputs/feed
    4. Others_________________________________
19. Do you have separate house for you poultry?
    1. Yes
    2. No
20. What is primary role of chicken production in your household?
    1. For sell to meet day to day financial need of the household.
    2. For consumption: 1. On holyday/festival 2. General
    3. Others_____________
21. What is primary role of egg production in your household?
    1. For sell to meet day to day financial need of the household.
    2. For consumption
    3. Hatching for reproduction
    4. Others________

22. Poultry and egg ownership, lost and sold

Poultry by Age and sex group and egg	Number currently owned	Current market value	No. lost during last 12 months due to diseases	No. lost during last 12 months due to predator /accidents	Number sold during last 12 months	Number consumed during last 12 months	Value of sold chickens/egg	Who owns it (A)
Hens								
Cocks								
Pullets								
Cockerels								
Young chickens								
Egg								
Total								

(A) 1-children, 2- adult-women, 3-adult-men, 4-youth

23. Role and responsibility of household members on poultry production and marketing: Identify key stages and then look at decision maker.

Activity	Responsibility (A)
Shelter construction	
Cleaning chicken house	
Provision of feed and water	
Purchasing of drugs	
Purchasing of replacement stock	

(A). 1=Adult men, 2= Adult women, 3= children

24. Poultry and egg selling place, target season, and reason by households

Type	Selling place (A)	Reason to choose these market place(B)	Main season at which you sell them(C)	Reason for sale (D)
Chicken/poultry				
Egg				

(A) 1=Neighbors, 2=Local collectors, 3=Near market, 4=Take to far markets 5= to restaurants 6= others
(B) 1=near home, 2= Good price, 3= Fear of diseases in big market, 4= Others
(C) 1= Christmas, 2= Easter, 3= Ramadan, 4= "Meskel", 5= new year, 6= dry season, 7= summer season, 8= any time need arise or the product available
(D) 1-To purchase food or other stuff, 2- to send children to school, 3-to cover hospital expense, 4- others specify_________

25. Poultry Income: Who decides, who keeps, who sells and what use

Type	Who decides to sell? (A)	Who takes to market? (A)	Who keeps income? (A)	How is income used? (B)
Chicken				
Egg				

(A). 1=Adult men, 2= Adult women, 3= children

(B). 1=Adult men, 2= Adult women, 3= children

Thank you!

Researchers' Initials ☐

Bird ID ☐

Date ☐

Camera number ☐

Latitude... **Longitude**...

1. Photograph bird id ☐ **2. Photograph of flock** ☐

Random selection of one cock and one hen, or two hens over 6 months;

3. Weight of basket....................................... **Weight of basket + bird**..

4. Bird details

Sex
Male ☐ Female ☐

Age.................
Known ☐ Estimated ☐

Origin
Homebred ☐ Directly from household in the same village ☐ Directly from household in a different village ☐ Purchased from market ☐

Length of time in flock (if not homebred)
< 1 month ☐ 1-3months ☐ 3-6months ☐ 6 -12 months ☐
>12 months ☐

Production (hens)
Laying ☐ Incubating eggs ☐ Brooding chicks ☐ Not in production ☐

Has this bird had any previous diseases? Yes ☐ No ☐

Has this bird had any treatment to treat or prevent disease? Yes ☐ No ☐

Other history? (Details of previous diseases, recent treatments, owner's comments on productivity)

☐

5. Photographs

Lateral ☐ Front ☐ Back ☐
Skin ☐ Detail of head ☐ Detail of leg ☐

6. Clinical Examination of Bird

i. Demeanor and appearance: Bright ☐ Quiet, depressed, ruffled ☐

ii. Breathing Normal ☐ Open-beak breathing ☐ Distressed, noisy ☐

iii. Neurological Normal ☐ Torticollis (twisted neck) ☐ Paralysis ☐

iv. Body condition score:				
(Circle one option)	**0**	**1**	**2**	**3**

Head

v. Discharge from eyes	Absent	☐	Present	☐		
vi. Discharge from nostrils	Absent	☐	Present	☐		
vii. Inside beak	Normal	☐	Lesions present	☐		
viii. Skin of face, comb, wattle	Normal	☐	Pale	☐	Swollen	☐
			Bruising	☐	Crusting scabs	☐

a. Head shape	Plain	☐	Crest	☐
b. Earlobe	Present	☐	Absent	☐

Feathers and skin

ix. Feathers	Normal	☐	Moulting	☐	Abnormal feather loss	☐
c. Feather type	Hard	☐	Soft	☐	Frizzle or curly	☐
					Silky or hookless	☐
d. Tail type	Normal	☐	Rumpless	☐	Henny feather	☐
					Long tail, non-moulting	☐

Under wings and side of keel

Body lice:	Total count of underside of **one** wing, including skin at base of flight feathers		....
	Timed count of:	One side of keel (30secs)	....

Back and rump

Body lice:	Timed count of:	Back (30secs)	....
		Rump (60secs)	....

Body lice:	Total count of underside of tail feathers	....

ix. Vent	Clean	☐	Soiled	☐

Any observations on any part of skin of body

x. Skin	Normal	☐	Bruising	☐	Nodules	☐
					Wounds	☐

Legs and feet

xii. Legs/feet	Normal	☐	Scaling	☐	Bruising	☐
			Wounds	☐	Joint swelling	☐

Scaly-leg Mites:	*0 – No visible signs*	☐
	1 – minor scaling (affecting bottom parts of legs only)	☐
	2 - large amount of scaling (whole leg infested).	☐

Legs and feet

e. Multiple spurs		Present ☐		Absent ☐
f. Hen spur	Vestigial ☐	Present ☐		Absent ☐
g. Polydactyl (additional toes)		Present ☐		Absent ☐
h. Heterodactyly (different number of toes on each foot)		Present ☐		Absent ☐
j.Syndactyly (webbed toes, two or more are fused together)		Present ☐		Absent ☐
k.Mottling on leg (uneven colour spots on leg)		Present ☐		Absent ☐

Other

l. Bantam type (dwarfism)	Present ☐	Absent ☐
Are there any other unusual morphological traits?	Yes ☐	No ☐

If yes, describe or note any other comments

Are there any other parasites? If yes, describe what and where from.

Collect and Label

Blood sample	1x Anticoag. tube ☐	FTA card ☐
Faecal sample (tick **one**)	From bird ☐	From environment ☐

Ectoparasites collected? yes no How many tubes?.................
(leg scrapings, feather ruffling, or freeze parasites with alcohol)

Have you taken additional photographs of this bird? yes no

Ring placed Yellow ☐
White ☐
Blue ☐ Ring Number ....

Body measurements [11]

Body part	Size (cm)	Body part	Size (cm)	Body part	Size (cm)
Comb width		Body length		Breast circum.	
Comb height		Back length		Shank length	
Wattle length		Keel length		Wingspan	

[11] *This question was part of an MSc student's survey on bird phenotypes, and was only included during the second season of sampling*

CH4D Follow-up Questionnaire for revisiting households	*Stick new bird label(s) here* ***only if bird(s) are re-sampled***
Date...	
Enumerator...	
Latitude...	*Longitude...*

Start by greeting the respondent in their language, and explain the following.

This questionnaire is a follow-up to the research study that you took part in 6 months ago, into the health problems of village poultry in rural Ethiopia. The study is conducted by International Livestock Research Institute, the Ethiopian Institute of Agricultural Research and the University of Liverpool in Great Britain. Please feel free to ask us if you would like more information or if there is anything that you do not understand.
We sampled 400 birds in several villages in this area and in another part of Ethiopia, and we are working on these samples to find out which the most important diseases in Habesha poultry are and why this affects some birds more than others. By doing this, we hope we can develop ways to reduce the problems with disease that will work in your village. We would like to find out what happened to the two of your birds that we sampled at our last visit. This will involve a few questions, which should take less than 10 minutes. We will record the answers on paper, and they will be stored in a safe place, where no-one else can see them. If you still have the birds, we would like to examine them again, and take another smaller blood sample. This will help us to see what has happened to your bird in the last few months. It is possible that your chicken could develop a bruise or an infection under the wing, where we take the blood sample, but the risk of this is very small. If you would like us to stop the examination or the sampling of your chickens at any time, you are free to tell us to stop without giving us a reason.
We would like to stress that you do not have to accept this invitation and should take part only if you want to. If you change your mind after you have answered the questions and would not like to be included in our study, we can destroy your answers or samples from your birds so they are not used. If you are worried for any reason, or have any problems with your birds after the sampling, please tell your Development Agent, who will contact us.

If the household has agreed to take part and for chickens to be sampled (if applicable), tick box. ☐

Part A

1. **Has there been an outbreak of disease in your flock since our last visit?** Yes...☐......**Go to Q.2**
   No....☐..**Go to Part B**

2. **Which month?...**

3. **What symptoms did the birds show?**

   Sudden death with no other signs....☐
   Paralysis/ loss of co-ordination........☐
   Depressed/ruffled...........................☐
   Diarrhoea..................☐
   Breathing problems...☐

   Other (please describe)..

4. **How many of your birds:**

Died	Had disease and recovered	Did not become sick

Part B

We would now like to ask some questions specifically about the birds we sampled at the last visit.

First bird **Ring colour**
Yellow........... ☐
White........... ☐
Blue.............. ☐

Ring Number...............................
(No number)......... ☐

1. Is the bird still in the flock?
Yes................ ☐ **................Complete a re-examination sheet**
No................. ☐ **....................................... Go to Question 2**

2. Why is the bird no longer in the flock?
Sold............................ ☐
Consumed.................. ☐
Culled[a]........................ ☐
Died due to disease[b]... ☐
Killed by a predator (witnessed)...................... ☐
Probably killed by a predator (not witnessed). ☐
Died due to an accident.................................. ☐
Missing for reason unknown........................... ☐

[a] **If it was culled, please describe why**...

[b] **If it died due to disease, what symptoms did it show?**
Sudden death with no other signs................. ☐
Paralysis/ loss of co-ordination...................... ☐
Depressed/ruffled... ☐
Diarrhoea............... ☐
Breathing problems.. ☐

Other(please describe)...

Second bird **Ring colour**
Yellow........... ☐
White........... ☐
Blue.............. ☐

Ring Number...............................
(No number)......... ☐

1. Is the bird still in the flock?
Yes................ ☐ **................Complete a re-examination sheet**
No................. ☐ **....................................... Go to Question 2**

2. Why is the bird no longer in the flock?
Sold............................ ☐
Consumed.................. ☐
Culled[a]........................ ☐
Died due to disease[b]... ☐
Killed by a predator (witnessed)...................... ☐
Probably killed by a predator (not witnessed). ☐
Died due to an accident.................................. ☐
Missing for reason unknown........................... ☐

[a] **If it was culled, please describe why**...

[b] **If it died due to disease, what symptoms did it show?**
Sudden death with no other signs................. ☐
Paralysis/ loss of co-ordination...................... ☐
Depressed/ruffled... ☐
Diarrhoea............... ☐
Breathing problems.. ☐

Other(please describe)...

CH4D - Re-examination Recording Sheet

Researchers' Initials ☐ **Bird ID** ☐

Date ☐ **Camera number** ☐

Latitude.. **Longitude..**

Ring identification: Yellow ☐ White ☐ Blue ☐

Ring Number ....

No number ☐

3. Weight of basket...................................... **Weight of basket + bird....................**

4. Bird details

Laying ☐ Incubating eggs ☐ Brooding chicks ☐ Not in production ☐

Has this bird had any diseases since last sampled?	Yes	☐	No	☐
Has this bird had any treatment to treat or prevent disease?	Yes	☐	No	☐

Please describe any disease, its severity, and treatment since last sampled

6. Clinical Examination of Bird

i. Demeanor and appearance:	Bright	☐	Quiet, depressed, ruffled	☐		
ii. Breathing	Normal	☐	Open-beak breathing	☐	Distressed,noisy	☐
iii. Neurological	Normal	☐	Torticollis (twisted neck)	☐	Paralysis	☐

iv. Body condition score: **(Circle one option)** 0 1 2 3

Head

v. Discharge from eyes	Absent	☐	Present	☐		
vi. Discharge from nostrils	Absent	☐	Present	☐		
vii. Inside beak	Normal	☐	Lesions present	☐		
viii. Skin of face, comb, wattle	Normal	☐	Pale	☐	Swollen	☐
			Bruising	☐	Crusting scabs	☐

Stick-tight Flea: Examination of face and wattles, total count

0	*none present*	☐
1	*1 to 10*	☐
2	*11 to 50*	☐
3	*over 50*	☐

Feathers and skin

ix. Feathers Normal ☐ Moulting ☐ Abnormal feather loss ☐

Under wings and side of keel

***Ticks**:* Examination and total count ....

Body lice: Total count of underside of **one** wing, including skin at base of flight feathers ....

Timed count of: One side of keel (30secs) ....

Back and rump

Body lice: Timed count of: Back (30secs) ....

Rump (60secs) ....

Body lice: Total count of underside of tail feathers ....

xi.Vent Clean ☐ Soiled ☐

Tropical Fowl Mites: vent region (an area approximately 6 cm in diameter immediately in front of the vent

0 *no mites visible,* ☐
1 *1–50 mites,* ☐
2 *51–500 mites,* ☐
3 *>500mites* ☐

Any observations on any part of skin of body

x. Skin Normal ☐ Bruising ☐ Nodules ☐ Wounds ☐

Legs and feet

xii. Legs/feet Normal ☐ Scaling ☐ Wounds ☐ Bruising ☐ Joint swelling ☐

***Scaly-leg Mites**:*

0 – No visible signs ☐
1 – minor scaling (affecting bottom parts of legs only) ☐
2 - large amount of scaling (whole leg infested). ☐

Any other comments

Collect and Label

Photographs (Side, front, back, close-up of head, close-up of feet) ☐

Blood sample 1x tubes ☐ 2x Smears ☐

Faecal sample (tick **one)** From bird ☐ From environment ☐

Have you taken additional photographs of this bird? Yes No

General Appendix B

Laboratory manuals

B.1 ELISA methods

B.2 HAI methods

B.3 Modified McMasters faecal egg count

CH4D laboratory method 2:

ELISA Assay for the detection of antibodies to *Salmonella* and Marek's disease virus

Updated November 2011.

CONTENTS

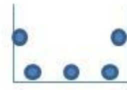
1. Introduction
2. Standard Indirect ELISA procedure
3. Checkerboard titrations for ELISA validation
4. Reagent preparation
5. Dilutions

Introduction

The key to all ELISA systems is the use of antibodies. These are proteins produced in animals in response to antigenic stimuli. Antibodies are specific chemicals that bind to the antigens used for their production; thus, they can be used to detect particular antigens if binding can be demonstrated. Conversely, specific antibodies can be measured by the use of defined antigens, and this forms the basis of many assays in diagnostic biology.

Indirect Elisa

1- Coat plate with antigen



4°C, overnight

Remove antigen

2- Block non-specific sites

37°C, 60mins

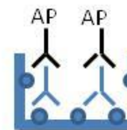
1 x wash

3- Add primary antiserum

37°C, 60mins

3 x washes

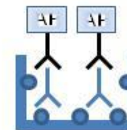
4- Add secondary enzyme linked antibody



37°C, 60mins

3 x washes

5- Add substrate



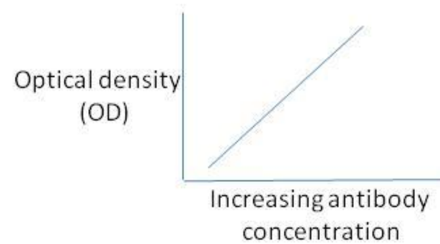
37°C, 15mins

7- Add stop solution

Colour change

8- Read plates spectrophotometrically

Primary antibody contains antigen specific antibodies



2 Standard ELISA procedure

	Antigen		Positive sera	Negative sera	Limits
Pasteurella[1]	Soluble lysate antigen	Diluted 1/80	Ovine PM vaccinated bird (EIAR)	Under validation	Under validation
Salmonella	Soluble lysate antigen	Diluted 1/80	Field serum Ethiopia	Field serum Ethiopia	Under validation
Marek's disease virus	Cell lysate from CV1988 infected CKC cells	Diluted 1/20	MDV vaccinated birds (EIAR)	Field serum Ethiopia	Under validation

ELISA Reagents	**Components**	**Per plate**
Coating buffer	Carbonate buffer pH9.6	10ml
Wash buffer (PBST)	PBS+ 0.05% Tween-20	150ml
Diluent	PBST + 3% skim milk	20ml
Substrate buffer	0.1M glycine buffer, pH 10.4, 1 mM MgCl2,1 mM ZnCl2	10ml
Substrate	Alkaline phosphatase yellow (pNPP) tablets dissolved in substrate buffer	2 tablets
Stop solution	3M NaOH	5ml
Primary antibody	Animal sera. Diluted 1:100 in diluents	variable
Secondary antibody	Anti-chicken IgG (stored at 4 degrees)	10ul

*** Ensure you have all reagents before proceeding.**

1. Add 100ul of antigen diluted in coating buffer to each well.
2. Cover with cling wrap and incubate at 4°C overnight in the fridge.
3. Remove the antigen. Bang the plate on absorbent material to ensure all liquid is removed.
4. Add 100ul of diluent, cover with cling wrap and incubate at 37°C for 1hr.
5. Remove diluent. Bang the plate on absorbent material to ensure all liquid is removed.
6. Add 100ul of pre-diluted serum (primary antibody) in duplicate (Diluted 1:100 in diluent).
7. Add 100ul of pre-diluted positive and negative control serum.
8. Cover with cling wrap and incubate at 37°C for 1hr.
9. Remove the primary antibody. Bang the plate on absorbent material to ensure all liquid is removed.
10. Wash wells three times with 200ul of PBST. Bang the plate on absorbent material to ensure all liquid is removed between each step.
11. Add 100ul of anti-Chicken IgY (secondary antibody) (Sigma A 9171) diluted 1:1000 in diluent.
12. Cover with cling wrap. Incubate at 37°C for 1hr.
13. Remove the secondary antibody. Bang the plate on absorbent material to ensure all liquid is removed.

14. Wash wells three times with 200ul of PBST. Bang the plate on absorbent material to ensure all liquid is removed between each step.
15. Add 100ul of Substrate. M**ake sure you add tablets to the buffer.**
16. Cover with cling wrap. Incubate at 37°C for 15 mins.
17. Add 50ul of stop solution.
18. Read plates at OD 405nm.

3 Checkerboard titration for Elisa validation

1. Add 200ul of antigen (pre-diluted in coating buffer or undiluted) to Columns 1 & 7
2. Add 100ul of coating buffer to columns 2-12 (not 7)
3. Perform two fold serial dilutions of the antigen in coating buffer by transferring 100ul from column 1 to column 2 and so on......Discard the 100ul for the last dilution in column 6. Repeat transferring 100ul from column 7 to column 8 and so on. Discard the 100ul for the last dilution in column 12
4. Incubate at 4°C overnight in the fridge
5. Remove the antigen
6. Add 100ul of Blocking solution and incubate at 37°C for 1hr
7. Remove blocking solution and wash wells once with 200ul of PBST
8. Add 200ul of prediluted serum (diluted 1:50) into row A
9. Add 100ul of diluent solution (PBST+3% Skim milk powder) into all remaining wells
10. Perform two fold serial dilutions of the antigen in coating buffer by transferring 100ul from row A to row B and so on......
11. Incubate at 37°C for 1hr
12. Remove the primary antibody and wash wells three times with 200ul of PBST (PBS +0.05% Tween-20)
13. Add 100ul of anti-Chicken IgY (Sigma A 9171) diluted 1:1000
14. Incubate at 37°C for 1hr
15. Remove the secondary antibody and wash wells three times with 200ul of PBST
16. Add 100ul of Substrate (Alkaline phosphatase yellow (pNPP) (Sigma A 3469)), incubate at 37°C for 15mins
17. Add 50ul of 3M NaOH to each well
18. Read plates at OD 405nm

Antigen Titration

	1	2	3	4	5	6	7	8	9	10	11	12
A	1/20	1/40	1/80	1/160	1/320	1/640	1/20	1/40	1/80	1/160	1/320	1/640
B	1/20	1/40	1/80	1/160	1/320	1/640	1/20	1/40	1/80	1/160	1/320	1/640
C	1/20	1/40	1/80	1/160	1/320	1/640	1/20	1/40	1/80	1/160	1/320	1/640
D	1/20	1/40	1/80	1/160	1/320	1/640	1/20	1/40	1/80	1/160	1/320	1/640
E	1/20	1/40	1/80	1/160	1/320	1/640	1/20	1/40	1/80	1/160	1/320	1/640
F	1/20	1/40	1/80	1/160	1/320	1/640	1/20	1/40	1/80	1/160	1/320	1/640
G	1/20	1/40	1/80	1/160	1/320	1/640	1/20	1/40	1/80	1/160	1/320	1/640
H	1/20	1/40	1/80	1/160	1/320	1/640	1/20	1/40	1/80	1/160	1/320	1/640

Antibody Titration (4 different samples. Sample 1 A:D 1-6)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1/50	1/50	1/50	1/50	1/50	1/50	1/50	1/50	1/50	1/50	1/50	1/50
B	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100
C	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200
D	1/400	1/400	1/400	1/400	1/400	1/400	1/400	1/400	1/400	1/400	1/400	1/400
E	1/50	1/50	1/50	1/50	1/50	1/50	1/50	1/50	1/50	1/50	1/50	1/50
F	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100
G	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200
H	1/400	1/400	1/400	1/400	1/400	1/400	1/400	1/400	1/400	1/400	1/400	1/400

4- Reagent preparation

Phosphate buffered saline (PBS)

		1 x Stock		10 x Stock	
		1000ml	500ml	1000ml	500ml
Sodium Chloride	NaCl	8g	4g	80g	40g
Potassium Chloride	KCl	0.2g	0.1g	2g	1g
Disodium hydrogen orthophosphate	Na_2HPO_4	1.44g	0.72g	14.4g	7.2g
Potassium dihydrogen phosphate	KH_2PO_4	0.24g	0.12g	2.4g	1.2g
Distilled water	dH_2O	800ml	400ml	800ml	400ml
Adjust the pH to 7.4					
Adjust to final volume		~200ml	~100ml	~200ml	~100ml

- Dissolve chemicals in 80% of the final volume of distilled water
- Adjust the pH to 7.4
- Adjust the volume to the final volume with distilled water
- Store at 4 °C and discard if microbial growth is detected.

* solutions can be sterilised using the pressure cooker for longer storage

TO MAKE PBS FROM A 10X STOCK- dilute 10x stock 1:10 with dH_2O
eg add 100ml of 10x stock to 900ml of dH_2O.

WASH BUFFER [PBST] (PBS + 0.05% Tween-20)

- Add 200ul of Tween-20 to 1L of PBS (20ul/100ml)
- Store at room temperature or 4 °C and discard if microbial growth is detected.

DILUENT (PBST+ 3% skim milk)

- Add 3g of skim milk powder to 100ml of PBST
- Store in the fridge for no more than 3 days

COATING BUFFER (carbonate buffer pH9.6)

		500ml	1000ml
Sodium bicarbonate	Na_2CO_3	2.65g	5.3g
Distilled water	dH_2O	400ml	800ml
Sodium	$NaHCO_3$	2.1g	4.2g
		pH to 9.6	
Adjust to final volume with dH_2O		~100ml	~200ml

- Dissolve Na_2CO_3 in the required volume of dH_2O (80% final vol.)
- Dissolve $NaHCO_3$ in the solution from step 1.
- pH to 9.60.
- Adjust the volume to the final volume with additional dH_2O
- Store at 4 °C and discard if microbial growth is detected.

* solutions can be sterilised using the pressure cooker for longer storage

SUBSTRATE substrate buffer
(0.1M glycine buffer, pH 10.4, 1 mM MgCl2,1 mM ZnCl2)

		500ml	1000ml
Glycine		3.75g	7.51g
Magnesium chloride	MgCl2	0.1g	0.20g
Zinc chloride	ZnCl2	0.06g	0.13g
Distilled water	dH_2O	400ml	800ml
		pH to 10.4	
Adjust to final volume with dH_2O		~100ml	~200ml

- Dissolve chemicals in the required volume of dH_2O (80% final vol.)
- Adjust pH to 10.40 (use solid NaOH pellets)
- Adjust the volume to the final volume with additional dH_2O
- Store at 4 °C and discard if microbial growth is detected.

* solution can be sterilised using the pressure cooker for longer storage

STOP solution (3M NaOH)

40g NaOH pellets per 100ml
Eg 120g NaOH pellets in 300ml distilled water

SUBSTRATE Alkaline phosphatase yellow (pNPP)

1. Remove the tablets 10 mins before making the solution
2. Add 1 tablet to 5ml of room temperature ELISA substrate buffer (2 tablets in 10ml)
3. Allow the tablet to dissolve prior to use
4. Use straight away and discard if solution turns yellow

5- Dilutions

How do i dilute the primary antiserum?

- I need to dilute the primary antiserum 1/100.

1/100 dilution of serum = 1 part serum in 99 parts of diluents
Eg 1 ul serum + 99ul of diluent; 5 ul serum in 495ul of diluent

- I need to dilute the primary antiserum 1/500.

1/500 dilution of serum=1 part serum in 499 parts of diluents
Eg 1 ul serum + 499ul of diluent; 2 ul serum in 998ul of diluent

How do i dilute the secondary antibody?

- I need to use a 1/1000 dilution of the secondary antibody.

1/1000 dilution of secondary antibody= 1 part secondary antibody in 999 parts of diluents
Eg 1 ul serum + 999ul of diluent; 10 ul serum in 9.990 ml of diluent

How do i dilute the coating antigen?

- I need to make 20mls of the coating antigen at a 1/80 dilution.

1/80dil. * 20ml= ml of antigen to add to 20mls (remember to subtract this volume)
Eg 1/80 * 20ml = 0.25ml (250ul) + 19.75 coating buffer

How do i make a 1 x PBS solution from a 10 x stock in 500ml?
Dilute the 10x stock 1:10
1/10 dilution of 10x stock = 1 part 10x stock plus 9 parts of diluents

Eg 50ml of 10x PBS + 450ml dH_20 or 100ml of 10x PBS + 900ml dH_2O

CH4D laboratory method 3:

Haemagglutination inhibition assay for the detection of antibodies to Newcastle disease virus.

Updated : November 2011

Reference : A basic laboratory manual for the small-scale production and testing of I-2 Newcastle disease vaccine. FAO Corporate repository http://www.fao.org/docrep/005/ac802e/ac802e0b.htm

CONTENTS

1- Introduction
2- Haemagglutination inhibition assay
3- Titration to establish haemagglutinin (HA) titre of a virus suspension
4- Preparation of Newcastle disease virus antigen for use in HI tests
5- Preparation of a 1% red blood cell solution
6- Re using micro titre plates

1. Introduction

All strains of Newcastle disease virus agglutinate chicken red blood cells. This is the result of the haemagglutinin part of the haemagglutinin/neuraminidase viral protein binding to receptors on the membrane of red blood cells. The linking together of the red blood cells, by the viral particles, results in clumping. This clumping is known as haemagglutination.

Haemagglutination is visible macroscopically and is the basis of haemagglutination tests to detect the presence of viral particles. Other viruses will also agglutinate chicken red blood cells. To demonstrate that the haemagglutinating agent is Newcastle disease virus, it is necessary to use a specific Newcastle disease virus antiserum to inhibit the haemagglutinating activity.

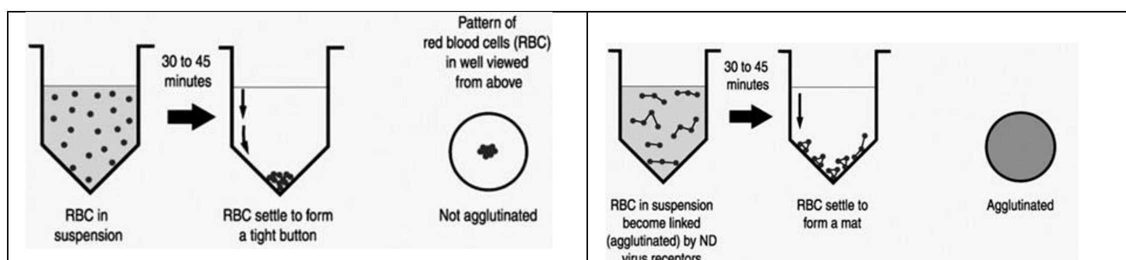


Figure 1 Haemagglutination of RBC by Newcastle disease virus

2. Haemagglutination inhibition assay to detected Newcastle antibodies in chicken serum

When serum containing antibodies against the haemagglutinin protein of Newcastle disease virus is mixed with Newcastle disease virus, the antibodies bind to the haemagglutinin protein in the envelope of the virus. This blocks the haemagglutinin protein from binding with the receptor site on chicken red blood cells. Thus the haemagglutination reaction between the virus and the red blood cells is inhibited. By performing two-fold serial dilutions on the serum prior to testing, the concentration of the serum antibodies can be expressed as an HI titre to the log base 2.

Materials

- Thawed serum samples in racks
- V-bottom microwell plates and covers
- PBS
- 1 percent washed red blood cells
- V-bottom reagent trough
- 50 µL single and multichannel pipettes and tips
- Microwell plate recording sheet.
- Newcastle disease virus antigen diluted to 4 HA units per 50 µL
- Standard positive and negative serum

Method

1. Fill in recording sheets to record how samples will be dispensed into microwell plates.
3. Dispense 50 µL of PBS into each well of the plates.

<u>Test serum</u>

4. Shake each serum sample and dispense 50 µL into the first well. Add 50uL of positive control sera and negative control sera in the last two rows respectively.
5. Use a multichannel pipette to make two-fold serial dilutions as required. Discard the last 50 µL. See Figure 2 for instructions on carrying out two-fold serial dilutions.
6. Add 50 µL of the 4HA dilution of antigen to each well, excluding the RBC control wells

<u>RBC control wells</u>

7. Add 50 µL of PBS (in place of virus) to all RBC control wells.

<u>Virus control and back titration</u>

8. Add 50 µL of the 4HA dilution of antigen to the Virus control neat well.
9. Dilute the 4HA dilution of antigen 1:2, 1:4, 1:8, 1:16 and 1:32 (two-fold dilutions)

This can be performed in tubes or wells of the plate. For 1:2 mix 100 µL of PBS with 100 µL of 4HA dilution of antigen. For 1:4 dilution, mix 100 µL of the 1:2 dilution with 100 µL of PBS............ etc

10. Add 50 µL of each dilutions (1:2, 1:4 etc) to the appropriate wells.

All samples
11. Gently tap the sides of the microwell plates to mix the reagents. Cover plates with a lid. Allow to stand for 30 minutes at room temperature.

12. Add 50 μL of 1 percent washed red blood cells to each well including the control wells.
13. Gently tap the sides of the microwell plates to mix the reagents. Allow to stand at room temperature for 45 minutes.

14. Read the settling patterns for each serum sample. Read the control serum well first then read the patterns in the other wells.

15. Record the pattern observed in each well on a microwell plate recording sheet. Determine the endpoint. This is the point where there is complete inhibition of haemagglutination (a button).

16. Record the antibody level for each serum sample. The antibody titre is the reciprocal of the last serum dilution to inhibit the agglutination.
Eg: if 1/16 dilution of serum is the last dilution where a button is seen (haemagglutination inhibition) that the titre is 16.

Plate layout test serum samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	1a	1b	1c	1d	1e	1f	9a	9b	9c	9d	9e	9f
B	2a	2b	2c	2d	2e	2f	10a	10b	10c	10d	10e	10f
C	3a	3b	3c	3d	3e	3f	11a	11b	11c	11d	11e	11f
D	4a	4b	4c	4d	4e	4f	12a	12b	12c	12d	12e	12f
E	5a	5b	5c	5d	5e	5f	13a	13b	13c	13d	13e	13f
F	6a	6b	6c	6d	6e	6f	14a	14b	14c	14d	14e	14f
G	7a	7b	7c	7d	7e	7f	+a	+b	+c	+d	+e	+f
H	8a	8b	8c	8d	8e	8f	-a	-b	-c	-d	-e	-f

Where a:2^1, b: 2^2, c: 2^3, d: 2^4, e: 2^5, f: 2^6 and samples 1:14 are tested.

2^1= 1:2 dilution (50% serum, 50% PBS)

PLATE LAYOUT CONTROL

	1	2	3	4	5	6	7	8	9	10	11	12
A	Neat	1:2	1:4	1:8	1:16	1:32	RBC	RBC	RBC	RBC	RBC	RBC

Virus control and back titration

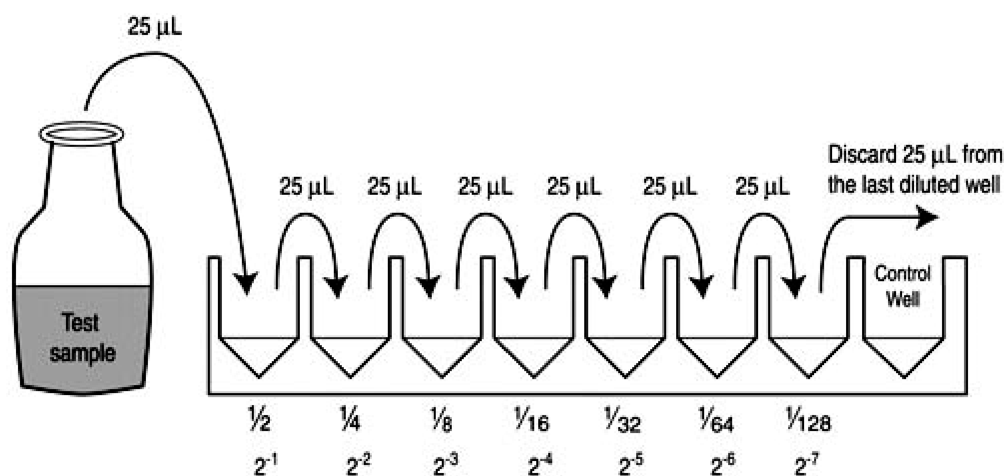
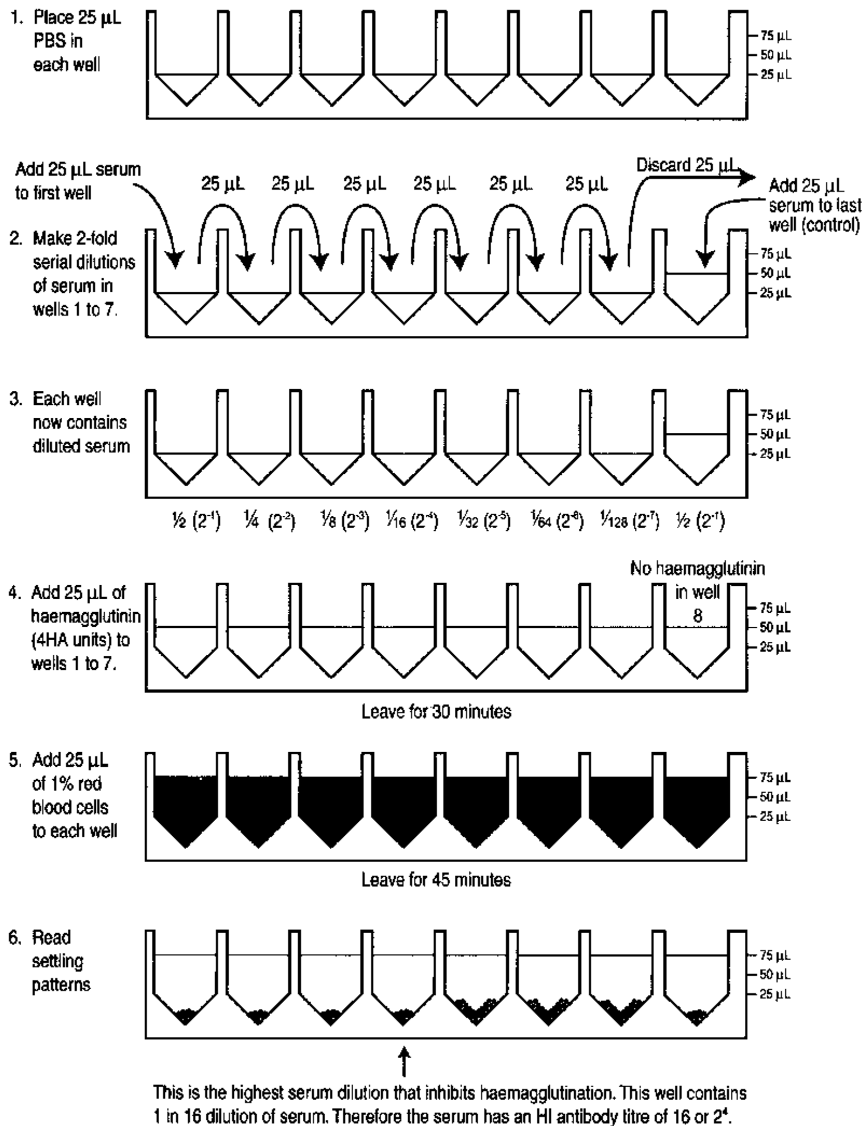


Figure 2 Two fold dilutions

(assay performed using 50ul)

Interpretation of results

- In the wells where antibodies are present there will be haemagglutination inhibition, the red blood cells will settle as a button.
- In the wells where antibodies are absent, the red blood cells will agglutinate.
- The end point of the titration is the well that shows complete haemagglutination inhibition. Sometimes it is not easy to determine. Look at the size of the button as an indication of the degree of haemagglutination inhibition. Use the control well as a point of comparison. Be consistent in determining the endpoint. The plates can be tilted. Compare the ‘tear drop shape of the RBC control well *no haemagglutination inhibition* to the test samples.
- The HI titre of the positive serum is no more than one dilution higher or lower than the assigned titre.
- Haemagglutination inhibition is not seen in the negative serum



* 50ul used instead of 25ul

3. Titration to establish haemagglutination (HA) titre of a virus suspension

The haemagglutination test is used to quantify the amount of Newcastle disease virus in a suspension. This is done by carrying out two-fold serial dilutions of the viral suspension in a microwell plate and then testing to determine an end point. This result can then be used to determine the amount of haemagglutinin in the suspension and is expressed as a HA titre.

Materials

- 96 well V-bottom microwell plate and cover
- 50 uL single and multi-channel micropipettes and tips
- PBS
- 1 percent chicken red blood cells
- Sample to be titrated
- Reagent troughs
- Microwell plate recording sheet.

Method

1. Record on recording sheets how samples will be dispensed in microwell plate.
2. Dispense 50 µL of PBS into each well of the microwell plate.
3. Place 50 µL of virus-containing solution (or solutions to test) in first well of each row of column 1. Samples can be tested in duplicate or triplicate if necessary.
4. Use a multichannel pipette to carry out two-fold serial dilutions across the plate until Column 11.
5. Add 50 µL of PBS to each well.
6. Add 50 µL of 1 percent red blood cells to each well including Column 12. The wells in this column are control wells that contain only PBS and red blood cells.
7. Gently tap sides of the plate to mix. Place a cover on the plate.
8. Allow the plate to stand for 45 minutes at room temperature.
9. Read and record the results in each well. All the control wells should be HA negative.
10. **HA negative:** A sharp button of red blood cells at the bottom of the V-bottom well.
11. **HA positive:** A hazy film of red blood cells, no button or a very a small button of red blood cells at the bottom of the V-bottom well.
12. Identify the end point. This will be the last well to show complete haemagglutination and contains one haemagglutinating unit.

Example plate layout (you may need to dilute up to 1/2048 to get an endpoint)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Neat	1:2	1:4	1:8	1:16	1:32	RBC	RBC	RBC	RBC	RBC	RBC

Definition of one HA unit

One HA unit in the haemagglutinin titration is the minimum amount of virus that will cause complete agglutination of the red blood cells. The last well that shows complete agglutination is the well that contains one HA unit.

Calculation of the HA titre of the test sample

The HA titre is the reciprocal of the dilution that produces one HA unit.
The endpoint titre is the last dilution of virus suspension which agglutinates the red blood cells. This dilution of the virus material contains.
For example: 1 HA unit.
The HA titre of the test sample is therefore the reciprocal of 1/64 = 64 = 2^6
The titre of the suspension of Newcastle disease virus can be expressed as 64 or 2^6 HA units in 50 uL.

4. Preparation of Newcastle disease virus antigen for use in HI tests

Antigen is prepared by inoculating embryonated eggs with a sample of Newcastle disease virus and harvesting the allantoic fluid four days later. A volume of 50 mL to 100 mL is adequate for a large number of tests. Centrifuge the sample at 1 200 *g* to clarify and remove any contaminating red blood cells. Store the antigen in one mL aliquots at -20°C.

Preparation of 4HA units of Newcastle disease virus antigen

The standard amount of Newcastle disease virus used in the haemagglutination inhibition (HI) test is 4HA units. It is necessary to prepare and test a suspension of Newcastle disease virus containing 4HA units in order to carry out the HI test. This involves a series of following steps.

1. Titrate the stored suspension of virus to be used as the antigen in the HI test.
2. Calculate the dilution factor required to produce 4 HA units. A simple way is to divide the HA titre by 4.
3. Apply the dilution factor and dilute the original suspension of antigen in PBS to produce an adequate volume of 4HA antigen to carry out the HI test. Allow 2.5 mL for each microwell plate.
4. Titrate the diluted (4HA) suspension of virus. This is a back titration to check the diluted antigen contains 4 HA units.
5. Read HA titre. It should equal 4HA units. If not adjust the dilution and titrate again.
6. Use the 4HA unit dilution of antigen in an HI test to test the standard positive and negative serum. The HI titre of the laboratory standard positive serum should equal the predetermined titre.

5. Preparation of a 1% red blood cell solution

Blood collection

Select chickens from a non-vaccinated stock (if available) otherwise from vaccinated chickens if that is all that is available. Collect blood from more than one chicken. A collection of 1.0 mL from each of three chickens will usually give between 8 to 10 mL of a 10 percent solution of washed red blood cells.

Materials

- Syringe and needle
- Ethanol to wipe wing vein
- Alsever’s solution.
- Sterile centrifuge tubes (15ml)
- Pipette.

1- Add 1ml of Alsever's (anticoagulant) solution to a syringe and collect 1ml from the wing vein of each of three birds.
2- Gently invert the syringe to mix the blood and anticoagulant.
3- After collecting the blood from all three chickens, remove the needles from the syringes and pool the blood samples in a sterile bottle with a lid.

Washing the red blood cells

After collection of the blood into an anticoagulant, the cells are washed and stored.

Materials

- Blood in anticoagulant
- DGV or PBS solution for washing.
- Sterile 15 mL centrifuge tube with a lid .
- Pasteur pipette or 10 mL graduated pipette with pipette filler.

Method

1. Transfer the blood to a container suitable for centrifugation.
2. Add PBS to fill the container. Mix gently.
3. Centrifuge at 500 *g* (~1000rpm) for 10 minutes.
4. Use a Pasteur pipette or a 10 mL glass pipette to remove the supernatant. Take care not to disturb the pellet of red blood cells.
5. Repeat Steps 2, 3, and 4 twice.

The cells have now formed a pellet after being washed three times and centrifuged.

Preparation of 10 percent red blood cells without using a micro-haematocrit centrifuge to measure packed cell volume (PCV)

1. Use a micropipette or glass pipette to remove one mL of the pellet of packed red blood cells after they have been washed as described above. Add 9 mL of sterile PBS to dilute to a 10 percent suspension.

2. Store the suspension at 4°C.

Note- As the cells settle under gravity, the storage solution should remain clear. Any red colour in the storage solution indicates haemolysis of the cells and the suspension is not suitable for use and should be discarded. Normally a 10 percent suspension in Alsever's can be kept for 2 -4 weeks at 4°C.

Alsever's Solution

Reagents

- Citric acid $C(OH)(COOH)(CH_2.COOH)_2.H_2O$ 0.055g
- Sodium Citrate $Na_3C_6H_5O_7.2H_2O$ 0.8g
- D-Glucose $C_6H_{12}O_6$ 2.05g
- Sodium chloride NaCl 0.42g
- Distilled water to make up to 100 mL

Method

1. Weigh out reagents into a conical flask.
2. Dissolve of distilled water and make up to100 mL.
3. Dispense into sterile 10 mL bottles.
4. Sterilize by autoclaving at 116°C for 10 minutes. Use slow exhaust.
5. Allow to cool, then tighten the lids and label the bottles.
6. Store in the refrigerator.

Reusing micro titre plates:

These plates cannot be autoclaved

(1) Disinfect by soaking overnight in a 2 percent chloride solution (Bleach).
(2) Wash and rinse three times in tap water
(3) Rinse three times in distilled water
(4) Dry, in the incubator and re use

CH4D Laboratory Protocols - Processing faecal samples: The Concentration McMaster Technique

1. Weigh out 3g of the faeces sample.
2. Add 42ml of tap water. (or 14ml for every 1g of faeces, if less than 3g is used)
3. Mix the faeces and the tap water thoroughly with a stirring device. (very hard lumps may need to soak for up to 30 minutes to break up, but this is not usually necessary)
4. Pour the faecal suspension through a tea strainer into a second container.
5. Transfer the faecal suspension into a test tube to the 10 ml mark.
6. Centrifuge the test tube for 7 minutes at 1200 RPM (revolutions per minute).
7. Remove the supernatant with a pipette, but be careful not to resuspend the sediment.
8. Add flotation fluid (stock: 1 litre water, 400g salt) up to the 4 ml mark.
9. Resuspend the sediment very careful, by sucking up and down in a Pasteur pipette several times, or using a whirlimix. Avoid making bubbles in the suspension, as these will make the egg counts less reliable.
10. Fill two McMaster counting chambers with the faecal suspension, immediately after resuspension of the sediment. Be careful to avoid air bubbles, and ensure the engraved side of the coverslip is in contact with the soloution.
11. Leave the filled McMaster chamber to rest on the table for 3-5 minutes before counting (minimum 3 minutes to allow all eggs to float, and maximum 10 minutes, as some eggs may be distorted in the flotation fluid).
12. Count the number of eggs in both counting fields and calculate the number of eggs per gram of faeces by multiplying the number of eggs by 20
13. After counting, the McMaster chamber should be washed under running tap water

Counting

1. Focus on the counting grid and count the different nematode eggs within the engraved area of both sides of the chamber. Use 10 x 10 magnification
2. When counting the engraved areas, the general rules for counting should be followed: all eggs inside the grid should be counted plus all eggs touching two sides of the grid (e.g. the upper and the left borderlines), while excluding all eggs touching the two other sides of the grid (e.g. the lower and the right borderlines).
3. Every type of nematode egg, cestode egg or coccidia oocyst should be counted separately.
4. Add the number of eggs in both chambers together, and multiply by 20 to give the number of eggs per gram.

NOTES

- Make sure the container is unambiguously labelled (disposable containers may be labelled in waterproof marking ink).
- After centrifuging and removing the supernatant, it is possible to interrupt the procedure by closing the tube and storing it in a refrigerator (approx. 4°C) for up to 7 days without any significant reduction in the egg counts. If many samples are to be handled simultaneously, this possibility for storage makes the laboratory work more flexible and rational, as 50-100 samples may be sieved and centrifuged in one step, thereafter they are stored until they are counted one by one.

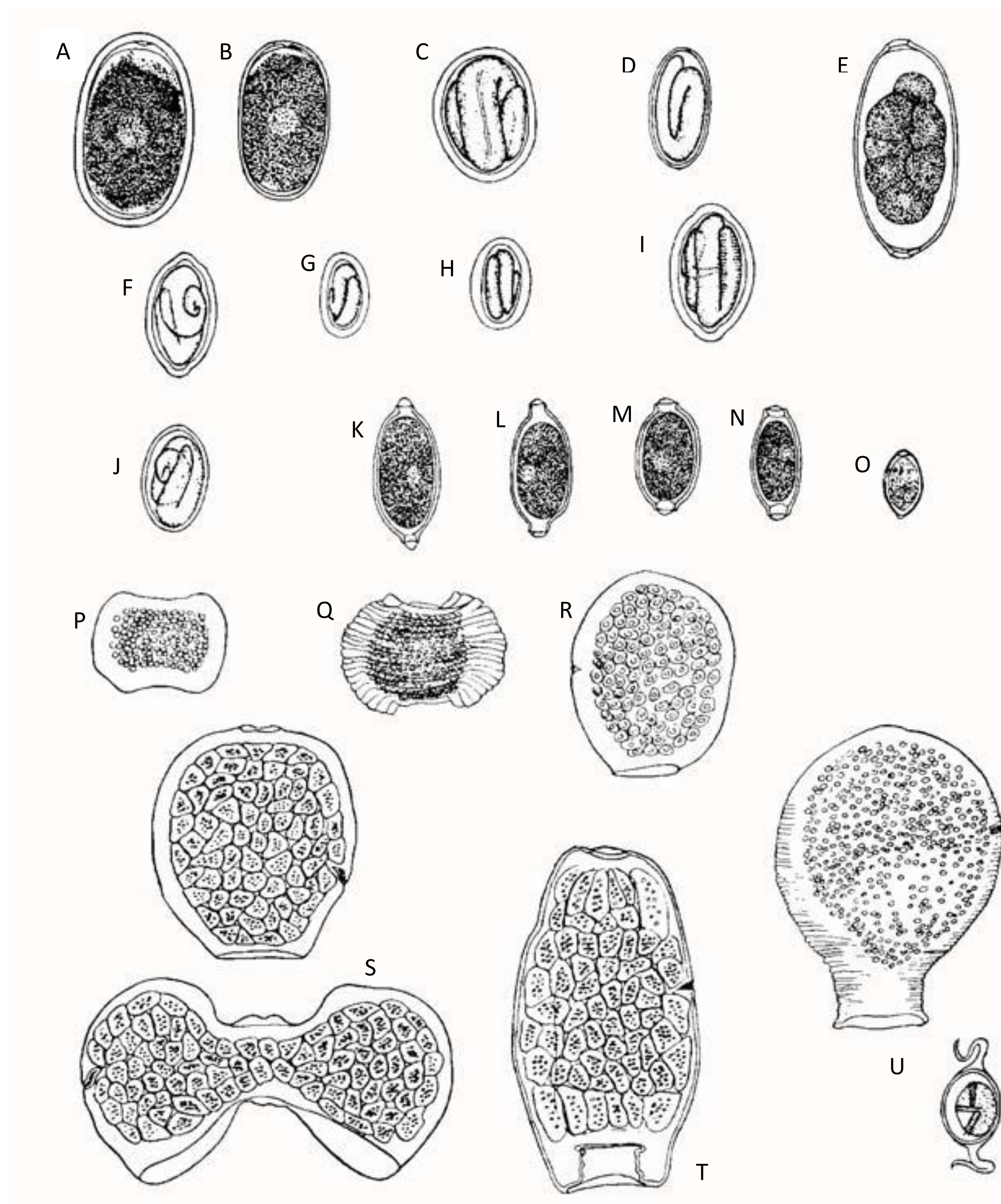


Figure 4.7 The most important helminth eggs and segments of cestodes. A: *Ascaridia galli* B: *Heterakis gallinarum* C: *Allodapa suctoria* D: *Strongyloides avium* E: *Syngamus trachea* F: *Tetrameres americana* G: *Acuaria* spp. H: *Acuaria hamulosa* I: *Gongylonema ingluvicola* J: *Oxyspirura mansoni* K: *Capillaria annulata* L: *Capillaria anatis* M: *Capillaria obsignata* N: *Capillaria contorta* O: *Prosthogonimus* spp. P - U: Segments of cestodes P: *Amoebotaenia cuneata* Q: *Hymenolepis carioca* R: *Raillietina cesticillus* S: *Raillietina echinobothrida* T: *Raillietina tetragona* U: Segment of *Choanotaenia infundibulum* and a single egg (Redrawn after Soulsby 1982).

Reference: Permin and Hanson (1998)

General Appendix C:

R code for figures and models

Chapter 2

```
#Figure 2.4 Distribution of IBD s:p ratios, showing the placement of
the cut-off, and the negative and positive controls

library(lme4)

setwd('M:/Documents/Poultry Disease/R stuff/Bird analysis/')
birds <- read.csv("basic bird.csv", header=T, sep=,)
ibd<-na.omit(birds[,c(1,61,62)])
y<-sort(birds$IBDV)

cutoff<-seq(min(y),max(y),0.005)
prev<-rep(0 ,length(cutoff))
res<- data.frame(cbind(cutoff,prev))
n<- length(cutoff)

 for(i in 1:n)
     {
    div<-ifelse(y>cutoff[i],1,0)
    pro<-prop.table(table(div))
    res$prev[i]<-pro[2]
    }

#select cut-off value u and determine prevalence v
    u<-0.306
   (v<-res[  which(abs( res$cutoff-u)==min(abs(res$cutoff-u))),2] )

plot(res$prev~res$cutoff, type="l",lwd=2,col="darkblue", xlab="s:p
ratio", ylab="Prevalence")
     x1<-c(u,u)
     y1<-c(-0.05,v)
     x2<-c(-0.5,u)
     y2<-c(v,v)

#cut-off line
      lines(x1,y1,lty=4, col="red", lwd=1.8)
      lines(x2,y2,lty=4, col="red", lwd=1.8)
      text(u,v+0.1,"cut-off value")
      text(u,v+0.05,"=0.306")

#positive and negative controls
(p<-res[  which(abs( res$cutoff-1)==min(abs(res$cutoff-1))),] )
        points(p[1],p[2], col="red", pch=16, cex=1.5)
(q<-res[  which(abs( res$cutoff-0)==min(abs(res$cutoff-0))),] )
         points(q[1],q[2], col="cyan", pch=16, cex=1.5)

legend(1,0.95,c("Positive control", "Negative
control"),cex=0.8,bty="n", pch=16,col=c("red","cyan"))

####################################################################
   # Figure 2.5 The change in prevalence with the alteration of the
cut-off value for the Salmonella data.

birds$Salmonella[birds$SalHigh==1]<-4.5
sal<-na.omit(birds[,c(1,67:70)])
y<-sort(sal$Salmonella)

cutoff<-seq(min(y),max(y),0.05)
prev<-rep(0 ,length(cutoff))
```

```
res<- data.frame(cbind(cutoff,prev))
for(i in 1: length(cutoff))
     {
    div<-ifelse(y>cutoff[i],1,0)
    pro<-prop.table(table(div))
    res$prev[i]<-pro[2]
    }

plot(res$prev~res$cutoff, type="l",lwd=2,col="darkblue", xlab="s:p
ratio", ylab="Prevalence")

#positive and negative controls
p<-  res[ which(abs( res$cutoff-1)==min(abs(res$cutoff-1))),]
points(p[1],p[2], col="red", pch=16, cex=1.2)
q<-  res[ which(abs( res$cutoff-0)==min(abs(res$cutoff-0))),]
points(q[1],q[2], col="cyan", pch=16, cex=1.2)

#choose sequence of cut-off values
uv<-c(0.3,0.5)

#plot chosen cut-offs against prevalence
       for (u in uv)
       {
       cufx<- rep(u,2)
       cufy<-c(par("usr")[3] ,res[which(abs( res$cutoff-
u)==min(abs(res$cutoff-u))),2] )
       lines( cufx,cufy,  type="l",lty=3, col="red", lwd=1)

       cofx<- c(par("usr")[1] ,u)
       cofy<- rep(res[which(abs( res$cutoff-u)==min(abs(res$cutoff-
u))),2],2)
       lines( cofx,cofy,  type="l",lty=3, col="red", lwd=1)
       }

legend(14.7,0.97,c("Positive control", "Negative
control"),cex=0.8,bty="n", pch=16,col=c("red","cyan"))

###################################################
#Figure 2.6 Sample OD values and s:p ratios for each plate for the
IBD ELISA

library(lattice)
library(latticeExtra)
setwd('M:/Documents/Poultry Disease/R Stuff/Serology/IBDV')
ibd<-read.csv("ibdfulltests.csv", header=T, sep=,)

n<-nrow(ibd)
ibd$ netOD<- ibd$MeanOD - ibd$Neg
ibd$netpos<-      ibd$Pos - ibd$Neg
ibd$ratio<- ibd$netOD/ibd$netpos
ibd$posrat<-rep(1,n)
ibd$negrat<-rep(0,n)

windows(20,10)
xyplot(ibd$ratio~ibd$MeanOD|ibd$PLATE.ID, pch=19, col="grey40",
xlab="Mean OD values", ylab="S:P ratio")   +
as.layer(xyplot(ibd$posrat~ibd$Pos|ibd$PLATE.ID, pch=19,
col="orangered" )) +
as.layer(xyplot(ibd$negrat~ibd$Neg|ibd$PLATE.ID, pch=19, col="cyan"
))
############################
```

```
#Figure 2.7  Values for the Pasteurella s:p ratios before and after
random-effect modelling to adjust for between-plate variation

pas<-na.omit(birds[,c(1,64:66)])
      (m3<-glmer(Pasteurella ~ 1 + (1|PMPlate), data=pas,REML = T,
na.action=na.omit, family="gaussian"))
      pas$Resid<-residuals(m3)
      birds$AdjPM<-pas$Resid[match(birds$UniqueID,pas$UniqueID)]

windows(20,10)
par(mfrow=c(2,2))
barplot(pas$Pasteurella, ylab="Raw", cex.lab=1.5)
hist(pas$Pasteurella,breaks=100, main="",xlab="",ylab="",
col="steelblue3")
barplot(pas$AdjPM, ylab="Plate-adjusted",cex.lab=1.5)
hist(pas$AdjPM,breaks=50, main="",xlab="",ylab="", col="steelblue3")
```

Chapter 3

```
#Figure 3.1 Farmer income derived from poultry compared to total
annual household income

setwd('M:/Documents/Poultry Disease/R stuff/HH analysis/')
sedata <- read.csv("socioeconomic data.csv", header=T, sep=,)

sedata$lv.inc.prop   <- 100*sedata$LivestockIncome/
sedata$TOTAlincome
sedata$ck.inc.prop   <- 100*sedata$PoultryIncome/ sedata$TOTAlincome
sedata$cp.inc.prop   <- 100*sedata$CropNetincome/ sedata$TOTAlincome
sedata$ot.inc.prop   <- 100*sedata$Otherincome/ sedata$TOTAlincome
sedata$incperhen<- sedata$PoultryIncome/(sedata$Hen + sedata$Cock)
sedata$a<-sqrt(sedata$incperhen/pi)
b<-c(50,100,200)
ba<-sqrt(b/pi)
fba<-0.1+0.25*ba

windows(12,8)

plot (sedata[which(sedata$RegionID=="H"),130]~
sedata[which(sedata$RegionID=="H"),96], xlim=c(0,35000), pch=19,
cex= 0.1+ 0.25*sedata[which(sedata$RegionID=="H"),134],col="red",
ylab="percentage of income contributed by poultry", xlab="Total
annual income (ETB)")

points (sedata[which(sedata$RegionID=="J"),130]~
sedata[which(sedata$RegionID=="J"),96],  pch=19, cex= 0.1+
0.25*sedata[which(sedata$RegionID=="J"),134],col="blue")

legend(35000,27,c("Horro","Jarso","","50 ETB", "100 ETB","200 ETB"),
pch=19, col=c("red","blue","white",rep("black",3)),
cex=1.5,pt.cex=c(1.5,1.5,0.1,fba),title.adj=1,xjust=1,bty="n",title=
"")

########################################
#Figure 3.2 Purposes of chicken and egg production in the two study
regions

#install.packages(VennDiagram)
library("VennDiagram")

draw.pairwise.venn(181,69,62,category=c("Sale","Consumption"),fill=c
(rgb(1,0,0,1),rgb(1,0,0,0.5)),

cat.just=c(list(c(0.5,1)),list(c(1,1))),cat.fontfamily=rep("sans",2)
,fontfamily=rep("sans",3), cex=2, cat.cex=1.8,cat.dist=-0.05,
ext.length=0.9)

draw.pairwise.venn(175,35,21,category=c("Sale","Consumption"),fill=c
(rgb(0,0,1,1),rgb(0,0,1,0.5)),

cat.just=c(list(c(0.5,1)),list(c(0.7,0.1))),cat.fontfamily=rep("sans
",2),fontfamily=rep("sans",3), cex=2, cat.cex=1.8,cat.dist=-0.05,
ext.length=0.9)
```

```
# Triple Venn diagrams were drawn by hand, as the programme cannot
handle 0 values
#####################################
#Figure 3.3  Farmer preferences for the time of year in which to
hatch eggs in Horro and Jarso regions

setwd('M:/Documents/Poultry Disease/R Stuff/HH analysis')
hhs<-read.csv("hhs.csv", header=T, sep=,)
  eggset<-na.omit( hhs[,c(1,3,4,5,6,54:65)] )
  reg<-table(eggset$RegionID)
  egg<- aggregate(eggset[,c(6:17)],
by=list(Reg=eggset$RegionID),sum)
  eggs<-as.data.frame(t(egg[,-1]))
  mo<-1:12
eggs<- data.frame(mo,eggs)
eggs$H.prop<- 100* eggs$V1 / rep( reg[1], length(eggs$V1))
eggs$J.prop<- 100* eggs$V2 / rep( reg[2], length(eggs$V2))

monames<-
c("Jan","Feb","Mar","Apr","May","Jun","Jul","Aug","Sep","Oct","Nov",
"Dec")

windows(10,5)
plot(eggs$mo,eggs$H.prop, type="l", xaxt="n", xlab="Month",
ylab="Percentage of Households",  col="red", lwd=2 )

points(eggs$mo,eggs$J.prop,col="blue",  type="l", lwd=2)
 axis(1,at=c(1:12),labels=monames)

legend("bottomright", c("Horro", "Jarso"), col=c("red", "blue"),
lwd=1,  bty="n")

#####################################################
#Figure 3.4 Farmers' estimates of hen reproductive parameters in
each region

setwd('M:/Documents/Poultry Disease/R stuff/HH analysis/')
eggs<-  read.csv("hhsseason2esk.csv", header=T, sep=,)

windows(12,4)
par(mfrow=c(1,4))
plot(eggs$RegionID,eggs$ClutchNo, ylab="Number of clutches per
year", col=c("red","blue"), names=c("Horro","Jarso"), cex.lab=1.8,
cex.axis=1.5)

plot(eggs$RegionID,eggs$CluLeng,ylab="Average length of clutch
(wks)",
col=c("red","blue"),names=c("Horro","Jarso"),cex.lab=1.8,cex.axis=1.
5)

plot(eggs$RegionID,eggs$InterClu,ylab="Average inter-clutch length
(wks)",
col=c("red","blue"),names=c("Horro","Jarso"),cex.lab=1.8,cex.axis=1.
5)

plot(eggs$RegionID,eggs$EggsClu,ylab="Average number of eggs per
clutch",
col=c("red","blue"),names=c("Horro","Jarso"),cex.lab=1.8,cex.axis=1.
5)
```

```
##################################################
#Figure 3.5  Production status of sampled hens by village and season
of sampling

setwd('M:/Documents/Poultry Disease/R stuff/Bird analysis/')
birds <- read.csv("basic bird.csv", header=T, sep=,)
x<- 17

(oba<-table
(birds[birds$VillageID=="H1A",x],birds[birds$VillageID=="H1A",9]) )
obp<-prop.table(oba[1:4,],2)
(obb<-table
(birds[birds$VillageID=="H1B",x],birds[birds$VillageID=="H1B",9]) )
obbp<-prop.table(obb[1:4,],2)
(obc<-table
(birds[birds$VillageID=="H2A",x],birds[birds$VillageID=="H2A",9]) )
obcp<-prop.table(obc[1:4,],2)
(obd<-table
(birds[birds$VillageID=="H2B",x],birds[birds$VillageID=="H2B",9]) )
obdp<-prop.table(obd[1:4,],2)

 (obe<-table
(birds[birds$VillageID=="J1A",x],birds[birds$VillageID=="J1A",9]) )
obep<-prop.table(obe[1:4,],2)
(obf<-table
(birds[birds$VillageID=="J1B",x],birds[birds$VillageID=="J1B",9]) )
obfp<-prop.table(obf[1:4,],2)
(obg<-table
(birds[birds$VillageID=="J2A",x],birds[birds$VillageID=="J2A",9]) )
obgp<-prop.table(obg[1:4,],2)
(obh<-table
(birds[birds$VillageID=="J2B",x],birds[birds$VillageID=="J2B",9]) )
obhp<-prop.table(obh[1:4,],2)

cols<- c( "lightsteelblue","steelblue" ,"steelblue4","firebrick")
windows(20,10)

par(fig=c(0,0.15,0,0.85))
oba<-barplot (obp[c(3,2,1,4),], col=cols,xaxt="n", ylab=
"Proportion", sub="H1A")
text( oba,par("usr")[3]-0.025 ,labels=levels(birds$SeasonID), xpd=T)
par(fig=c(0.1,0.25,0,0.85),new=T)

oba<-barplot (obbp[c(3,2,1,4),], col=cols, sub="H1B",xaxt="n",
yaxt="n", ylab= "")
text( oba,par("usr")[3]-0.025 ,labels=levels(birds$SeasonID), xpd=T)

par(fig=c(0.25,0.4,0,0.85),new=T)
barplot (obcp[c(3,2,1,4),], col=cols,sub="H2A", xaxt="n",yaxt="n",
ylab= "")
text( oba,par("usr")[3]-0.025 ,labels=levels(birds$SeasonID), xpd=T)

par(fig=c(0.35,0.5,0,0.85),new=T)
barplot (obdp[c(3,2,1,4),], col=cols, sub="H2B", xlab=
"",xaxt="n",yaxt="n", ylab= "")
text( oba,par("usr")[3]-0.025 ,labels=levels(birds$SeasonID), xpd=T)

par(fig=c(0.5,0.65,0,0.85), new=T)
barplot (obep[c(3,2,1,4),], col=cols,sub="J1A", xlab=
"",xaxt="n",yaxt="n", ylab= "")
```

```
text( oba,par("usr")[3]-0.025 ,labels=levels(birds$SeasonID), xpd=T)

par(fig=c(0.6,0.75,0,0.85),new=T)
oba<-barplot (obfp[c(3,2,1,4),], col=cols, sub="J1B",xaxt="n",
yaxt="n", ylab= "")
text( oba,par("usr")[3]-0.025 ,labels=levels(birds$SeasonID), xpd=T)

par(fig=c(0.75,0.9,0,0.85),new=T)
barplot (obgp[c(3,2,1,4),], col=cols,sub="J2A", xaxt="n",yaxt="n",
ylab= "")
text( oba,par("usr")[3]-0.025 ,labels=levels(birds$SeasonID), xpd=T)
par(fig=c(0.85,1,0,0.85),new=T)
barplot (obhp[c(3,2,1,4),], col=cols, sub="J2B", xlab=
"",xaxt="n",yaxt="n", ylab= "")
text( oba,par("usr")[3]-0.025 ,labels=levels(birds$SeasonID), xpd=T)

par(fig=c(0,1,0,1), mar=c(0,0,0,0))
   legend(3,1.1, c("Laying", "Incubating eggs","Rearing chicks","Not
in production"), fill=c( "lightsteelblue","steelblue" ,
"steelblue4", "firebrick"), ncol=2, bty="n" , cex=1.6)

###################################################
#Figure 3.6  Distribution of household flock sizes in Horro and
Jarso

setwd('M:/Documents/Poultry Disease/R Stuff/HH analysis')
hhs<-read.csv("hhs.csv", header=T, sep=,)
hhs$Flock<-rowSums(hhs[,c(18:22)])

jres<-matrix(0,5,45)
N<-c(1:45)

for (i in 1:45)
  {
  B<- hhs[which(hhs$RegionID=="J" & hhs$Flock==i),c(18:22)]
  jres[,i]<-mean(B)
  N[i]<-nrow(B)
  }

jres[,which(jres[1,]=="NaN")] <-0
V<-colSums(jres)
jres2<-prop.table(jres,2)

for (i in 1:45)
  {
   jres2[ ,i]<- jres2[ ,i]*N[i]
   }

hres<-matrix(0,5,45)
M<-1:45

for (i in 1:45)
  {
  C<- hhs[which(hhs$RegionID=="H" & hhs$Flock==i),c(18:22)]
  hres[,i]<-mean(C)
  M[i]<-nrow(C)
  }

hres[,which(hres[1,]=="NaN")] <-0
V<-colSums(hres)
hres2<-prop.table(hres,2)
```

```
for (i in 1:45)
  {
   hres2[ ,i]<- hres2[ ,i]*M[i]
   }

cols<-c("darkred","orangered","gold","lightblue","steelblue3")

windows(20,12)
par(fig=c(0,1,0.62,1),mar=c(0,5,2,4),oma=c(5,0,0,0))
barplot(hres2,main="", col=cols,ylab="Horro", cex.lab=1.5,xaxt="n",
xlab="", ylim=c(0,40), space=0)
legend("topright",  names(B), bty="n", fill=cols, cex=1.2)
par(fig=c(0,1,0,0.58) , new=T,mar=c(2,5,0,4))
barplot(jres2,main="", col=cols,ylab="Jarso",
cex.lab=1.5,xlab="",ylim=c(0,60),space=0,cex.names=0.8,
names.arg=c(1:45), axis.lty=1)

###############################################
 #Figure 3.7  Age distributions of females and males sampled in each
region

setwd('M:/Documents/Poultry Disease/R stuff/Bird analysis/')
birds <- read.csv("basic bird.csv", header=T, sep=,)
  labs<-c("Females","Males","Females","Males")
  windows(9,6)
  boxplot(birds$Age~ birds$Sex*birds$Region, col=c("red", "red",
"blue","blue"), ylab="Age (months)",names=labs )
  legend("topright",c("Horro","Jarso"),fill=c( "red", "blue"),
bty="n")

#####################################################
  #Figure 3.8  Plots of (a) weight and (b) wattle length against age
for females and males in different regions

setwd('M:/Documents/Poultry Disease/R stuff/Bird analysis/')
birds <- read.csv("basic bird.csv", header=T, sep=,)
setwd('M:/Documents/Poultry Disease/R stuff/Bird analysis/')
Esk <- read.csv("Esk.csv", header=T, sep=,)

windows(15,12)
par(fig=c(0,0.4,0.5,1))
plot( birds[which(birds$RegionID=="H" & birds$Sex=="F"),20]
~birds[which(birds$RegionID=="H" & birds$Sex=="F"),14],
pch=4,col="red",cex.lab=1.3, ylab="Weight (kg)", xlab="Age
(months)",xlim=c(0,72), ylim=c(0,3), main="Females")
abline(lm( birds[which(birds$RegionID=="H" & birds$Sex=="F"),20]
~birds[which(birds$RegionID=="H" & birds$Sex=="F"),14]),col="red")
points( birds[which(birds$RegionID=="J" & birds$Sex=="F"),20] ~birds
[which(birds$RegionID=="J" & birds$Sex=="F"),14],
pch=4,col="steelblue1")
abline(lm( birds[which(birds$RegionID=="J" & birds$Sex=="F"),20]
~birds[which(birds$RegionID=="J" &
birds$Sex=="F"),14]),col="steelblue1")

par(fig=c(0.4,0.8,0.5,1), new=T)
plot( birds[which(birds$RegionID=="H" & birds$Sex=="M"),20]
~birds[which(birds$RegionID=="H" & birds$Sex=="M"),14],
pch=4,col="darkred",cex.lab=1.3,ylab="Weight (kg)", xlab="Age
(months)", xlim=c(0,72), ylim=c(0,3), main="Males")
```

```
abline(lm( birds[which(birds$RegionID=="H" & birds$Sex=="M"),20]
~birds[which(birds$RegionID=="H" &
birds$Sex=="M"),14]),col="darkred")
points(birds[which(birds$RegionID=="J" & birds$Sex=="M"),20] ~birds
[which(birds$RegionID=="J"&birds$Sex=="M"),14],
pch=4,col="steelblue3")
abline(lm( birds[which(birds$RegionID=="J" & birds$Sex=="M"),20]
~birds[which(birds$RegionID=="J" &
birds$Sex=="M"),14]),col="steelblue3")

par(fig=c(0,0.4,0,0.5), new=T)
plot( Esk[which(Esk$RegionID=="H" & Esk$Sex=="F"),36]
~Esk[which(Esk$RegionID=="H" & Esk$Sex=="F"),9],
pch=19,col="red",cex.lab=1.3,ylab="Wattle length (cm)",
xlab="Age",xlim=c(0,72), ylim=c(0,8), main="")
abline(lm( Esk[which(Esk$RegionID=="H" & Esk$Sex=="F"),36]
~Esk[which(Esk$RegionID=="H" & Esk$Sex=="F"),9]),col="red")
points( Esk[which(Esk$RegionID=="J" & Esk$Sex=="F"),36]
~Esk[which(Esk$RegionID=="J" & Esk$Sex=="F"),9],
pch=19,col="steelblue1",ylab="Wattle length", xlab="Age (months)")
abline(lm( Esk[which(Esk$RegionID=="J" & Esk$Sex=="F"),36]
~Esk[which(Esk$RegionID=="J" & Esk$Sex=="F"),9]),col="steelblue1")


par(fig=c(0.4,0.8,0,0.5), new=T)
plot( Esk[which(Esk$RegionID=="H" & Esk$Sex=="M"),36]
~Esk[which(Esk$RegionID=="H" & Esk$Sex=="M"),9],
pch=19,col="darkred",cex.lab=1.3,ylab="Wattle length (cm)",
xlab="Age (months)", xlim=c(0,72), ylim=c(0,8), main="")
abline(lm( Esk[which(Esk$RegionID=="H" &
Esk$Sex=="M"),36]~Esk[which(Esk$RegionID=="H" & Esk$Sex=="M"),9])
,col="darkred")
points( Esk[which(Esk$RegionID=="J" & Esk$Sex=="M"),36]
~Esk[which(Esk$RegionID=="J" & Esk$Sex=="M"),9],
pch=19,col="steelblue3")
abline(lm( Esk[which(Esk$RegionID=="J" &
Esk$Sex=="M"),36]~Esk[which(Esk$RegionID=="J" & Esk$Sex=="M"),9])
,col="steelblue3")

par(fig=c(0.8,1,0,1), mar=c(2,0,2,0),xpd=T)
legend( 5,0.5,c("Horro", "Jarso" ), pch=19, col=c("darkred",
"steelblue"), bty="n", cex=1.6)

###############################
#Figure 3.9  Proportion of farmers who confine birds in each month
of the year

setwd('M:/Documents/Poultry Disease/R Stuff/HH analysis')
hhs<-read.csv("hhs.csv", header=T, sep=,)
res<-matrix(0,12,8)
for(i in 36:47)
 {
 z<-prop.table( table(hhs[,i],hhs$VillageID),2)
 res[i-35,]<-z[2,]
 }
mo<-1:12
res<-cbind(mo,res)
cols<-
c("red","red","darkred","darkred","blue","blue","steelblue1","steelb
lue1")
lin<-c(  1,6,1,6,1,6,1,6)
```

```
windows(12,8)
plot(res[,1],res[,2], type="l", xaxt="n", xlab="Month",
ylim=c(0,0.5),ylab="Proportion of Households", main="", col="red",
lwd=2 )
for(i in 3:9)
 {
 points(res[,1],res[,i],col=cols[i-1],  type="l", lwd=2, lty=lin[i-
1])
 }

axis(1,at=c(1:12),labels=monames)
legend(11,0.5,c("H1", "H2","J1","J2"), col=cols[c(1,3,5,7)], lwd=1,
title="Marketshed", bty="n"  )
legend(11,0.38,c("A", "B"), lty=c(1,6), lwd=1, title="Village",
bty="n"  )

########################################
#Figure 3.10   Farmers' estimates of the amount of time their
chickens spend mixing with those from other households
setwd('M:/Documents/Poultry Disease/R Stuff/HH analysis')
hhs<-read.csv("hhs.csv", header=T, sep=,)
contacts<-na.omit(hhs[,c(1,3:6,48,49)])
drys<-prop.table(table(contacts$DryConta,
contacts$VillageID)[1:6,],2)
wets<-prop.table(table(contacts$WetConta,
contacts$VillageID)[1:6,],2)
pal<-c( "steelblue3","lightblue","yellow3", "gold3"
,"darkorange3","firebrick3")

windows(18,12)
 par(fig=c(0,0.5,0.2,1) )
 barplot (drys[c(1:6),], beside=F, ylim=c(0,1), main="Dry Season",
col=pal)
 par(fig=c(0.5,1,0.2,1), new=T)
 barplot (wets[c(1:6),], beside=F, ylim=c(0,1), main="Wet Season",
col=pal)
 par(fig=c(0,1,0,0.25), mar=c(0,5,0,5))
 legend("top", c("No mixing", "Rarely","A few times a week for less
than one hour", "A few times a week for more than one hour",
    "Every day for less than one hour", "Every day for more than one
hour"),cex=1.4,bty="n", ncol=2, fill=pal)

 ########################################
#Figure 3.11 and 3.12 Distribution of flock sizes for different
sampling seasons

setwd('M:/Documents/Poultry Disease/R Stuff/HH analysis')
hhs<-read.csv("hhs.csv", header=T, sep=,)
hhs$Flock<-rowSums(hhs[,c(18:22)])
 S<-levels(hhs$SeasonID)
Time<-c("May 2011", "Oct 2011", "May 2012", "Oct 2012")
cols<-c("darkred","orangered","gold","lightblue","steelblue3")
posx<-
matrix(c(0.1,0.55,0.55,1,0.55,1,0.55,1,0.1,0.55,0.1,0.55,0.55,1,
0.1, 0.55) ,4,4)
hres<-matrix(0,5,45)
    M<-1:45

windows(20,12)
par(mfrow=c(2,2))
```

```
for(j in 1:4)
 {
  for (i in 1:45)
  {
  B<- hhs[which(hhs$RegionID=="H" & hhs$SeasonID==S[j] &
hhs$Flock==i),c(18:22)]
  hres[,i]<-mean(B)
  M[i]<-nrow(B)
  }

hres2<-prop.table(hres,2)
for (i in 1:45)
  {
   hres2[ ,i]<- (hres2[ ,i]*M[i]) /sum(M)
   }

par(fig=posx[,j], new=T)
barplot(hres2,main="", col=cols,ylab="",  sub=Time[j],xlab="",
ylim=c(0,0.3), space=0,cex.names=0.8, names.arg=c(1:45), axis.lty=1)
   }

par(fig=c(0.08,1,0,1), new=T)
plot(hhs$Flock,type="n",axes=F, xlab="Flock size", ylab="Proportion
of flocks sampled", cex.lab=1.8)
legend("topright",  names(B), bty="n", fill=cols, cex=1.2)

N<-c(1:45)
jres<-matrix(0,5,45)

windows(20,12)
par(mfrow=c(2,2))

for(j in 1:4)
 {
  for (i in 1:45)
  {
  B<- hhs[which(hhs$RegionID=="J" & hhs$SeasonID==S[j] &
hhs$Flock==i),c(18:22)]
  jres[,i]<-mean(B)
  N[i]<-nrow(B)
  }

jres2<-prop.table(jres,2)
for (i in 1:45)
  {
   jres2[ ,i]<- (jres2[ ,i]*N[i]) /sum(N)
   }

par(fig=posx[,j], new=T)
barplot(jres2,main="", col=cols,ylab="",
sub=Time[j],xlab="",ylim=c(0,0.3),space=0,cex.names=0.8,
names.arg=c(1:45), axis.lty=1)
    }

par(fig=c(0.08,1,0,1), new=T)
plot(hhs$Flock,type="n",axes=F, xlab="Flock size", ylab="Proportion
of flocks sampled", cex.lab=1.8)
legend("topright",  names(B), bty="n", fill=cols, cex=1.2)


#############
```

```
#Appendix 3.3 and 3.4
setwd('M:/Documents/Poultry Disease/R stuff/Bird analysis/')
birds <- read.csv("basic bird.csv", header=T, sep=,)
 m4<-glmer(  Age~ RegionID*relevel(Sex,"M")+ (1|FarmUnID),
data=birds,REML=T,  na.action=na.omit, family="gaussian")
summary(m4)

m5<-glmer(BirdWt~ SeasonID*RegionID+ Sex*Age*RegionID+
relevel(as.factor (BCS),"3")+(1|FarmUnID),data=birds,REML=T,
na.action=na.omit, family="gaussian")
summary(m5)

#Figure 3.13 Diagnostic plots of model residuals from regression
model for bird weight
windows(12,4)
par(mfrow=c(1,3))
plot( fitted(m4),residuals(m4), xlab="Fitted", ylab="Residuals",
main="Bird Weight residuals" )
qqnorm(residuals(m4),main="Bird level")
qqnorm(ranef(m4)$FarmUnID[[1]],main="Farm effects")


##############################
#Appendix 3.5
setwd('M:/Documents/Poultry Disease/R stuff/Bird analysis/')
Esk <- read.csv("Esk.csv", header=T, sep=,)

(m1<-glmer( WattleL~RegionID*Sex*Age+ (1|FarmUnID) ,data=Esk,
na.action=na.omit, family="gaussian"))

#Figure 3.14 Diagnostic plots of model residuals from regression
model for bird wattle length
windows(   12,4)
par(mfrow=c(1,3))
plot( fitted(m1),residuals(m1), xlab="Fitted", ylab="Residuals",
main="Bird Wattle length residuals" )
qqnorm(residuals(m1),main="Bird level")
qqnorm(ranef(m1)$FarmUnID[[1]],main="Farm effects")
```

Chapter 4

```
#Figure 4.1  Fates of birds at the time of follow-up visit

setwd('M:/Documents/Poultry Disease/Epidemiology/Spreadsheets/csv
files/')
HHS <- read.csv("Household level survival.csv", header=T, sep=,)

tabD<-as.data.frame(aggregate(HHS[,c(6:13)],
by=list(Region=HHS$RegionID, Season=HHS$SeasonID),sum))
tabD$Total<-rowSums(tabD[,c(3:10)])

    proptab<-tabD
    for(i in c(3:10))
    {
    for (j in c(1:6))
    {
    proptab[j,i]<- proptab[j,i]/proptab[j,11] *100
    }
    }

gra<- as.matrix(cbind(proptab[c(1:6),c(3:5)],
rowSums(proptab[c(1:6),c(6:9)]) ,proptab[c(1:6),10] ))
nom<-c("Survived",  "Died of disease", "Eaten by predator", "Other
fate*", "Lost to follow-up")
cols<- c( "red3", "blue3",  "firebrick3", "royalblue2",
"indianred1", "cornflowerblue")

windows(12,6)
barplot(gra, col=cols, beside=T, names.arg=nom,ylim=c(0,70),
ylab="percentage" )
legend("topright", c("Season A Horro","Season A Jarso", "Season B
Horro", "Season B Jarso","Season C Horro","Season C Jarso"),
fill=cols, bty="n")


##################################################
#Figure 4.2  Prevalence of reported household outbreaks in each
month for 2 regions

setwd('M:/Documents/Poultry Disease/R Stuff/Outbreaks')
mons<- read.csv("months_1.csv", header = T, sep= ",")

(mons2<-strptime(mons$Month, format="%d/%m/%Y"))
mons2<-as.POSIXlt(mons2)
e<-cbind(mons2,mons)

windows (title="ab",20,12)
par(fig=c(0,1,0.66,1),mar=c(0,5,5,4),oma=c(5,0,0,0))
plot(  e$mons2, e$Hprev, col="red", type="l",lwd=3,xaxt="n",
xlab="", ylab="", ylim=c(0,20))
      lines(e$mons2, e$Hallprev, col="red", lty=5)
      lines (e$mons2, e$Jprev, col="blue",lwd=3)
      lines (e$mons2, e$Jallprev, col="blue",lty=5)
      legend("topleft","a) All respondents",  bty="n")

legend("topright", c("Horro","Jarso","All deaths", "Outbreaks of 2
or more birds"), lwd=c(2,2,1,3),
```

```
lty=c(1,1,5,1),col=c("red","blue","black","black"),
title="",ncol=2, bty="n", cex=0.8)


par(fig=c(0,1,0.52,0.6598),mar=c(0,5,0,4) , new=T)
hma<- plot(  e$mons2, e$HA, col="red", type="l",lwd=2, xaxt="n",
xlab="", ylab="",  ylim=c(0,15),yaxt="n")
axis(4, cex.axis=0.8)
      lines(e$mons2, e$Harep, col="red", lty=5,lwd=2)
      lines (e$mons2, e$JA, col="blue",lwd=2)
      lines (e$mons2, e$Jarep, col="blue",lty=5,lwd=2)
      legend("topleft","b) Season A respondents", cex=0.8, bty="n")
      legend("topright", c("Initial period", "Follow-up period
"), lwd=2, lty=c(1,2),col=c("black","black"),  title="",ncol=2,
bty="n", cex=0.8)

par(fig=c(0,1,0.39,0.52),mar=c(0,5,0,4) , new=T)
hma<- plot(  e$mons2, e$HB, col="red",type="l",lwd=2, xaxt="n",
xlab="", ylab="",  ylim=c(0,15),cex.axis=0.8)
      lines(e$mons2, e$Hbrep, col="red",lty=5,lwd=2)
      lines (e$mons2, e$JB, col="blue",lwd=2)
      lines(e$mons2, e$Jbrep, col="blue",lty=5,lwd=2)
      legend("topleft","c) Season B respondents", cex=0.8, bty="n")

par(fig=c(0,1,0.26,0.39),mar=c(0,5,0,4) , new=T)
hma<- plot(  e$mons2, e$HC, col="red", type="l",lwd=2, xaxt="n",
xlab="", ylab="", cex.lab=0.8, ylim=c(0,15),yaxt="n")
axis(4,cex.axis=0.8)
      lines(e$mons2, e$Hcrep, col="red", lty=5, lwd=2)
      lines (e$mons2, e$JC, col="blue", lwd=2)
      lines(e$mons2, e$Jcrep, col="blue", lty=5, lwd=2)
      legend("topleft","d) Season C respondents", cex=0.8, bty="n")

par(fig=c(0,1,0,0.26),mar=c(5,5,0,4) , new=T)
hma<- plot(  e$mons2, e$HD, col="red",type="l",lwd=2,
xaxt="n",xlab="Month", ylab="",  ylim=c(0,15),cex.axis=0.8)
lines (e$mons2, e$JD, col="blue", lwd=2)
legend("topleft","e) Season D respondents", cex=0.8, bty="n")

to<- (par("usr")[2] - par("usr")[1] )/31
      p<-rep(0,32)
      for (i in 1:length(p))
      {
      p[i]<- par("usr")[1]+ ((i-1)*to )
      }
labs<-c("May 2010", "Sept 2010", "Jan 2010", "May 2011", "Sept
2011", "Jan 2011", "May 2012", "Sept 2012")

axis(1,at=p[c(2,6,10,14,18,22,26,30)],labels=labs, cex.axis=0.8)


#######################
# Figure 4.4 GAM plots showing the change in reporting with respect
to time elapsed
library(mgcv)

setwd('M:/Documents/Poultry Disease/R stuff/My dataframes/')
source("outbreaks ch4.r")

ltim$BOB<- ifelse(ltim$Loss>1,1,0)
```

```
g1<-gam(BOB~s(Elapse), data=ltim[which(ltim$SeasonID=="A"),],
method="REML", family=binomial)
g2<-gam(BOB~s(Elapse), data=ltim[which(ltim$SeasonID=="B"),],
method="REML", family=binomial)
g3<-gam(BOB~s(Elapse), data=ltim[which(ltim$SeasonID=="C"),],
method="REML", family=binomial)
g4<-gam(BOB~s(Elapse), data=ltim[which(ltim$SeasonID=="D"),],
method="REML", family=binomial)

windows(20,10)
par(mfrow=c(2,2))
plot(g1, jit=T, col="darkred", ylab="",ylim=c(-5,2),lwd=2, xlab="",
sub="Season A",cex.sub=1.5)
plot(g2,jit=T, col="red", lwd=2,ylab="",ylim=c(-
5,2),xlab="",sub="Season B" , cex.sub=1.5)
plot(g3, jit=T, col="orangered", lwd=2,ylab="",ylim=c(-5,2),
xlab="", sub="Season C",cex.sub=1.5)
plot(g4,jit=T, col="orange", lwd=2,ylab="",ylim=c(-
5,2),xlab="",sub="Season D", cex.sub=1.5 )

###########################################################
#Figure 4.5  Reported numbers of bird deaths on each farm by month
farmer reported that outbreak occurred

setwd('M:/Documents/Poultry Disease/R Stuff/Outbreaks')
hhs = read.csv("hhdis.csv", header = T, sep= ",")
reps = read.csv("repsoutbreak.csv", header = T, sep= ",")

setwd('M:/Documents/Poultry Disease/R Stuff/Outbreaks')
mons<- read.csv("months_1.csv", header = T, sep= ",")
mop<-levels  (mons$Month)
mop<-mop[c(9,12,15,18,21,24,27,29,1,3,5,7,10,13,16,19,22,25,28,30,
2,4,6,8,11,14,17,20,23,26)]

try<-data.frame (rep(0,1),rep(0,1))
names(try)<-c("Loss", "Month")
HA<-hhs[which(hhs$SeasonID=="A" & hhs$RegionID=="H"),]
HA1<-HA[,c( 24:31,20:23)]

for(i in 1:12)
  {
 bib<-HA1[which(HA1[,i]>0),  i]
  a<-length(bib)
  try[i,1]<-a
  try[i,2]<-mop[i]
   }
   try$Mono<-c(1:12)
long<-as.data.frame(lapply(try, function(x)rep(x,try$Loss)))

 bib<-HA1[which(HA1[,1]>0),  1]
 b<-data.frame( bib)
 names(b)<-"bib"
 for(i in 2:12)
 {
   bib<-HA1[which(HA1[,i]>0),  i]
    a<-as.data.frame(bib)
    b<-rbind(b,a)
    }
       HA<-cbind(b,long[,c(2,3)])
     names(HA)<-c("Loss","Month","Mono")
```

```
  #-----------
 tryB<-data.frame (rep(0,1),rep(0,1))
 names(tryB)<-c("Loss", "Month")
 HB<-hhs[which(hhs$SeasonID=="B" & hhs$RegionID=="H"),]
 HB1<-HB[,c( 30:31,20:29)]
   for(i in 1:12)
  {
 bib<-HB1[which(HB1[,i]>0),  i]
  a<-length(bib)
  tryB[i,1]<-a
  tryB[i,2]<-mop[i+6]
  }
 tryB$Mono<-c(7:18)
 longB<-as.data.frame(lapply(tryB, function(x)rep(x,tryB$Loss)))
 b<-data.frame( HB1[which(HB1[,2]>0),  2])
 names(b)<-"bib"
  for(i in 3:12)
 {
   bib<-HB1[which(HB1[,i]>0),  i]
 a<-as.data.frame(bib)
 b<-rbind(b,a)
    }
 HB<-cbind(b,longB[,c(2,3)])
 names(HB)<-c("Loss","Month","Mono")

      #-----------------------------
       tryC<-data.frame (rep(0,1),rep(0,1))
   names(tryC)<-c("Loss", "Month")
    HC<-hhs[which(hhs$SeasonID=="C" & hhs$RegionID=="H"),]
  HC1<-HC[,c( 24:31,20:23)]
   for(i in 1:12)
  {
 bib<-HC1[which(HC1[,i]>0),  i]
  a<-length(bib)
  tryC[i,1]<-a
  tryC[i,2]<-mop[i+12]
  }
   tryC$Mono<-c(13:24)
longC<-as.data.frame(lapply(tryC, function(x)rep(x,tryC$Loss)))
   b<-data.frame( HC1[which(HC1[,1]>0),  1])
 names(b)<-"bib"
   for(i in 2:12)
 {
   bib<-HC1[which(HC1[,i]>0),  i]
    a<-as.data.frame(bib)
    b<-rbind(b,a)
    }
    HC<-cbind(b,longC[,c(2,3)])
     names(HC)<-c("Loss","Month","Mono")

   #-------
tryD<-data.frame (rep(0,1),rep(0,1))
names(tryD)<-c("Loss", "Month")
HD<-hhs[which(hhs$SeasonID=="D" & hhs$RegionID=="H"),]
HD1<-HD[,c( 30:31,20:29)]
for(i in 1:12)
  {
 bib<-HD1[which(HD1[,i]>0),  i]
  a<-length(bib)
  tryD[i,1]<-a
  tryD[i,2]<-mop[i+18]
```

```
   }
  tryD$Mono<-c(19:30)
  longD<-as.data.frame(lapply(tryD, function(x)rep(x,tryD$Loss)))
   b<-data.frame( HD1[which(HD1[,3]>0),  3])
  names(b)<-"bib"
   for(i in 4:12)
  {
    bib<-HD1[which(HD1[,i]>0),  i]
     a<-as.data.frame(bib)
     b<-rbind(b,a)
     }
HD<-cbind(b,longD[,c(2,3)])
names(HD)<-c("Loss","Month","Mono")

  #-----------
HR<-na.omit(reps[which(reps$RegionID=="H"),c(16,9)])
mib<-as.data.frame(mop)
mib$Mono<-c(1:30)
HR$Mono<-mib$Mono[match(HR$Month,mib$mop)]
names(HR)<- c("Loss","Month","Mono")
HALL<-rbind(HA,HB,HC,HD,HR)
HALL$RegionID<-"H"

        ####################
try<-data.frame (rep(0,1),rep(0,1))
names(try)<-c("Loss", "Month")
JA<-hhs[which(hhs$SeasonID=="A" & hhs$RegionID=="J"),]
JA1<-JA[,c( 24:31,20:23)]
for(i in 1:12)
  {
 bib<-JA1[which(JA1[,i]>0),  i]
  a<-length(bib)
  try[i,1]<-a
  try[i,2]<-mop[i]
   }
try$Mono<-c(1:12)
long<-as.data.frame(lapply(try, function(x)rep(x,try$Loss)))
b<-data.frame( JA1[which(JA1[,3]>0),  3])
names(b)<-"bib"
for(i in 4:12)
 {
   bib<-JA1[which(JA1[,i]>0),  i]
    a<-as.data.frame(bib)
    b<-rbind(b,a)
    }
JA<-cbind(b,long[,c(2,3)])
names(JA)<-c("Loss","Month","Mono")

  #-----------
tryB<-data.frame (rep(0,1),rep(0,1))
names(tryB)<-c("Loss", "Month")
JB<-hhs[which(hhs$SeasonID=="B" & hhs$RegionID=="J"),]
JB1<-JB[,c( 30:31,20:29)]
for(i in 1:12)
  {
 bib<-JB1[which(JB1[,i]>0),  i]
  a<-length(bib)
  tryB[i,1]<-a
  tryB[i,2]<-mop[i+6]
  }
    tryB$Mono<-c(7:18)
```

```
 longB<-as.data.frame(lapply(tryB, function(x)rep(x,tryB$Loss)))
   b<-data.frame( JB1[which(JB1[,2]>0),  2])
 names(b)<-"bib"
  for(i in 3:12)
 {
   bib<-JB1[which(JB1[,i]>0),  i]
    a<-as.data.frame(bib)
    b<-rbind(b,a)
    }
JB<-cbind(b,longB[,c(2,3)])
names(JB)<-c("Loss","Month","Mono")

      #------------------------------
tryC<-data.frame (rep(0,1),rep(0,1))
names(tryC)<-c("Loss", "Month")
JC<-hhs[which(hhs$SeasonID=="C" & hhs$RegionID=="J"),]
JC1<-JC[,c( 24:31,20:23)]
for(i in 1:12)
  {
 bib<-JC1[which(JC1[,i]>0),  i]
  a<-length(bib)
  tryC[i,1]<-a
  tryC[i,2]<-mop[i+12]
  }
   tryC$Mono<-c(13:24)
longC<-as.data.frame(lapply(tryC, function(x)rep(x,tryC$Loss)))
b<-data.frame( JC1[which(JC1[,1]>0),  1])
names(b)<-"bib"
for(i in 2:12)
 {
   bib<-JC1[which(JC1[,i]>0),  i]
    a<-as.data.frame(bib)
    b<-rbind(b,a)
    }
JC<-cbind(b,longC[,c(2,3)])
     names(JC)<-c("Loss","Month","Mono")

   #-------
          tryD<-data.frame (rep(0,1),rep(0,1))
   names(tryD)<-c("Loss", "Month")
    JD<-hhs[which(hhs$SeasonID=="D" & hhs$RegionID=="J"),]
  JD1<-JD[,c( 30:31,20:29)]
   for(i in 1:12)
  {
 bib<-JD1[which(JD1[,i]>0),  i]
  a<-length(bib)
  tryD[i,1]<-a
  tryD[i,2]<-mop[i+18]
  }
    tryD$Mono<-c(19:30)
 longD<-as.data.frame(lapply(tryD, function(x)rep(x,tryD$Loss)))
  b<-data.frame( JD1[which(JD1[,1]>0),  1])
 names(b)<-"bib"
  for(i in 2:12)
 {
   bib<-JD1[which(JD1[,i]>0),  i]
    a<-as.data.frame(bib)
    b<-rbind(b,a)
    }
    JD<-cbind(b,longD[,c(2,3)])
     names(JD)<-c("Loss","Month","Mono")
```

```
  #-----------
  JR<-na.omit(reps[which(reps$RegionID=="J"),c(16,9)])
  mib<-as.data.frame(mop)
    mib$Mono<-c(1:30)
   JR$Mono<-mib$Mono[match(JR$Month,mib$mop)]
    names(JR)<- c("Loss","Month","Mono")
         JALL<-rbind(JA,JB,JC,JD,JR)
         JALL$RegionID<-"J"
    ##################################
 summary( HALL$Loss)
 summary( JALL$Loss)

 (meds<- aggregate( HALL$Loss, by=list(M=HALL$Mono),median))
 (meds2<- aggregate( JALL$Loss, by=list(M=JALL$Mono),median))
 windows(20,15)
par(fig=c(0,1,0.5,1),mar=c(0,5,5,4),oma=c(5,0,0,0))
stripchart(c(0,0,JALL$Loss,0)~c(1,2,JALL$Mono,30), vertical=T,
pch=16, col=rgb(0,0,1,0.3),xaxt="n",ylim=c(0,50), ylab="Losses")
points(meds2$M,meds2$x, type="l", col="blue")
legend("left", "Jarso",bty="n")

par(fig=c(0,1,0,0.5) ,mar=c(5,5,0,4), new=T)
stripchart(HALL$Loss~HALL$Mono, vertical=T,
pch=16,col=rgb(1,0,0,0.3),group.names=mop[1:30],ylim=c(0,50),ylab="L
osses")
points(meds$M,meds$x, type="l", col="red")
legend("topleft", "Horro",bty="n")

########################
# Regression model code
library(mgcv)
library(lme4)

setwd('M:/Documents/Poultry Disease/R stuff/MyFunctions/')
source("Regression outputs.r")

setwd('M:/Documents/Poultry Disease/R stuff/My dataframes/')
source("outbreaks ch4.r")


ltim$BOB<- ifelse(ltim$Loss>1,1,0)

g1<-gam(BOB~s(Elapse), data=ltim[which(ltim$SeasonID=="A"),],
method="REML", family=binomial)
g2<-gam(BOB~s(Elapse), data=ltim[which(ltim$SeasonID=="B"),],
method="REML", family=binomial)
g3<-gam(BOB~s(Elapse), data=ltim[which(ltim$SeasonID=="C"),],
method="REML", family=binomial)
g4<-gam(BOB~s(Elapse), data=ltim[which(ltim$SeasonID=="D"),],
method="REML", family=binomial)

  windows(20,10)
  par(mfrow=c(2,2))
plot(g1, jit=T, col="darkred", ylab="",ylim=c(-5,2),lwd=2,
xlab="",sub="Season A", cex.sub=1.5)
plot(g2,jit=T, col="red", lwd=2,ylab="",ylim=c(-
5,2),xlab="",sub="Season B" , cex.sub=1.5)
plot(g3, jit=T, col="orangered", lwd=2,ylab="",ylim=c(-
5,2),xlab="",sub="Season C",cex.sub=1.5)
```

```
plot(g4,jit=T, col="orange", lwd=2,ylab="",ylim=c(-
5,2),xlab="",sub="Season D",  )

   ltim$lapse2<-ltim$Elapse
   ltim$lapse2[which(ltim$lapse2> -5 )] <- -4

 (m6<-glmer(BOB ~ relevel(RegionID,"J")+ Rain +
relevel(SeasonID,"B")* lapse2+ as.factor(AskTime)+ (1|FarmUnID) ,
data=ltim,REML = T, na.action=na.omit, family="binomial"))
        log.reg.outputs(m6)

  (m3<-glmer(Loss>1 ~ relevel(RegionID,"J")  +(1|FarmUnID) ,
data=ltim[which(ltim$Loss>0),],REML = T, na.action=na.omit,
family="binomial"))
  log.reg.outputs(m3)

 (m4<-glmer(Loss ~ relevel(RegionID,"J")+ Rain  +as.factor(AskTime)+
(1|FarmUnID) , data=ltim[which(ltim$Loss>1 & ltim$Loss<60),],REML =
T, na.action=na.omit, family="poisson"))
     log.reg.outputs(m4)


###############################################
#Appendix 4.2
library(lme4)
setwd('M:/Documents/Poultry Disease/R stuff/MyFunctions/')
source("Regression outputs.r")
ltim$lapse2<-ltim$Elapse
ltim$lapse2[which(ltim$lapse2> -5 )] <- -4

(m6<-glmer(BOB ~ relevel(RegionID,"J")+ Rain +
relevel(SeasonID,"B")*  lapse2+ as.factor(AskTime)+ (1|FarmUnID) ,
data=ltim,REML = T, na.action=na.omit, family="binomial"))

log.reg.outputs(m6)

###########################################

#Appendix 4.3

(m3<-glmer(Loss>1 ~ relevel(RegionID,"J")  +(1|FarmUnID) ,
data=ltim[which(ltim$Loss>0),],REML = T, na.action=na.omit,
family="binomial"))

log.reg.outputs(m3)

(m4a<-glmer(Loss ~ relevel(VillageID,"J2B")+as.factor(AskTime)+
relevel(SeasonID,"C")*Rain + relevel(SeasonID,"C")*lapse2+
(1|FarmUnID) , data=ltim[which(ltim$Loss>1) ,],REML = T,
na.action=na.omit, family="poisson"))

log.reg.outputs(m4a)

(m4b<-glmer(Loss ~ relevel(RegionID,"J")+ Rain  +as.factor(AskTime)+
(1|FarmUnID) , data=ltim[which(ltim$Loss>1 & ltim$Loss<60),],REML =
T, na.action=na.omit, family="poisson"))

log.reg.outputs(m4b)

##################################
```

```
#Figure 4.6  Reported outbreaks showing age groups affected across
different months

MOnames<-
c("Jan","Feb","Mar","Apr","May","Jun","Jul","Aug","Sep","Oct",
"Nov","Dec")
age<- hhs[,c(1,3,6, 15:19,8:14,20:31, 36:47)]

Jan<-age[which(age[,4]>0),c(1:3,4,4+12,4+24) ]
names(Jan)[4:6]<-c("Month" ,"Loss","AgeGp")
Jan[,4]<-MOnames[4-3]
b<-Jan

for(i in 5:15)
        {
       Jan<-age[which(age[,i]>0),c(1:3,i,i+12,i+24) ]
      names(Jan)[4:6]     <-c("Month" ,"Loss","AgeGp")
       Jan[,4]<-MOnames[i-3]
       b<-rbind(b,Jan)
       }
nrow(b)

cols<-c("darkred","red","orange","gold","chartreuse3","steelblue3",
"slateblue")
mos<-c("J","F","M","A","M","J","J","A","S","O","N","D")
cols2<-rep(cols[1:7],each=12)

z<- table(b$Month,b$AgeGp )
zz<-z[c(5,4,8,1,9,7,6,2,12,11,10,3),-1]
yy<-t(zz)

windows(12,8)
par(mfrow=c(2,1))
barplot(yy, beside=T,names=MOnames, col=cols, space=c(0,2))
legend("topright",labl[-1],fill=cols,bty="n", cex=0.9)

barplot(zz, beside=T,names=rep(mos,7),cex.names=0.5, col=cols2,
space=c(0,2))
```

Chapter 5

```
library(mgcv)
library(lme4)
library(MASS)
library(RgoogleMaps)
library(geoR)

 setwd('M:/Documents/Poultry Disease/R stuff/MyFunctions/')
 source("Regression outputs.r")
 source("spatial test function oct2012.R")

setwd('M:/Documents/Poultry Disease/R stuff/Survival analysis/')
suv <- read.csv("multioutcome.csv", header=T, sep=,)

names(suv)

suv$LiceTot<- rowSums(suv[,c(22:26)])
suv$outcome<-rowSums (suv[,c(7:13)])

suv$Finben<-  rowSums(suv[,c(10:11)])
suv$Finloss<-  rowSums(suv[,c(9,12,13)])
 suv$Goodoc<-ifelse(suv$Survival==1 |suv$Finben==1,1,0)
  suv$Season<-as.factor(ifelse(suv$SeasonID=="B","Dry","Wet") )
       mydat<-suv[which(suv$outcome==1),]

 #################################
 #Intraclass correlation coefficients
 (Shh0<-glmer(Survival ~ 1+(1|RegionID/MarkShID/VillageID/FarmUnID),
data=suv,REML = T, na.action=na.omit, family="gaussian"))
       summary(Shh0)
 (Shhbin<-glmer(Survival ~
1+(1|RegionID/MarkShID/VillageID/FarmUnID), data=suv,REML = T,
na.action=na.omit, family="binomial"))
       summary(Shhbin)
(Shh1<-glmer(Survival ~ RegionID+(1|FarmUnID), data=suv,REML = T,
na.action=na.omit, family="gaussian"))
       anova(Shh0,Shh1)
       (Shh2<-glmer(Survival ~ VillageID+(1|FarmUnID), data=suv,REML
= T, na.action=na.omit, family="gaussian"))
       anova(Shh2,Shh1)

(Dhh0<-glmer(Disease ~ 1+(1|RegionID/MarkShID/VillageID/FarmUnID),
data=suv,REML = T, na.action=na.omit, family="gaussian"))
       summary(Dhh0)
       (Dhhbin<-glmer(Disease ~
1+(1|RegionID/MarkShID/VillageID/FarmUnID), data=suv,REML = T,
na.action=na.omit, family="binomial"))
       summary(Dhhbin)
(Dhh1<-glmer(Disease ~ VillageID+(1|FarmUnID), data=suv,REML = T,
na.action=na.omit, family="gaussian"))
       anova(Dhh0,Dhh1)
(Dhh2<-glmer(Disease ~ RegionID+(1|FarmUnID), data=suv,REML = T,
na.action=na.omit, family="gaussian"))
       anova(Dhh2,Dhh1)

(Lhh0<-glmer(Finloss ~ 1+(1|RegionID/MarkShID/VillageID/FarmUnID),
data=suv,REML = T, na.action=na.omit, family="gaussian"))
       summary(Lhh0)
```

```
        (Lhhbin<-glmer(Finloss ~
1+(1|RegionID/MarkShID/VillageID/FarmUnID), data=suv,REML = T,
na.action=na.omit, family="binomial"))
        summary(Lhhbin)
(Lhh1<-glmer(Finloss ~ RegionID+(1|FarmUnID), data=suv,REML = T,
na.action=na.omit, family="gaussian"))
        anova(Lhh0,Lhh1)
        (Lhh2<-glmer(Finloss ~ VillageID+(1|FarmUnID), data=suv,REML
= T, na.action=na.omit, family="gaussian"))
        anova(Lhh2,Lhh1)

(Bhh0<-glmer(Finben ~ 1+(1|RegionID/MarkShID/VillageID/FarmUnID),
data=suv,REML = T, na.action=na.omit, family="gaussian"))
        summary(Bhh0)
        (Bhhbin<-glmer(Finben ~
1+(1|RegionID/MarkShID/VillageID/FarmUnID), data=suv,REML = T,
na.action=na.omit, family="binomial"))
        summary(Bhhbin)
(Bhh1<-glmer(Finben ~ VillageID+(1|FarmUnID), data=suv,REML = T,
na.action=na.omit, family="gaussian"))
        anova(Bhh0,Bhh1)
(Bhh2<-glmer(Finben ~ RegionID+(1|FarmUnID), data=suv,REML = T,
na.action=na.omit, family="gaussian"))
        anova(Bhh2,Bhh1)

     #########################
   #Figure 5.2
  (Shh9<-glmer(Survival ~ relevel(VillageID,"H2B")+(1|FarmUnID),
data=mydat,REML = T, na.action=na.omit, family="binomial",nAGQ=2))
   tab1<-          log.reg.outputs(Shh9)

  (Dhh7<-glmer(Disease ~ relevel(VillageID,"H2B")+(1|FarmUnID),
data=mydat,REML = T, na.action=na.omit, family="binomial",nAGQ=2))
   tab2<-          log.reg.outputs(Dhh7)

  (Phh4<-glmer(Finloss ~ relevel(VillageID,"H2B") +(1|FarmUnID),
data=mydat,REML = T, na.action=na.omit, family="binomial",nAGQ=2))
   tab3<-          log.reg.outputs(Phh4)

  (Ehh6<-glmer(Finben ~ relevel(VillageID,"H2B") +(1|FarmUnID),
data=mydat,REML = T, na.action=na.omit, family="binomial",nAGQ=2))
   tab4<-          log.reg.outputs(Ehh6)

res<-data.frame(rep(0,40),rep(0,40),rep(0,40))
names(res)<-c("OR","LCI","UCI")
           for ( i in 1:8)
           {
          p<- rbind(tab1[i,3:5],tab2[i,3:5],tab3[i,3:5],tab4[i,3:5]
)
          res[(5*i- 3):(5*i),]<- p
          }


   library(plotrix)
   windows(14,6)
         par(fig=c(0.15,1,0,1),mar=c(4,0,2,10), xpd=T)
          plotCI(log(res[7:40,1]),pch=19, col=c("blue", "red",
"orange","chartreuse3","white"),ylim=c(-5,7),yaxt="n",
ylab="",xaxt="n", xlab="",
ui=log(res[7:40,3]),li=log(res[7:40,2]),slty=1 , sfrac=0.005)
         axis (4)
```

```
        mtext("log odds ratio", side=4, padj=4)
        x<-seq(5,30,5)
         for (i in 1:7)
         {
         lines(c(x[i],x[i]),c(-5,5), lty=3,col="grey60")
         }
         lines(c(15,15),c(-5,7), lty=2)
               lines(c(0,35),c(0,0), lty=3)

         y<-seq(2.5,32.5,5)
         mtext(levels(mydat$VillageID)[c(1:3,5:8)], side=1, at=y)
         mtext(c("Horro","Jarso"), side=1, at=c(5,25),
cex=1.2,padj=2)
         mtext("H2B",side=2, at=0, cex=1.2, las=2, adj=2)
         mtext("Reference",side=2, at=-6, cex=1.2, las=2, adj=1.2)
         mtext("village",side=2, at=-6.6, cex=1.2, las=2, adj=1.5)
          legend(38,7,legend=c("Survival",
"Disease","Loss","Gain"),cex=0.9,pch=19,lty=1, col=c("blue", "red",
"orange","chartreuse3"), bty="n")

##############################################
 # Village/Season interaction models
(Shh1<-glmer(Survival ~ RegionID+(1|FarmUnID), data=suv,REML = T,
na.action=na.omit, family="binomial"))
(Shh2<-glmer(Survival ~ Season*RegionID+(1|FarmUnID), data=suv,REML
= T, na.action=na.omit, family="binomial"))  # best fit
       anova(Shh2,Shh1)
(Shh3<-glmer(Survival ~
Season*relevel(VillageID,"H1B")+(1|FarmUnID), data=suv,REML = T,
na.action=na.omit, family="binomial"))
       anova(Shh2,Shh3)
(Shh4<-glmer(Survival ~ relevel(SeasonID,"B")*RegionID+(1|FarmUnID),
data=suv,REML = T, na.action=na.omit, family="binomial"))
       anova(Shh2,Shh4)


(Dhh1<-glmer(Disease ~ relevel(VillageID,"J2A")+(1|FarmUnID),
data=suv,REML = T, na.action=na.omit, family="binomial", nAGQ=2))
(Dhh3<-glmer(Disease ~ Season*relevel(VillageID,"J2A")+(1|FarmUnID),
data=suv,REML = T, na.action=na.omit, family="binomial", nAGQ=2))
       anova(Dhh1,Dhh3)
(Dhh4<-glmer(Disease ~
relevel(SeasonID,"B")+relevel(VillageID,"J2A")+(1|FarmUnID),
data=suv,REML = T, na.action=na.omit, family="binomial", nAGQ=2)) #
best fit
       anova(Dhh1,Dhh4)


(Lhh1<-glmer(Finloss ~ RegionID+(1|FarmUnID), data=suv,REML = T,
na.action=na.omit, family="binomial"))
(Lhh2<-glmer(Finloss ~ VillageID+(1|FarmUnID), data=suv,REML = T,
na.action=na.omit, family="binomial"))
       anova(Lhh2,Lhh1)
(Lhh3<-glmer(Finloss ~ Season*relevel(VillageID,"J2A")+(1|FarmUnID),
data=suv,REML = T, na.action=na.omit, family="binomial"))# best fit
       anova(Lhh2,Lhh3)
(Lhh4<-glmer(Finloss ~ relevel(SeasonID,"B")*VillageID+(1|FarmUnID),
data=suv,REML = T, na.action=na.omit, family="binomial"))
       anova(Lhh4,Lhh3)
```

```
(Bhh1<-glmer(Finben ~ VillageID+(1|FarmUnID), data=suv,REML = T,
na.action=na.omit, family="binomial"))
(Bhh2<-glmer(Finben ~ RegionID+(1|FarmUnID), data=suv,REML = T,
na.action=na.omit, family="binomial"))
anova(Bhh1,Bhh2)
(Bhh3<-glmer(Finben ~ Season*relevel(VillageID,"J2A")+(1|FarmUnID),
data=suv,REML = T, na.action=na.omit, family="binomial"))  # best
fit
       anova(Bhh3,Bhh1)
(Bhh4<-glmer(Finben ~ relevel(SeasonID,"B")*VillageID+(1|FarmUnID),
data=suv,REML = T, na.action=na.omit, family="binomial"))
       anova(Bhh3,Bhh4)

####################
  #Figure 5.3
  suv$sv<-paste(suv$VillageID,suv$Season)
   oc<-   aggregate(  suv[,c(7,59,8,60)],na.rm=TRUE,
by=list(v=suv$sv),sum)
    windows(12,7)
         par(mar=c(10,4,2,10), xpd=T)
   barplot(   prop.table(t(oc[,-1]),2),  col=c("blue","chartreuse3",
"red", "orange"), space=c(1,0,1,0,1,0,1,0,2,0))
   y<-  c(2,5,8,11,15,18,21,24)
   mtext(levels(mydat$VillageID), side=1, at=y,padj=2,cex=1.2)
    mtext("Village", side=1, at=28,padj=2,cex=1.2)
   mtext(rep(c("Dry"),8), side=1, at=y-0.5)
   mtext(rep(c("Wet"),8), side=1, at=y+0.5)
    mtext("Season", side=1, at=28)
    mtext(levels(mydat$MarkShID), side=1,
at=c(3.5,9.5,16.5,22.5),padj=4,cex=1.5)
     mtext("Marketshed", side=1, at=28,padj=4,cex=1.5)
      mtext(c("Horro", "Jarso", "Region"), side=1, at=c(6.5,19.5
,28),padj=6,cex=1.8)
  legend(26,0.95,legend=c("Survival",
"Disease","Loss","Gain"),cex=0.9, fill=c("blue", "red",
"orange","chartreuse3"), bty="n")

  #########################

 mydatH<-mydat[which(mydat$RegionID=="H"),]
  mydatH$Age<-ifelse(mydatH$Age>40,36,mydatH$Age)
  mydatH$recpur<-ifelse(mydatH$LastBird==0,1,0)

 #Horro Approach 1
 Shh9<-glmer(Survival ~ relevel(VillageID,"H2B")+AdjPM*
Age+Sex+(1|FarmUnID), data=mydatH,REML = T, na.action=na.omit,
family="binomial",nAGQ=2)
summary(Shh9)
(tab1<- log.reg.outputs(Shh9))

death<-
na.omit(mydatH[which(mydatH$Survival==0),c(1:6,63,14,8,59,60)]  )
colSums( death[,9:11])

Ys<-c(t(death[,9:11]))
xs<-c(t(matrix(as.factor(death$Sex),nrow(death),3)))
xv<-c(t(matrix(as.factor(death$VillageID),nrow(death),3)))
xb<-c(t(matrix(death$recpur,nrow(death),3)))
rs<- c(t(matrix(death$FarmUnID,nrow(death),3)))
cat<-c(t(matrix(1:3,nrow(death),3,T)))
```

```
chck<-cbind(xs,xb,xv,rs,cat,Ys)
head(chck)

ref.model1<-glmer(Ys ~ -1+as.factor(cat)+
as.factor(cat):relevel(as.factor(xv),"H2B")+ as.factor(cat):xb+ (-
1+as.factor(cat)|rs) , REML = T, na.action=na.omit,
family="poisson")
log.reg.outputs(ref.model1)

#Fig 5.4
fixef(Shh9)
 model<-Shh9
summary(mydatH$Age)
summary(mydatH$AdjPM)

hl1<-seq(-2,4,length.out=100)

Age1<-rep(6, times =100)
age.hl.6<-
fixef(model)[1]+fixef(model)[6]*Age1+fixef(model)[5]*hl1+fixef(model
)[8]*Age1*hl1

Age1<-rep(12,times =100)
age.hl.12<-
fixef(model)[1]+fixef(model)[6]*Age1+fixef(model)[5]*hl1+fixef(model
)[8]*Age1*hl1

Age1<-rep(24, times =100)
age.hl.24<-
fixef(model)[1]+fixef(model)[6]*Age1+fixef(model)[5]*hl1+fixef(model
)[8]*Age1*hl1

Age1<-rep(36, times =100)
age.hl.36<-
fixef(model)[1]+fixef(model)[6]*Age1+fixef(model)[5]*hl1+fixef(model
)[8]*Age1*hl1

out<-as.data.frame(cbind( exp(age.hl.6)/(1+exp(age.hl.6)),
exp(age.hl.12)/(1+exp(age.hl.12)),
exp(age.hl.24)/(1+exp(age.hl.24)),
exp(age.hl.36)/(1+exp(age.hl.36))))

c1<-c("darkred", "red", "orange","gold")

windows(10,6)
par(mfrow=c(1,1))
plot(y=out[,1],x=hl1+ 1.021, type="l", col=c1[1], lwd=2,
ylim=c(0,1),ylab="Probability of survival", xlab="Pasteurella s:p
ratio")
lines(y=out[,2],x=hl1+ 1.021, type="l", col=c1[2], lwd=2)
lines(y=out[,3],x=hl1+ 1.021, type="l", col=c1[3], lwd=2)
lines(y=out[,4],x=hl1+ 1.021, type="l", col=c1[4], lwd=2)

 legend("bottomleft", c("6 mths", "12 mths", "24 mths", "36
mths"),bty="n", lwd=2,col=c1)



################
```

```
#Approach 2

mydatHtmp<-na.omit(mydatH[, c(1:6,15,61,62,63)])
table(mydatHtmp$Goodoc)

(Dhh7<-glmer(Goodoc ~
relevel(VillageID,"H2B")*Season+Age+recpur+(1|FarmUnID),
data=mydatHtmp,REML = T, na.action=na.omit,
family="binomial",nAGQ=2))
(tab2<- log.reg.outputs(Dhh7))

good <-
na.omit(mydatH[which(mydatH$Goodoc==1),c(1:6,62,54,27,15,14,7,59)]
)
table(good$Survival)
(Dhh8<-glmer(Survival ~
Sex+Age*AdjPM+as.factor(Cnemido>1)+(1|FarmUnID), data=good,REML = T,
na.action=na.omit, family="binomial",nAGQ=2))
(tab2<-log.reg.outputs(Dhh8))

 bad <-na.omit(mydatH[which(mydatH$Goodoc==0),c(1:6,62,8,60)]  )
 table(bad$Finloss)
 (Dhh9<-glmer(Finloss ~ -1+relevel(VillageID,"H2B")+(1|FarmUnID),
data=bad,REML = T, na.action=na.omit, family="binomial",nAGQ=8))
 (tab3<-log.reg.outputs(Dhh9))

 #Approach 3
all<-
na.omit(mydatH[which(mydatH$outcome==1),c(1:5,62,27,63,54,14,15,7,8,
59,60)]  )
colSums( all[,12:15])

Ys<-c(t(all[,12:15]))
xs<-c(t(matrix(as.factor(all$Sex),nrow(all),4)))
xv<-c(t(matrix(as.factor(all$VillageID),nrow(all),4)))
xb<-c(t(matrix(all$recpur,nrow(all),4)))
xt<-c(t(matrix(as.factor(all$Season),nrow(all),4)))
xa<-c(t(matrix(all$Age,nrow(all),4)))
xp<-c(t(matrix(all$AdjPM,nrow(all),4)))
xl<-c(t(matrix(all$Cnemido,nrow(all),4)))

rs<- c(t(matrix(all$FarmUnID,nrow(all),4)))
cat<-c(t(matrix(1:4,nrow(all),4,T)))

chck<-cbind(xs,xa,xl,xb,xv,xp,rs,cat,Ys)
head(chck)

ref.model2<-glmer(Ys ~ -1+as.factor(cat)+
as.factor(cat):relevel(as.factor(xv),"H2B")
+as.factor(cat):xt+
as.factor(cat):as.factor(xl>0)+as.factor(cat):xb+
as.factor(cat):xs+as.factor(cat):xa*  as.factor(cat):xp+
(-1+as.factor(cat)|rs) , REML = T, na.action=na.omit,
family="poisson")

summary(ref.model2)
log.reg.outputs(ref.model2)



###########################
```

```
#Jarso

 mydatJ<-mydat[which(mydat$RegionID=="J"& mydat$PooSourc==0),]
  mydatJ$Cocount<-ifelse(mydatJ$Coccidia<150,mydatJ$Coccidia*0.02,3)

  #Approach 1
     (Shh8<-glmer(Survival ~ relevel(LayState,"L")+Season+AdjMDV +
Cocount+(1|FarmUnID), data=mydatJ,REML = T, na.action=na.omit,
family="binomial",nAGQ=2))
     ( tab1<-log.reg.outputs(Shh8))

 mydatJ<-mydat[which(mydat$RegionID=="J"),]
 death<-
na.omit(mydatJ[which(mydatJ$Survival==0),c(1:5,62,14,8,59,60)]  )
 colSums( death[,8:10])

Ys<-c(t(death[,8:10]))
xs<-c(t(matrix(as.factor(death$Sex),nrow(death),3)))
xv<-c(t(matrix(as.factor(death$VillageID),nrow(death),3)))

rs<- c(t(matrix(death$FarmUnID,nrow(death),3)))
cat<-c(t(matrix(1:3,nrow(death),3,T)))

chck<-cbind(xs,xv,rs,cat,Ys)
head(chck)

ref.model0<-glmer(Ys ~ -1+as.factor(cat)+as.factor(cat):xs+
as.factor(cat):relevel(as.factor(xv),"J1A")+(-1+as.factor(cat)|rs) ,
REML = T, na.action=na.omit, family="poisson")
summary(ref.model0)
log.reg.outputs(ref.model0)


#Approach 2
 mydatJ<-mydat[which(mydat$RegionID=="J"& mydat$PooSourc==0),]
  mydatJ$Cocount<-ifelse(mydatJ$Coccidia<150,mydatJ$Coccidia*0.02,3)
table(mydatJ$Goodoc)

 (Dhh11<-glmer(Goodoc
~relevel(VillageID,"J1A")+Season+relevel(LayState,"L")*AdjMDV+Cocoun
t+(1+Season+AdjMDV|FarmUnID), data=mydatJ,REML = T,
na.action=na.omit, family="binomial",nAGQ=4))
   (tab2<- log.reg.outputs(Dhh11))

  (Dhh10<-glmer(Goodoc
~relevel(VillageID,"J1A")+Season+relevel(LayState,"L")*AdjMDV+Cocoun
t+(1+AdjMDV|FarmUnID), data=mydatJ,REML = T, na.action=na.omit,
family="binomial"))
  anova(Dhh10,Dhh11)

   mydatJ<-mydat[which(mydat$RegionID=="J"),]
   good <-
na.omit(mydatJ[which(mydatJ$Goodoc==1),c(1:6,62,27,14,7,59)]  )

      table(good$Survival)
       (Dhh8<-glmer(Survival ~
Sex+as.factor(Cnemido>0)+relevel(VillageID,"J1B")+Season+(1|FarmUnID
), data=good,REML = T, na.action=na.omit, family="binomial",nAGQ=2))
      ( tab2<- log.reg.outputs(Dhh8))

    bad <-na.omit(mydatJ[which(mydatJ$Goodoc==0),c(1:6,62,8,60)])
```

```
      names(bad)
      table(bad$Finloss)

       (Dhh9<-glmer(Finloss ~ 1+(1|FarmUnID), data=bad,REML = T,
na.action=na.omit, family="binomial",nAGQ=20))
      (tab2<-log.reg.outputs(Dhh9))


####################################################
 #Approach 3
mydatJ<-mydat[which(mydat$RegionID=="J"& mydat$PooSourc==0),]
  mydatJ$Cocount<-ifelse(mydatJ$Coccidia<150,mydatJ$Coccidia*0.02,3)
all<-
na.omit(mydatJ[which(mydatJ$outcome==1&mydatJ$PooSourc==0),c(1:5,62,
27,63,56,14,7,8,59,60)]  )
colSums( all[,11:14])

Ys<-c(t(all[,11:14]))
xs<-c(t(matrix(as.factor(all$Sex),nrow(all),4)))
xv<-c(t(matrix(as.factor(all$VillageID),nrow(all),4)))
xc<-c(t(matrix(all$Cocount,nrow(all),4)))
xt<-c(t(matrix(as.factor(all$Season),nrow(all),4)))
xm<-c(t(matrix(all$AdjMDV,nrow(all),4)))
xl<-c(t(matrix(as.factor(all$Cnemido>0),nrow(all),4)))

rs<- c(t(matrix(all$FarmUnID,nrow(all),4)))
cat<-c(t(matrix(1:4,nrow(all),4,T)))

chck<-cbind(xs,xl,xt,xv,xm,xc,rs,cat,Ys)
head(chck)

ref.model6<-glmer(Ys ~ -
1+as.factor(cat)+as.factor(cat):xs+as.factor(cat):relevel(as.factor(
xv),"J1A")+as.factor(cat):xt+as.factor(cat):xc+ (-
1+as.factor(cat)|rs) , REML = T, na.action=na.omit,
family="poisson")
summary(ref.model6)

log.reg.outputs(ref.model6)

#########################################
names(mydatJ)
farm<- na.omit( mydatJ[, c(1:6,62,21,56,63,61)])
farm$Latitude<-  mydatJ$Latitude[    match(
farm$UniqueID,mydatJ$UniqueID)]
farm$Longtude<-  mydatJ$Longtude[    match(
farm$UniqueID,mydatJ$UniqueID)]

 #Figure 5.5
 (Dhh11<-glmer(Goodoc
~relevel(VillageID,"J1A")+Season+relevel(LayState,"L")*AdjMDV+Cocoun
t+(1+Season+AdjMDV|FarmUnID), data=farm,REML = T, na.action=na.omit,
family="binomial",nAGQ=4))
head(ranef(Dhh11)[[1]] )
 head(coef(Dhh11)$FarmUnID)
 hist(coef(Dhh11)$FarmUnID[,5], breaks=20, main="Jarso farm-level
Season coefficients")

farmplot<-unique(farm[,c(2:7,12,13)])
farmplot<- cbind(farmplot,  coef(Dhh11)[[1]])
hist(farmplot$SeasonWet, breaks=20)
```

```
windows(12,6)
plot(farmplot$SeasonID,farmplot$AdjMDV, col=c("darkred", "orange",
"orangered"))


###########################################
#Appendix B
J1<-farmplot[which(farmplot$MarkShID=="J2"),]
  windows(10,10)
      H1s<-PlotOnStaticMap( lat=J1$Latitude, lon=J1$Longtude,
zoom=7, size = c(640,640), GRAYSCALE  = FALSE,
               add=FALSE, FUN = points, pch="",mar=c(0,0,0,0),
NEWMAP = TRUE, TrueProj = TRUE, axes= FALSE, verbose = 1)
                PlotOnStaticMap(
MyMap=H1s,lat=J1[J1$SeasonID=="A",7], lon=J1[J1$SeasonID=="A",8],
zoom=14, size = c(640,640), GRAYSCALE  = FALSE,
               add=T, FUN = points,col=c("darkred"),
pch=19,cex=0.75*(1.2+J1[J1$SeasonID=="A",13]),mar=c(0,0,0,0), NEWMAP
= F, TrueProj = TRUE, axes= FALSE, verbose = 1)
                PlotOnStaticMap(
MyMap=H1s,lat=J1[J1$SeasonID=="C",7], lon=J1[J1$SeasonID=="C",8],
zoom=14, size = c(640,640), GRAYSCALE  = FALSE,
               add=T, FUN = points,col=c("orangered"),
pch=19,cex=0.75*(1.2+J1[J1$SeasonID=="C",13]),mar=c(0,0,0,0), NEWMAP
= F, TrueProj = TRUE, axes= FALSE, verbose = 1)


     legend("topright", c("Season A", "Season C"),
col=c("darkred","orangered"), pch=19 , bty="n" , cex=1.5, pt.cex=1.5
)


J1.Wet<-na.omit(farmplot[farmplot[,2]=="J1A" &
farmplot[,6]=="Wet",c(7,8,9)] )
test1 <- spatial.test(J1.Wet, u.vector=seq(0,1,0.001), nsim=10000)

J1.Wet<-na.omit(farmplot[farmplot[,2]=="J1B" &
farmplot[,6]=="Wet",c(7,8,9)] )
test1 <- spatial.test(J1.Wet, u.vector=seq(0,1,0.001), nsim=10000)

J1.Wet<-na.omit(farmplot[farmplot[,2]=="J2A" &
farmplot[,6]=="Wet",c(7,8,9)] )
test1 <- spatial.test(J1.Wet, u.vector=seq(0,1,0.001), nsim=10000)

J1.Wet<-na.omit(farmplot[farmplot[,2]=="J2B" &
farmplot[,6]=="Wet",c(7,8,9)] )
test1 <- spatial.test(J1.Wet, u.vector=seq(0,1,0.001), nsim=10000)
```

Chapter 6

```
library(vegan)
library(lattice)
library("MASS")
library(car)
library(RColorBrewer)

setwd('M:/Documents/Poultry Disease/R stuff/Multivariate analysis/')
sero <- read.csv("svepm data.csv", header=T, sep=,)
sero2<-na.omit(sero[,c(1:6,31:37,23)])

# unscaled data matrix
test0 <- as.data.frame(sero2[,7:14])

# scaled by mean / sd
test1 <- as.data.frame(scale(sero2[,7:14]))

# scaled by min/max
test2<- as.data.frame(matrix(0,nrow(sero2),8))
 for(i in 7:14)
 {
a <- ((sero2[,i])-min(sero2[,i]))/ (max(sero2[,i])-min(sero2[,i]) )
test2[,(i-6)]<-a
 }

 names(test2)<-names(sero2) [7:14]

# scaled by log transformations of IBD, Salmonella and PM and square
root transforations of counts
test3<- as.data.frame(matrix(0,nrow(sero2),8))
 for(i in 7:9)
 {
a <- log(2+sero2[,i])
test3[,(i-6)]<-a
 }

 test3[,4]<-sero2$AdjMDV
 test3[,8]<-sero2$Cnemido
 for(i in 11:13)
 {
a <- sqrt(sero2[,i])
test3[,(i-6)]<-a
 }

  names(test3)<-names(sero2) [7:14]

 #  standardise by Hellinger transformation of parasite counts and
min/max scaling of serological valuessummary(
 chord<-decostand(sero2[,11:13],"hel")
 test4 <-cbind   (test2[,1:4],chord, test2$Cnemido)
 names(test4)[8]<-"Cnemido"


 # dichotomise data
  setwd('M:/Documents/Poultry Disease/R stuff/Bird analysis/')
birds <- read.csv("basic bird.csv", header=T, sep=,)
```

```
prop.table(table(birds$AdjIBD> 0.225))
prop.table(table(birds$AdjPM> -0.41))
prop.table(table(birds$AdjSal> -0.84))
prop.table(table(birds$AdjMDV> 0.125))

 bin<-  (sero2[,7:14])
 0.306-mean(birds$IBDV, na.rm=T)
bin$IBDpos<-ifelse(bin$AdjIBD>0.27,1,0)
 0.41-   mean(birds$Pasteurella, na.rm=T)
bin$PMpos<-ifelse(bin$AdjPM> -0.52,1,0)
 mean(birds$Salmonella, na.rm=T)  -0.84
bin$Salpos<-ifelse(bin$AdjSal> -0.68,1,0)
0.125+    mean(birds$MDVnet, na.rm=T)
bin$MDVpos<-ifelse(bin$AdjMDV> 0.34,1,0)
bin$Ascpos<-ifelse(bin$AscType> 0,1,0)
bin$Eimpos<-ifelse(bin$Coccidia> 0,1,0)
bin$Licepos<-ifelse(bin$LiceTot> 0,1,0)
bin$SLpos<-ifelse(bin$Cnemido> 0,1,0)
test5<-bin[,9:16]
 names(test5)<-names(test4)


#################################
# Figure 6.1 Correlations between disease variables

panel.hist <- function(x, ...)
{
    usr <- par("usr"); on.exit(par(usr))
    par(usr = c(usr[1:2], 0, 1.5) )
    h <- hist(x, plot = FALSE)
    breaks <- h$breaks; nB <- length(breaks)
    y <- h$counts; y <- y/max(y)
    rect(breaks[-nB], 0, breaks[-1], y, col = "cyan", ...)
}

panel.cor <- function(x, y, digits = 2, prefix = "", cex.cor, ...)
{
    usr <- par("usr"); on.exit(par(usr))
    par(usr = c(0, 1, 0, 1))
    r <- abs(cor(x, y))
    txt <- format(c(r, 0.123456789), digits = digits)[1]
    txt <- paste(prefix, txt)
    if(missing(cex.cor)) cex.cor <- 0.8/strwidth(txt)
    text(0.5, 0.5, txt, cex = cex.cor * ((r)^(1/3)))
}

labs<-c("IBD", "Pasteurella", "Salmonella","MDV","Ascaridida",
"Coccidia","Lice")

windows(18,18)
pairs(sero2[,7:13], labels=labs, cex.labels=1.5,font.labels=
3,lower.panel=panel.smooth, upper.panel=panel.cor, diag.panel=
panel.hist, pch=21, bg=rgb(.2,.2,.2,.1))

#####################################
#Figure 2 Variation in the dataset represented by the PCA axes
sero.pca<-rda(test1, scale=T)
summary(sero.pca)

ev<-(sero.pca$CA$eig)
ev[ev>mean(ev)]
```

```
windows(12,6)
par(mfcol=c(1,1))
barplot(ev,main="Eigenvalues", col="bisque", las=2)
abline(h=mean(ev), col="red", lwd=2)
legend("topright", "Average Eigenvalue", lwd=2, col=2, bty="n",
cex=1.5)

########################################
Figure 6.3 Biplots of PCA

library(rgl)
d<-data.frame(summary(sero.pca)[2])
plot3d(d[,1], d[,2], d[,3], size=5)

labs<-c("IBD", "Pasteurella", "Salmonella","MDV",
"Eimeria","Ascaridida","Lice","Scaly leg")
pos1<-c( 4,4,4,2,1,2,2,1)
pos2<-c( 4,4,4,2,2,1,3,1)
pos3<-c( 1,3,1,2,2,2,3,2)

windows(15,6)
par(mfrow=c(1,3))

biplot(sero.pca, main="a)", cex.main=2, cex.lab=1.7,
display="sites",  xlim=c(-1.5,4))

test<-scores(sero.pca,choices=1:2,scaling=2,display="sp")
   arrows(0,0,test[,1],test[,2],length=0,lty=1,lwd=1)
text(  test[,1],test[,2],labs,cex=1.5,offset=1,pos=pos1)

biplot(sero.pca, main="b)",choices=c(1,3), cex.main=2, cex.lab=1.7,
display="sites", xlim=c(-2,4))

test<-scores(sero.pca,choices=c(1,3),scaling=2,display="sp")
   arrows(0,0,test[,1],test[,2],length=0,lty=1,lwd=1)
text(  test[,1],test[,2],labs,cex=1.5,offset=1,pos=pos2)

biplot(sero.pca, main="c)", choices=c(2,3),cex.main=2, cex.lab=1.7,
display="sites", ylim=c(-3,2.5))

test<-scores(sero.pca,choices=c(2,3),scaling=2,display="sp")
   arrows(0,0,test[,1],test[,2],length=0,lty=1,lwd=1)
text(  test[,1],test[,2],labs,cex=1.5,offset=1,pos=pos3)

###############################
#Figure 6.4  Biplots after binarisation of the response data
pos7<-c( 1,1,4,2,1,1,2,1)
pos8<-c( 3,4,4,2,3,3,1,1)
pos9<-c( 2,3,3,2,3,3,2,1)

sero.pca<-rda(test5, scale=T)

windows(15,6)
 par(mfrow=c(1,3))

biplot(sero.pca, main="a)", cex.main=2, cex.lab=1.7,
display="sites", xlim=c(-1.5,4))
test<-scores(sero.pca,choices=1:2,scaling=2,display="sp")
   arrows(0,0,test[,1],test[,2],length=0,lty=1,lwd=1)
text(  test[,1],test[,2],labs,cex=1.5,offset=1,pos=pos7)
```

```
biplot(sero.pca, main="b)",choices=c(1,3), cex.main=2, cex.lab=1.7,
display="sites", xlim=c(-2,4))

test<-scores(sero.pca,choices=c(1,3),scaling=2,display="sp")
   arrows(0,0,test[,1],test[,2],length=0,lty=1,lwd=1)
text(  test[,1],test[,2],labs,cex=1.5,offset=1,pos=pos8)

biplot(sero.pca, main="c)", choices=c(2,3),cex.main=2, cex.lab=1.7,
display="sites", ylim=c(-3,3))

test<-scores(sero.pca,choices=c(2,3),scaling=2,display="sp")
   arrows(0,0,test[,1],test[,2],length=0,lty=1,lwd=1)
text(  test[,1],test[,2],labs,cex=1.5,offset=1,pos=pos9)

##########################
#Figure 6.5 Biplots after Hellinger transformation of the parasite
data
pos4<-c( 4,4,3,2,3,4,1,3)
pos5<-c( 4,4,4,1,2,1,3,3)
pos6<-c( 4,3,3,2,3,1,3,3)

sero.pca<-rda(test4, scale=T)
 windows(15,6)
 par(mfrow=c(1,3))

biplot(sero.pca, main="a)", cex.main=2, cex.lab=1.7,
display="sites", xlim=c(-1.5,4))

test<-scores(sero.pca,choices=1:2,scaling=2,display="sp")
   arrows(0,0,test[,1],test[,2],length=0,lty=1,lwd=1)
 text(  test[,1],test[,2],labs,cex=1.5,offset=1,pos=pos4)

biplot(sero.pca, main="b)",choices=c(1,3), cex.main=2, cex.lab=1.7,
display="sites", xlim=c(-2,4))

test<-scores(sero.pca,choices=c(1,3),scaling=2,display="sp")
   arrows(0,0,test[,1],test[,2],length=0,lty=1,lwd=1)
 text(  test[,1],test[,2],labs,cex=1.5,offset=1,pos=pos5)

biplot(sero.pca, main="c)", choices=c(2,3),cex.main=2, cex.lab=1.7,
display="sites", ylim=c(-3.5,3))

test<-scores(sero.pca,choices=c(2,3),scaling=2,display="sp")
   arrows(0,0,test[,1],test[,2],length=0,lty=1,lwd=1)
 text(  test[,1],test[,2],labs,cex=1.5,offset=1,pos=pos6)

############################
 #RDA

sero2<-na.omit(sero[,c(1,2,3,5,6,9:11,13:16,17,19:22,23,31:37)])
sero2$Season<-
ifelse(sero2$SeasonID=="A"|sero2$SeasonID=="C","Dry","Wet")
sero2$VS<- paste(sero2$VillageID, sero2$SeasonID)
 YT <- as.data.frame(scale(sero2[,c(18:25)]))
 W<-  sero2[,c(2,6:17,27)]

 bdsrda<-rda(YT~.,W[,-1])
 summary(bdsrda)
 coef(bdsrda)
  anova.cca(bdsrda,step=1000)
```

```
  anova.cca(bdsrda,by="axis",step=1000)
  d<-data.frame(summary(bdsrda)[2])
  RsquareAdj(bdsrda)$adj.r.squared

#variable selection to be retained in the model - takes a bit of
time to run
(step.forward<- ordistep(  rda(YT~1,data=W[,-1]) ,
scope=formula(bdsrda),direction="forward",pstep=1000))

#model partialling out household variation
parsrda<-rda(YT ~ relevel(LayState,"E") + BirdWt+
Age+BCS+Source+VS+Condition(FarmUnID), data = W  )

summary(parsrda)

anova.cca(parsrda,step=1000)
anova.cca(parsrda,by="axis",step=1000)

(step.forward<- ordistep(  rda(YT~1,data=W[,c(1,9:14)]) ,
scope=formula(parsrda),direction="forward",pstep=1000))

#construction of most parsimonius RDA models
sero3<-na.omit(sero[,c(1,2,9:10,14,17,21,23,31:37)])

YT <- as.data.frame(scale(sero3[,c(8:15)]))
W2<-   sero3[,c(1:7)]

hhsrda<-rda(YT ~ relevel(LayState,"E") + BirdWt+  ChckSick
+GrowSick+HseSpray, data = W2  )

parsrda<-rda(YT ~ relevel(LayState,"E") + BirdWt+
Condition(FarmUnID), data = W2  )


##############
#Figure 6.6 Triplots

windows(18,10)
vars1<- c("Rearing","In lay","Not in lay","Male","Weight","Chick
deaths", "Grower deaths", "Spray")
pos1<-c( 1,3,4,2,1,3,3,3)
labs<-c("Scaly leg","IBD", "Pasteurella", "Salmonella","MDV",
"Eimeria","Ascaridida","Lice")
pos2<-c( 2,3,4,4,2,4,3,2)

par(mfrow=c(1,2))
plot(hhsrda,type="n",scaling=2,choices=c(1,2), main="Without
household as fixed effect",lwd=2,display=c("lc"),  col="darkblue",
xlim=c(-1.1,1.2),ylim=c(-0.6,0.6))

test<-scores(hhsrda,choices=c(1,2),scaling=2,display="bp")
 arrows(0,0,test[,1],test[,2],length=0,lty=4,lwd=2)
text(  test[,1],test[,2],vars1, offset=1,pos=pos1, cex=0.85, font=3)

test<-scores(hhsrda,choices=c(1,2),scaling=2,display="sp")
 arrows(0,0,test[,1],test[,2],length=0,lty=1,lwd=2)
text(  test[,1],test[,2],labs, offset=0.5, pos=pos2)

   pos3<-c( 1,4,4,2,1)
   pos4<-c( 1,4,4,4,3,4,1,2)
```

```
plot(parsrda,type="n", scaling=2,choices=c(1,2), main="Including
household as fixed effect",lwd=2,display="lc",xlim=c(-1,0.9),
ylim=c(-1,1))

test<-scores(parsrda,choices=c(1,2),scaling=2,display="bp")
arrows(0,0,test[,1],test[,2],length=0,lty=4,lwd=2)
text(  test[,1],test[,2],vars1[1:5],font=3, offset=1, cex=0.9,
pos=pos3)

test<-scores(parsrda,choices=c(1,2),scaling=2,display="sp")
arrows(0,0,test[,1],test[,2],length=0,lty=1,lwd=2)
text(  test[,1],test[,2],labs, offset=0.5, pos=pos4)

  ###################################################

#Appendix 6.1
sero.pca<-rda(test1, scale=T)
summary(sero.pca)

#Appendix 6.2
 summary(hhsrda)

 #Appendix 6.3
 summary(parsrda)
```

Chapter 7

```
library(binom)
library(lme4)
library(mgcv)
library(RgoogleMaps)
library(geoR)
library(RColorBrewer)
library (lqmm) # Needs R package 3.1 or above

setwd('M:/Documents/Poultry Disease/R stuff/MyFunctions/')
 source("Regression outputs.r") #Code for summarising model outputs
 source("spatial test function oct2012.R") #Code for spatial test

setwd('M:/Documents/Poultry Disease/R stuff/Survival analysis/')
suv <- read.csv("multioutcome.csv", header=T, sep=,)

setwd('M:/Documents/Poultry Disease/R stuff/Multivariate analysis/')
sero <- read.csv("svepm data.csv", header=T, sep=,)

setwd('M:/Documents/Poultry Disease/R stuff/Bird analysis/')
birds <- read.csv("basic bird.csv", header=T, sep=,)

birds$SalHigh<-ifelse(birds$Salmonella>2,1,0)

for(i in c(75:76,78:79) )
{
birds[which(is.na(birds[,i])),i]<-0
}
birds$LiceTot<-rowSums(birds[,75:79])

 birds$Season<-"Dry"
  birds[which(birds$SeasonID=="A"), 84]<-"Wet"
  birds[which(birds$SeasonID=="C"), 84]<-"Wet"
  table(birds$Season, birds$SeasonID)

  xx<-suv[match(birds$UniqueID,suv$UniqueID),c(1,32:45,49:52)]
  yy<-sero[match(birds$UniqueID,sero$UniqueID),c(1,13:16)]

birds<-cbind(birds,xx[,2:18], yy[2:5])

setwd('M:/Documents/Poultry Disease/R Stuff/')
blood <- read.csv("Plasma record.csv", header=T, sep=,)
birds$CKCL<-    blood$CKCL[match(birds$UniqueID,blood$SampleID)]

##################################
#Table 7.1
sum(complete.cases(  birds$AdjPM))

binom.confint(x=sum(birds$AdjPM+mean(birds$Pasteurella,na.rm=T)>0.61
, na.rm=T),  sum(complete.cases(  birds$AdjPM)), conf.level=0.95,
method="wilson")

binom.confint(x=sum(birds$AdjPM+mean(birds$Pasteurella,na.rm=T)>0.3,
na.rm=T),  sum(complete.cases(  birds$AdjPM)), conf.level=0.95,
method="wilson")

binom.confint(x=sum(birds$AdjPM+mean(birds$Pasteurella,na.rm=T)>0.5,
```

```
na.rm=T),  sum(complete.cases(  birds$AdjPM)), conf.level=0.95,
method="wilson")

binom.confint(x=sum(birds$AdjPM+mean(birds$Pasteurella,na.rm=T)>3,
na.rm=T),  sum(complete.cases(  birds$AdjPM)), conf.level=0.95,
method="wilson")

sum(complete.cases(  birds$AdjSal))

binom.confint(x=sum(birds$AdjSal+mean(birds$Salmonella,na.rm=T)>0.34
, na.rm=T),  sum(complete.cases(  birds$AdjSal)), conf.level=0.95,
method="wilson")

binom.confint(x=sum(birds$AdjSal+mean(birds$Salmonella,na.rm=T)>0.3,
na.rm=T),  sum(complete.cases(  birds$AdjSal)), conf.level=0.95,
method="wilson")

binom.confint(x=sum(birds$AdjSal+mean(birds$Salmonella,na.rm=T)>0.5,
na.rm=T),  sum(complete.cases(  birds$AdjSal)), conf.level=0.95,
method="wilson")

binom.confint(x=sum(birds$AdjSal+mean(birds$Salmonella,na.rm=T)>3,
na.rm=T),  sum(complete.cases(  birds$AdjSal)), conf.level=0.95,
method="wilson")

sum(complete.cases(  birds$IBDV))

binom.confint(x=sum(birds$IBDV>0.285, na.rm=T),  sum(complete.cases(
birds$AdjIBD)), conf.level=0.95, method="wilson")

binom.confint(x=sum(birds$AdjIBD+mean(birds$IBDV,na.rm=T)>0.265,
na.rm=T),  sum(complete.cases(  birds$AdjIBD)), conf.level=0.95,
method="wilson")

sum(complete.cases(  birds$AdjMDV))

binom.confint(x=sum(birds$AdjMDV+mean(birds$MDVnet,na.rm=T)>0.29,
na.rm=T),  sum(complete.cases(  birds$AdjMDV)), conf.level=0.95,
method="wilson")

binom.confint(x=sum(birds$AdjMDV+mean(birds$MDVnet,na.rm=T)>0.3,
na.rm=T),  sum(complete.cases(  birds$AdjMDV)), conf.level=0.95,
method="wilson")

binom.confint(x=sum(birds$AdjMDV+mean(birds$MDVnet,na.rm=T)>0.5,
na.rm=T),  sum(complete.cases(  birds$AdjMDV)), conf.level=0.95,
method="wilson")

binom.confint(x=sum(birds$AdjMDV+mean(birds$MDVnet,na.rm=T)>3,
na.rm=T),  sum(complete.cases(  birds$AdjMDV)), conf.level=0.95,
method="wilson")

################################
#Figure 7.1

windows(12,8)
par(mfcol=c(2,2))

bob<-seq(-0.5,13.7,0.2)
pal<-c(rep("steelblue3",4),"gold",rep("orangered2",14),
rep("darkred",58))
```

```
hist(birds$AdjPM +mean(birds$Pasteurella,na.rm=T), breaks= bob,
col=pal, xlab="Pasteurella s:p ratio", main="", xlim=c(-0.5,10),
ylim=c(0,200))

bob<-seq(-0.5,19.1,0.2)
pal<-c(rep("steelblue3",4),"gold",rep("orangered2",14),
rep("darkred",58))

hist(birds$AdjSal +mean(birds$Salmonella,na.rm=T), breaks= bob,
col=pal, xlab="Salmonella s:p ratio", main="",xlim=c(-0.5,10),
ylim=c(0,200))

bob<-seq(-0.254,1.486,0.02)
pal<-c(rep("steelblue3",27),"gold",rep("orangered2",56),
rep("darkred",52))

hist(birds$AdjIBD , breaks= bob, col=pal, xlab="IBD s:p ratio",
main="", xlim=c(-0.26,1.5),ylim=c(0,200))

legend("topright",c("Negative", "Equivocal", "Positive","High
positive"),fill=c("steelblue3","gold","orangered2","darkred"),
bty="n")

bob<-seq(-2.3,3.8,0.1)
pal<-c(rep("steelblue3",26),rep("gold",2),rep("orangered2",14),
rep("darkred",58))

hist(birds$AdjMDV +mean(birds$MDVnet,na.rm=T), breaks= bob, col=pal,
xlab="MDV s:p ratio", main="",xlim=c(-2.5,4))

###########################
   #Figure 7.2
     (P0<-glmer(Pasteurella ~
1+(1|RegionID/MarkShID/VillageID/FarmUnID)+(1|PMPlate),
data=birds[which(birds$Pasteurella<10),],REML = T,
na.action=na.omit, family="gaussian"))

   (S0<-glmer(Salmonella ~
1+(1|RegionID/MarkShID/VillageID/FarmUnID)+(1|SalPlate),
data=birds[which(birds$Salmonella<10),],REML = T, na.action=na.omit,
family="gaussian"))

   (I0<-glmer(IBDV ~
1+(1|RegionID/MarkShID/VillageID/FarmUnID)+(1|IBDPlate),
data=birds,REML = T, na.action=na.omit, family="gaussian"))

   (M0<-glmer(MDVnet ~
1+(1|RegionID/MarkShID/VillageID/FarmUnID)+(1|MDVPlate),
data=birds,REML = T, na.action=na.omit, family="gaussian"))


   (P0b<-glmer(Pasteurella >0.5~
1+(1|RegionID/MarkShID/VillageID/FarmUnID)+(1|PMPlate),
data=birds[which(birds$Pasteurella<10),],REML = T,
na.action=na.omit, family="binomial"))

   (S0b<-glmer(Salmonella>0.5 ~
1+(1|RegionID/MarkShID/VillageID/FarmUnID)+(1|SalPlate),
data=birds[which(birds$Salmonella<10),],REML = T, na.action=na.omit,
family="binomial"))
```

```
   (I0b<-glmer(IBDV >0.285~
1+(1|RegionID/MarkShID/VillageID/FarmUnID)+(1|IBDPlate),
data=birds,REML = T, na.action=na.omit, family="binomial"))

   (M0b<-glmer(MDVnet>0.3 ~
1+(1|RegionID/MarkShID/VillageID/FarmUnID)+(1|MDVPlate),
data=birds,REML = T, na.action=na.omit, family="binomial"))

res<-matrix(NA,6,4)
row.names(res)<-c("Plate","Region","Market Shed","Village","Farm",
"Bird")

res[,1]<-c( as.numeric(VarCorr(P0)[c(2,5,4,3,1)]) ,
attr(VarCorr(P0), "sc")^2 )
res[,2]<-c( as.numeric(VarCorr(S0)[c(2,5,4,3,1)]) ,
attr(VarCorr(S0), "sc")^2 )
res[,3]<-c( as.numeric(VarCorr(I0)[c(2,5,4,3,1)]) ,
attr(VarCorr(I0), "sc")^2 )
res[,4]<-c( as.numeric(VarCorr(M0)[c(2,5,4,3,1)]) ,
attr(VarCorr(M0), "sc")^2 )

resb<-matrix(NA,5,4)
row.names(resb)<-c("Plate","Region","Market Shed","Village","Farm")

 resb[,1]<-as.numeric(VarCorr(P0b)[c(2,5,4,3,1)])
  resb[,2]<- as.numeric(VarCorr(S0b)[c(2,5,4,3,1)])
   resb[,3]<-as.numeric(VarCorr(I0b)[c(2,5,4,3,1)])
    resb[,4]<-as.numeric(VarCorr(M0b)[c(2,5,4,3,1)])

 resb1<-resb
 resb1<-rbind( resb1,rep(pi,4))
 row.names(resb1)[6]<-"Residual"

res1<-res
 res1[,1:4]<- 100*(prop.table(res[,1:4],2))
 resb1[,1:4]<- 100*(prop.table(resb1[,1:4],2))

allres<-cbind(res1[,1],resb1[,1],res1[,2],resb1[,2],res1[,3],
resb1[,3],res1[,4],resb1[,4])

cols<-brewer.pal(5,"Reds")

  xtext<-c( -allres[1,c(1,4,5,7)]-3)
  ytext<-seq(16.89,2,-4.5)
  bartext<-c("Pasteurella","Salmonella","IBDV","MDV")
  barfonts<-c(3,3,1,1)
  gap<-rep(c(2,0.5),4)

windows(15,8)
par(fig=c(0.21,1,0,1), mar=c(5,0,5,10), xpd=T)
  a<-barplot(allres[-1,c(8:1)], beside=F, horiz=T, col=cols,
space=gap, main="Percentage of variance contibuted", xlim=c(0,100))
   legend(105,20,  rownames(res)[2:6],fill=cols, title="Levels",
bty="n")
  par(fig=c(0,0.20,0,1), mar=c(5,5,5,0), new=T)
   b<-barplot( -allres[1,c(8:1)],  horiz=T, col="steelblue3",
space=gap, main="ELISA Plate variance", axes=F)
    text(xtext,ytext,bartext,font=barfonts, adj=1,cex=1.2)
     text(xtext[1:2]+1,ytext[1:2]+0.3,c(1,2), adj=1,cex=0.8)
```

```
#########################################
Pasteurella model

(m4<-lmer(log(1+Pasteurella) ~
(Outbreak>0)*RegionID+(RainFeed=="N")+SeasonID
+RegionID*BirdWt+relevel(LayState,"L")+AdjSal*(AdjSal<1)+(1|PMPlate)
+ (1|FarmUnID), data=full,REML = T, na.action=na.omit))

summary(m4)

lin.reg.outputs(m4)


#########################################
Salmonella model

full<-na.omit(birds[ ,c(1,5:9,17,67:68,66,13,74,63,84,106)])

ms11<-glmer(log(1+Salmonella)
~Season+relevel(VillageID,"J2B")+AdjIBD+Sex+AdjMDV+ Season* AdjPM
+Season*CKCL +(1|SalPlate)+ (1|FarmUnID), data=full,REML = T,
na.action=na.omit, family="gaussian")

summary(ms11)

lin.reg.outputs(ms11)

#####################################
#Figure 7.3

model<-ms11
 fixef(model)
summary(full$AdjPM)
 hl1<-seq(-2,11,length.out=100)
sD<-fixef(model)[1]+fixef(model)[13]*hl1
sW<-
fixef(model)[1]+fixef(model)[13]+fixef(model)[13]*hl1+fixef(model)[1
5]*hl1

c1<-c("darkred", "orange", "black", "black")

par(mfrow=c(1,1))
plot(sD,x=hl1+ 1.021, type="l", col=c1[1], lwd=2, ylab="Predicted
Salmonella titre", xlab="Explanatory s:p ratio", ylim=c(0,3),
xlim=c(-2,12))
lines(sW,x=hl1+ 1.021, type="l", col=c1[2], lwd=2)

summary(full$CKCL)
 hl1<-seq(-2,12,length.out=100)
cD<-fixef(model)[1]+fixef(model)[14]*hl1
cW<-
fixef(model)[1]+fixef(model)[14]+fixef(model)[14]*hl1+fixef(model)[1
6]*hl1

lines(cD,x=hl1, type="l", col=c1[1], lwd=2,lty=2 )
lines(cW,x=hl1, type="l", col=c1[2], lwd=2, lty=2)

legend("bottomright", c("Dry", "Wet","Pasteurella", "Cell
lysate"),bty="n", lwd=2,col=c1,lty=c(1,1,1,2))
```

```
##########################
Table 7.4

a<-  chisq.test(table( birds$VillageID, birds$Salmonella>2))
prop.table( a$observed,1) [,2]

###############################
#Quantile regression

full<-na.omit(birds[ ,c(1,5:9,17,67:68,66,13,74,63,84,106)])

res<-as.data.frame(matrix(0,8,18))
res[,1]<-seq(0.2,0.9,0.1)

seres<-as.data.frame(matrix(0,8,18))
seres[,1]<-seq(0.2,0.9,0.1)

for(i in 1:nrow(res))
{
salqm<-
lqmm(fixed=Salmonella~Season+relevel(VillageID,"J2B")+AdjIBD+Sex+
AdjMDV+Season* AdjPM +Season*CKCL , random=~1,group=SalPlate,
tau=res[i,1], nK=7, type="normal", data=full)
s<-summary(salqm)
res[i,2:17]<-coef(salqm)
res[i,18]<-VarCorr(salqm)
seres[i,2:17]<-s$tTable[,2]
}

names(res)<-c("tau",names(coef(salqm)),"Plate")
names(seres)<-c("tau",names(coef(salqm)),"Plate")

s<- summary(salqm)
s
s$tTable
res

ms12<-lmer(log(1+Salmonella)
~Season+relevel(VillageID,"J2B")+AdjIBD+Sex+ AdjMDV+Season* AdjPM
+Season*CKCL +(1|SalPlate), data=full,REML = T, na.action=na.omit)
summary(ms12)

village<-levels(birds$VillageID)
nom<-
c("","Intercept","Season",village[1:7],"IBD","Sex","MDV","Pasteurell
a","Cell lysate", "Pasteurella:Season interaction", "Cell lysate:
Season interaction")


#Figure 7.4
windows (12,20)
par(mfrow=c(4,2))
for(i in c(2,4:10))
{
plot(res$tau,res[,i], ylim=c(-0.5,1.5),type="l",main=nom[i],
xlab="Quantile", ylab="Parameter estimate")
lines(res$tau, res[,i]+(2*seres[,i]), lty=2)
lines(res$tau, res[,i]-(2*seres[,i]), lty=2)
lines(x=c(0.1,1), y=c(rep(fixef(ms12)[i-1],2)), lty=3, col="red")
lines(x=c(0.1,1), y=c(0,0), lty=3)
}
```

```
yl<-c(0,0,-0.5,0,0,0,0,0,0,0,-1,-1,-0.5,-0.5,-0.5,-0.5,-1)
yu<-c(0,0,1,0,0,0,0,0,0,0,2,0.5,0.5,1,1,1,0.5)

Figure 7.5
windows (12,20)
par(mfrow=c(4,2))
for(i in c(3,11:17))
{
plot(res$tau,res[,i], ylim=c(yl[i],yu[i]),type="l",main=nom[i],
xlab="Quantile", ylab="Parameter estimate")
lines(res$tau, res[,i]+(2*seres[,i]), lty=2)
lines(res$tau, res[,i]-(2*seres[,i]), lty=2)
lines(x=c(0.1,1), y=c(rep(fixef(ms12)[i-1],2)), lty=3, col="red")
lines(x=c(0.1,1), y=c(0,0), lty=3)
}

########################################
IBD model

full<-na.omit(birds[  ,c(1,5:9,61,62,70,100)])
  (m3<-lmer(IBDV ~
AdjSal+GrowSick+(VillageID=="H1A")+SeasonID+(1|IBDPlate)+
(1|FarmUnID), data=full,REML = T, na.action=na.omit))
lin.reg.outputs(m3)

########################################
Figure 7.6

full$Latitude<-  birds$Latitude[    match(
full$UniqueID,birds$UniqueID)]
full$Longtude<-  birds$Longtude[    match(
full$UniqueID,birds$UniqueID)]

farm<-unique(full[,c(2:6,11,12)])
nrow(farm)

farmeff<-ranef (m3)$FarmUnID[[1]]
summary(farmeff)
farm<-cbind(farm,farmeff)

#plot farm effects by market shed
library(RgoogleMaps)

H1<-farm[which(farm$MarkShID=="H1"),]

windows(10,10)

H1s<-PlotOnStaticMap( lat=H1$Latitude, lon=H1$Longtude,  zoom=7,
size = c(640,640), GRAYSCALE  = FALSE,add=FALSE, FUN = points,
pch="", mar=c(0,0,0,0), NEWMAP = TRUE, TrueProj = TRUE, axes= FALSE,
verbose = 1)

PlotOnStaticMap( MyMap=H1s,lat=H1[H1$SeasonID=="A",6],
lon=H1[H1$SeasonID=="A",7],   zoom=14, size = c(640,640), GRAYSCALE
= FALSE,  add=T, FUN = points,col=c("darkred"), pch=19,
cex=4*(0.1+3*H1[H1$SeasonID=="A",8]), mar=c(0,0,0,0), NEWMAP = F,
TrueProj = TRUE, axes= FALSE, verbose = 1)
```

```
PlotOnStaticMap( MyMap=H1s,lat=H1[H1$SeasonID=="B",6],
lon=H1[H1$SeasonID=="B",7],   zoom=14, size = c(640,640), GRAYSCALE
= FALSE,add=T, FUN = points,col=c("orangered"),
pch=19,cex=4*(0.1+3*H1[H1$SeasonID=="B",8]),mar=c(0,0,0,0), NEWMAP =
F, TrueProj = TRUE, axes= FALSE, verbose = 1)

PlotOnStaticMap( MyMap=H1s,lat=H1[H1$SeasonID=="C",6],
lon=H1[H1$SeasonID=="C",7],   zoom=14, size = c(640,640), GRAYSCALE
= FALSE,add=T, FUN = points,col=c("orange"),
pch=19,cex=4*(0.1+3*H1[H1$SeasonID=="C",8]),mar=c(0,0,0,0), NEWMAP =
F, TrueProj = TRUE, axes= FALSE, verbose = 1)

PlotOnStaticMap( MyMap=H1s,lat=H1[H1$SeasonID=="D",6],
lon=H1[H1$SeasonID=="D",7],   zoom=14, size = c(640,640), GRAYSCALE
= FALSE, add=T, FUN = points,col=c("yellow1"),
pch=19,cex=4*(0.1+3*H1[H1$SeasonID=="D",8]),mar=c(0,0,0,0), NEWMAP =
F, TrueProj = TRUE, axes= FALSE, verbose = 1)

legend("topright", c("Season A", "Season B", "Season C", "Season
D"), col=c("darkred","orangered","orange", "yellow1"), pch=19 ,
bty="n" , cex=1.5, pt.cex=0.75 )


#Spatial test

test<-data.frame(farm$VillageID, farm$MarkShID,
farm$SeasonID,farm$Longtude,farm$Latitude,  farm$farmeff)

head(test)
H1.A<-na.omit(test[test[,2]=="H1" ,4:6])
H1.A<-test[test[,1]=="H1A" & test[,3]=="B",4:6]
test1 <- spatial.test(H1.A, u.vector=seq(0,1,0.001), nsim=10000)

########################################
MDV model

birds$MDV<-ifelse (birds$MDVxreact==1,NA,birds$MDVnet)
full<-na.omit( birds[,c(1,5:9,71,17,70,107)])
full$sv<-paste(full$VillageID,full$SeasonID)

mm2<-lmer(MDV ~ relevel(as.factor(sv),"J1B
D")+relevel(LayState,"N/A")+AdjSal*(AdjSal<0.5)+(1|MDVPlate)+
(1|FarmUnID), data=full,REML = T, na.action=na.omit)

summary(mm2)
lin.reg.outputs(mm2)

#Figure 7.7
full1<-na.omit( birds[,c(1,5:9,74)])
  full1$sv<-paste(full1$VillageID,full1$Season)
  full1$MDpos<-
ifelse(full1$AdjMDV+mean(birds$MDVnet,na.rm=T)>0.5,1,0)
  x<-aggregate(  full1$MDpos, by=list(v=full1$sv),sum)
  n<-aggregate(  complete.cases(full1$MDpos),
by=list(v=full1$sv),sum)
  x<-cbind(x,n[,2])
  x$pp<-x[,2]/x[,3]

   cols<-c("darkred","orangered","orange", "yellow1")
   res<-matrix(x[,4],4,8)
```

```
windows(12,8)
  barplot(res, beside=T,col=cols, names.arg=levels(birds$VillageID))
   legend("topleft", c("Season A", "Season B", "Season C", "Season
D"), fill=c("darkred","orangered","orange", "yellow1"),  bty="n"  )

#########################################
Figure 7.8

(A0b<-glmer(AscType >0~ 1+(1|RegionID/MarkShID/VillageID/FarmUnID),
data=birds,REML = T, na.action=na.omit, family="binomial"))

(B0b<-glmer(Cestodes >0~ 1+(1|RegionID/MarkShID/VillageID/FarmUnID),
data=birds[which(!birds$SeasonID=="A"),],REML = T,
na.action=na.omit, family="binomial"))

(C0b<-glmer(Coccidia>0 ~ 1+(1|RegionID/MarkShID/VillageID/FarmUnID),
data=birds,REML = T, na.action=na.omit, family="binomial"))
(I0b<-glmer(LiceTot >0~ 1+(1|RegionID/MarkShID/VillageID/FarmUnID),
data=birds,REML = T, na.action=na.omit, family="binomial"))

(M0b<-glmer(Cnemido>0 ~ 1+(1|RegionID/MarkShID/VillageID/FarmUnID),
data=birds,REML = T, na.action=na.omit, family="binomial"))

  resb<-matrix(NA,4,5)
  row.names(resb)<-c("Region","Market Shed","Village","Farm")

 resb[,1]<-as.numeric(VarCorr(A0b)[c(4:1)])
  resb[,2]<- as.numeric(VarCorr(B0b)[c(4:1)])
  resb[,3]<- as.numeric(VarCorr(C0b)[c(4:1)])
   resb[,4]<-as.numeric(VarCorr(I0b)[c(4:1)])
    resb[,5]<-as.numeric(VarCorr(M0b)[c(4:1)])

resb1<-resb
rs<-(pi^2)/3
 resb1<-rbind( resb1,rep(rs,5))
 row.names(resb1)[5]<-"Residual"
resb1<- 100*(prop.table(resb1,2))

cols<-brewer.pal(5,"Reds")
  xtext<-rep(100,5)
  ytext<-seq(15.5,2,-3)
  bartext<-c("Ascaridida","Cestodes","Eimeria","Lice","Scaly leg")
  barfonts<-c(1,1,3,1,1)
  gap<-rep(c(2,0.5),4)

windows(12,8)
par(fig=c(0,1,0,1), mar=c(5,5,5,10), xpd=T)

a<-barplot(resb1[,c(5:1)], beside=F, horiz=T, col=cols, space=2,
main="Percentage of variance contibuted", xlim=c(0,100))

legend(105,15,  rownames(resb1),fill=cols, title="Levels", bty="n")

text(xtext,ytext,bartext,font=barfonts, adj=1,cex=1.2)Salmonella
model



##################################
```

```
    # Parasite statistical tests

moment.k<-function(x)
     {
     m<-mean(na.omit(x))
      v<- var(na.omit(x))
      l<- length(na.omit(x))
      p<-v/l
      (m^2 - p)/(v-m)
      }

       D.stat<-function(x)
    {
     y<-sort(na.omit(x))
        m<-mean(na.omit(x))
        l<- length(na.omit(x))
         b<-rep(0,l)
         d<-rep(0,l)

        for(i in 1:l)
        {
        b[i]<-sum(y[1:i])
        d[i]<-mean(y)*i
        }

       B<- sum(b)
       D<-sum(d)
        1-(B/D)
        }

 D.stat(birds$AscType)
 moment.k(birds$AscType)

z<-80
H<- birds[which(birds$RegionID=="H"),z]
J<- birds[which(birds$RegionID=="J"),z]

ks.test(H,J)

#########################################
Ascarid model

full<-na.omit(  birds[,c(1,5:9,13, 14, 74,70,54,90,  80,81)])

(m1b<-
glmer(AscType>0~Sex+(Cnemido>0)+(Coccidia>0)+AdjMDV*relevel(VillageI
D,"H2B")+ (1|FarmUnID), data=full,REML = T, na.action=na.omit,
family="binomial"))

  log.reg.outputs(m1b)

(mc5<-
glmer(AscType~Sex*RegionID+AdjSal+RegionID+(Coccidia>0)*RegionID +
(1|RegionID/FarmUnID),  data=full[which(full$AscType>0
&full$AscType<500),],REML = T, na.action=na.omit, family="poisson"))

   log.reg.outputs(mc5)



#########################################
```

```
Cestode models

full<-na.omit(  birds[which(!birds$SeasonID=="A"),c(1,5:9,66,82)])

(m4b<-glmer(Cestodes>0 ~ AdjPM+
relevel(RegionID,"J")*relevel(SeasonID,"C") +(1|FarmUnID),
data=full,REML = T, na.action=na.omit, family="binomial"))
          log.reg.outputs(m4b)

full<-na.omit(  birds[which(birds$RegionID=="J"&
!birds$SeasonID=="A"),c(1,5:9,13,74,82,21,54,81)])

(m3Jb<-glmer(Cestodes
~relevel(Sex,"M")*(Coccidia>0)+(Cnemido>0)+AdjMDV+relevel(Sex,"M")*B
CS +(1|FarmUnID), data=full[which(full$Cestodes>0),],REML = T,
na.action=na.omit, family="poisson"))

log.reg.outputs(m3Jb)

full<-na.omit(  birds[which(birds$RegionID=="H"&
!birds$SeasonID=="A"),c(1,5:9,13,82,21,54,66,74,81)])

(m3Hb<-glmer(Cestodes ~relevel(Sex,"M")*(Coccidia>0)+(Cnemido>0)
+relevel(Sex,"M")*AdjMDV+AdjPM+relevel(Sex,"M")*BCS+relevel(Sex,"M")
*relevel(SeasonID,"C") +(1|FarmUnID),
data=full[which(full$Cestodes>0),], REML = T, na.action=na.omit,
family="poisson"))

log.reg.outputs(m3Hb)

########################################
Coccidia models

full<-na.omit(  birds[which(birds$PooSourc==0),
c(1,5:9,14,70,82,81)])

cb8<-glmer(Coccidia>0~relevel(VillageID,"H1B")+SeasonID*Age+AdjSal+
(Cestodes>0)+(1|FarmUnID),  data=full,REML = T, na.action=na.omit,
family="binomial")

log.reg.outputs(cb8)


full<-na.omit(  birds[which(birds$PooSourc==0),
c(1,5:9,13,14,70,82,81,80,54)])
full$  Sal.sq<-full$AdjSal^2

c4<-glmer(Coccidia~RegionID+Sex+Age*(Age>9)+AdjSal+Sal.sq+
(Cestodes>0)+RegionID*AscType+(Cnemido>1)+(1|FarmUnID),
data=full[which(full$Coccidia>0),],REML = T, na.action=na.omit,
family="poisson")

log.reg.outputs(c4)



##########################################
Figure 7.9

  nom<-c("Ascaridida","Coccidia","Cestodes")
     fact<-c(20,2,20)
```

```
     windows(14,24)
     par(mfrow=c(3,1))
    for (z in c(80,82,81))
    {
     H<- birds[which(birds$RegionID=="H"),z]
      J<- birds[which(birds$RegionID=="J"),z]

      x<-H
       y<-sort(na.omit(x))
         l<- length(na.omit(x))
         b<-rep(0,l)

        for(i in 1:l)
        {
        b[i]<-sum(y[1:i])
         }

plot(b*fact[z-79],1:l*100/l, type="l", col="red",lwd=2,
ylab="Percentage of birds", xlab="Cumulative number of parasites
shed", main=nom[z-79], xlim=c(0,1.05*fact[z-
79]*(max(sum(na.omit(H)),sum(na.omit(J))))) )

         x<-J
       y<-sort(na.omit(x))
        m<-mean(na.omit(x))
        l<- length(na.omit(x))
         b<-rep(0,l)
         d<-rep(0,l)

        for(i in 1:l)
        {
        b[i]<-sum(y[1:i])
        d[i]<-mean(y)*i
        }
         lines(b*fact[z-79],1:l*100/l, type="l", col="blue", lwd=2)

          }

legend("bottomright" , c("Horro","Jarso"), col=c("red","blue"),
lwd=2, bty="n")


##########################################
Figure 7.10

windows(10,6)

    for (z in 83)
    {
     H<- birds[which(birds$RegionID=="H"),z]
      J<- birds[which(birds$RegionID=="J"),z]

      x<-H
       y<-sort(na.omit(x))
         l<- length(na.omit(x))
         b<-rep(0,l)

        for(i in 1:l)
        {
        b[i]<-sum(y[1:i])
         }
```

```
plot(b,1:l*100/l, type="l", col="red",lwd=2, ylab="Percentage of
birds", xlab="Cumulative number of parasites observed", main="Lice",
xlim=c(0,1.05*(max(sum(na.omit(H)),sum(na.omit(J))))) )

         x<-J
       y<-sort(na.omit(x))
        m<-mean(na.omit(x))
        l<- length(na.omit(x))
         b<-rep(0,l)
         d<-rep(0,l)

        for(i in 1:l)
        {
        b[i]<-sum(y[1:i])
        d[i]<-mean(y)*i
        }
         lines(b,1:l*100/l, type="l", col="blue", lwd=2)
          }

legend("bottomright" , c("Horro","Jarso"), col=c("red","blue"),
lwd=2, bty="n")

################################################
#Lice models

full<-na.omit(  birds[,c(1,5:9,13,14,102:105,83)])

lb3<-glmer(LiceTot>0~RegionID+SeasonID+Sex+HseSmoke+(1|FarmUnID),
data=full,REML = T, na.action=na.omit, family="binomial")

   summary(lb3)
   log.reg.outputs(lb3)

full<-na.omit(  birds[,c(1,5:9,13,14,20,21,66,102:105,83)])

l3<-glmer(LiceTot~Sex+Age*(Age<24)+BirdWt*Sex*BCS+AdjPM+NoPara+
(1|FarmUnID), data=full[which(full$LiceTot>0),],REML = T,
na.action=na.omit, family="poisson")

summary(l3)
log.reg.outputs(l3)


################################################
#Hyperkeratosis models

full<-na.omit(  birds[,c(1,5:9,13,17,81,98,105,54)])
full1<-na.omit(  birds[,c(1,5:9,13,80,81,87,89,2,3,102:106,54)])
full1$Pullets<-ifelse(full1$Pullets>6,6,full1$Pullets)

l1<-glmer(Cnemido>0~relevel(RegionID,"J")*relevel(SeasonID,"D")
+as.factor(DeadDisp)+(Coccidia>0)+relevel(LayState,"N")+HseSmoke+(1|
FarmUnID), data=full,REML = T, na.action=na.omit, family="binomial")

lb1<-glmer(Cnemido>1~BrdSpray+relevel(SeasonID,"A")+Chicks+(
AscType>0) +Sex+(1|FarmUnID), data=full1[which(full1$Cnemido>0),],
REML = T, na.action=na.omit, family="binomial")

log.reg.outputs(l1)
log.reg.outputs(lb1)
```

General Appendix D:

Published paper

This text box is where the unabridged thesis included the following third party copyrighted material:

Bettridge, J.M., Lynch, S.E., Brena, M.C. , Melese, K., Dessie, T., Terfa, Z. G., Desta, T.T. , Rushton, S. Hanotte, O., Kaiser, P., Wigley, P., Christley, R.M. (2014). Infection-interactions in Ethiopian village chickens. *Preventive Veterinary Medicine*. http://dx.doi.org/10.1016/j.prevetmed.2014.07.002