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der Tierärztlichen Hochschule Hannover
und dem Research Department des Wildfowl and Wetlands Trust
in Slimbridge, United Kingdom

Control and Management of Avian Tuberculosis in a Captive Collection of Wildfowl at the Wildfowl and Wetlands Trust Centre in Llanelli, using an Enzyme-linked Immunosorbent Assay as Diagnostic Aid

INAUGURAL – DISSERTATION
Zur Erlangung des Grades einer
Doktorin der Veterinärmedizin
(Dr. vet. med.)
durch die Tierärztliche Hochschule Hannover

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Hannover 2001

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Tag der mündlichen Prüfung: 23.11.2001

Für meine Eltern und meine Schwester

AIDS	Acquired Immunodeficiency Syndrome
BACTEC	Becton and Dickinson diagnostic instrument system
B-cell	Bursa Fabricii derived lymphocyte
b.i.d.	twice a day
°C	degree Celsius
CD3/CD4+/CD8+	cluster of differentiation (antigens on the surface of immune cells)
DNA	desoxyribonuclein acid
EDTA	ethylene diamine tetracetate
ELISA	enzyme-linked immunosorbent assay
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPL	glycopeptidolipid
HIV	Human Immunodeficiency Virus
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
IFNγ	interferon-gamma
Ig	immunoglobulin
IL	interleukin
kDa	kilodalton
µl	microliter
LAM	lipoarabinomannan
LPS	lipopolysaccharide
M	molar
MAI complex	*Mycobacterium avium* - *Mycobacterium intracellulare* complex
MD	megadaltons
MOTT	mycobacteria other than tuberculous mycobacteria
ml	millilitre
mm	millimeter
nm	nanometer
µm	micrometer
NK	natural killer cell
NTM	non-tuberculous mycobacteria
OPD	o-phenylenediamine.
pH	pondus hydrogenii (measurement for hydrogen ion concentration)
PCR	polymerase chain reaction
PPEM	potential pathogenic environmental mycobacteria
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
S	sedimentation coefficient (Svedberg)
s.i.d	once a day
T-cell	thymus-lymphocyte
TGF	transforming growth factor
TMB	3,3',5,5'-tetramethylbenzidine
TNF	tumor necrosis factor
WWT	Wildfowl and Wetlands Trust

A. Introduction

The Wildfowl and Wetlands Trust (WWT) was founded in 1946 by Sir Peter Scott. The aim is to conserve wetlands for wildlife and people via education, research, direct conservation actions and the use of wildfowl in captive breeding and reintroduction programmes. To these ends the Trust maintains several captive collections of wildfowl and manages protected areas of wetlands as reserves to provide refuges for wild birds. The first reserve and captive collection were established at Slimbridge in Gloucestershire, where the headquarters of the Trust remain. Eight other Trust centres have opened since, of which six also hold captive wildfowl, and two providing refuges for wild birds only. Wild birds are encouraged within the grounds of each collection as well as on the surrounding reserves. Captive birds are pinioned and kept in open pens. WWT programmes have successfully bred and reintroduced a number of threatened species to the wild.

The WWT centre at Llanelli, Carmarthenshire, South Wales, is situated on the estuary of the river Loughor. The reserve comprises approximately 300 acres, of which 45 acres are enclosed by a ground-predator-proofed fence and houses a collection of some 1200 wildfowl. Stocking the centre with birds began 1989 and it was opened to the public in 1991.

Avian tuberculosis, caused by *Mycobacterium avium,* has been a major cause of death in adult birds within all WWT collections for several years and is responsible for approximately 40% of annual mortality in the WWT centre at Slimbridge (ASH and CROMIE, 1998). For this reason the WWT centre at Llanelli was stocked entirely with birds hatched on site, or with very young birds (less than three weeks old) supplied from other WWT centres in an attempt to prevent an outbreak of avian tuberculosis. No adult birds have been imported into the collection, with the exception of the flamingos. Flamingos are difficult to hand rear as they rely on ‘crop milk’ from the parents. Since they have found to be largely resistant to avian tuberculosis (BROWN & PICKERING, 1992), any risk in supplying adult birds was thought to be outweighed by the advantage of avoiding the need to hand rear. The risk of transmission of *Mycobacterium avium* from parent bird to chick via the egg is considered to be insignificant (CROMIE, 1991). The low incidence of avian tuberculosis in flamingos is confirmed by findings of CROMIE (1991) and BEEHLER (1990).

The Loughor estuary was already known as an area which attracted significant numbers of wildfowl, the majority of which frequented the shore opposite the site proposed for the centre (PAINTER, 1995). The site itself was farmland with no large wet areas. The reserve area was therefore assumed to be free from any significant level of contamination with *Mycobacterium avium*. Late in the summer of 1994, however, the first cases of avian tuberculosis occurred in different areas of the grounds (PAINTER, 1995).

Avian tuberculosis was first described by CRISP in 1868. By the turn of the century the causative organism had been identified as distinct from that responsible for tuberculosis in humans and was termed *Mycobacterium avium* (GRANGE et al., 1990). Avian tuberculosis is global in its distribution and probably affects all species of birds, although there are variations in the susceptibility of species (GRANGE et al., 1990). The disease has been described frequently in wild birds, particularly in those species of gregarious or carnivorous habits. Among captive collections of birds, avian tuberculosis is the most commonly encountered bacterial infection (CROMIE, 1991). The significance of an outbreak of avian tuberculosis in a collection is considerable due to the potential public health hazard, the economic losses, and the difficulty of replacing certain threatened species.

The disease is characterised by unthriftiness, chronicity, death and persistence in a flock or aviary once established. Signs that a bird is infected are only seen late in the course of the disease, usually shortly before death. Therefore, birds can spread the disease for a long period before disease is detected. The unnaturally high stocking densities found in captive collections and the permanent presence of birds on the same ground allow high levels of contamination to accumulate. Once established in a population the disease is very challenging to eradicate due to difficulty in diagnosis and the resistant, tenacious nature of the bacillus. Avian tuberculosis can therefore easily become a permanent problem in a collection resulting in a high incidence of mortality among adult birds and reduced fertility. The risk of spreading infection means that birds cannot be moved between collections or reintroduced to the wild. There is also a significant risk that infection will be spread to wild birds using the same site (DERICKSON and PICKETT, 1991; BEEHLER, 1990).

In light of the above considerations the need for a useful diagnostic technique to identify the infected individuals is obvious. This thesis reports the development of an improved enzyme-linked immunosorbent assay (ELISA) in an attempt to meet this need. Reliable ELISA results would identify infectious birds and permit the culling of positive birds in order to reduce the build up of infection at the site. It must be borne in mind, however, that total elimination of avian tuberculosis from a collection is not a realistic aim. To gain more information about the distribution of avian tuberculosis at the WWT centre at Llanelli, gross post mortem data from the last ten years have been analysed in consideration of species, age, sex, habitat, stocking density and physical environment. Management and control of the disease will entail a continuing programme of monitoring to try and remove infected individuals at an early stage.

Enzyme linked immunosorbent assay (ELISA) is a labelling technique which demonstrates the presence or absence of antigen or antibody. The technique was first published in 1971 by two independent groups, one in Sweden (ENGVALL and PERLMANN, 1971) and the other in Holland (VAN WEEMEN and SCHUURS, 1971). The first commercial ELISA kit became available in 1976, from Organon Teknika (Eppelheim, Germany), and was used to detect hepatitis B surface antigen. In this case the 'antibody sandwich' method was used to perform an assay. For detection of anti-mycobacterial antibodies in wildfowl, secreted antigens from *Mycobacterium avium* serotypes one and three, from *Mycobacterium vaccae,* and sonicated antigens originating from serotype one and from *Mycobacterium fortuitum* have been used and are coated to the solid phase (PAINTER, 1996; CROMIE et al., 1991). During sample incubation the antibody, if present, binds to the solid phase. After removal of unbound material by washing, an anti-duck immunoglobulin G conjugated with the enzyme horseradish peroxidase is added, which will bind to the antibody. The enzyme can be detected at very low concentrations by the addition of a substrate capable of yielding a detectable signal in the form of flourescence, luminescence or as in this case, colour.

The aim of this thesis is to assess the ELISA's diagnosis accuracy, to develop guidelines for its interpretation and to assess its value as a tool for the ante mortem diagnosis and control of avian tuberculosis in a collection. An important aspect is the useful practicability of the diagnostic technique.

The epidemiological analysis of data of the WWT centre at Llanelli from 1989 to 1999 elucidates the factors affecting the incidence of the disease and the distribution of avian tuberculosis within the grounds. Based on those findings a management and control plan has been developed.

The findings of this thesis shall contribute to the WWT aims to control the disease in its collections via a three pronged attack, namely: vaccination of captive birds, identification and removal of infected individual prior to them becoming faecal excretors of bacilli, and practical collection and environmental management against the disease.

B. Literature review

1. Avian immune system

The immune system is constructed by several integrated components such as non-specific defence and specific defence, which consist of humoral immune system, cell-mediated immune system and the development of tolerance. The interaction of cells and hormone-like mediators or secretions connect the components of the defence system by activating and suppressing each other (GERLACH, 1994b). Immune responsiveness is mainly genetically controlled, however nutrition, environmental factors, the developmental stage of the bird, and products of the endocrine system all influence in the effectiveness of the avian immune response to a great degree (VON KOLLIAS, 1986).

Research about the avian immune system mainly refers to the immune system of chickens. Initial comparisons between the immune systems of chickens, ducks and geese indicate significant similarities (GERLACH, 1994b). On the other hand, one has to bear in mind that the immune system of chickens is well studied but may not be entirely representative of the *Anseriformes*. For example, there are structural differences in antibodies of some of the more 'primitive' birds, such as waterfowl, when compared to those from chicken (HIGGINS and WARR, 1993) and there is no differentiation between T-cell and B-cell populations in ducks found (HIGGINS and CHUNG, 1986). It needs to be pointed out that this chapter reviews mainly the immunology of chickens, therefore, detailed information about the differences between the immune system of chicken and waterfowl is given in paragraph 1.2.1.2.

1.1.Non-specific immune defence

1.1.1. Non-cellular components

Skin and mucosal linings of the intestinal, respiratory, urinary and reproductive tracts act as primary physical barriers to potentially pathogenic micro-organisms and establish environments that are best suited to commensal organisms with a low pathogenicity or none at

all. Colonisation by other, less well adapted and frequently more pathogenic organisms are thereby effectively inhibited (SANDER, 1995; GERLACH, 1994b).

The normal or autochthonous flora in the intestinal tract is species-specific and functions by taking up the available space, occupying receptors and acting competitively against invaders by various mechanisms such as production of a low pH environment, of inhibitory metabolites and of bacteriocins. Furthermore, the so-called lymphoepithelial system is involved in capturing and processing antigen from the mucosal surface and so-called globlet cells on the mucosal surfaces secrete tenacious mucus, which contains lysozyme with its antibacterial and antiviral activities, and immunoglobulin A. Foreign material is transported out of the respiratory tact by cilia on the respiratory mucosa (GERLACH, 1994b).

The complement system and its activation by the classical and alternate pathways have been described in avian species. The classic pathway is activated by the presence of antibody-antigen complexes or non-specifically by the properdin system The complement cascade finishes by developing the ‘membrane attack complex’, which is not as yet defined in the avian system (SANDER, 1995; NEUMANN and KALETA, 1992). Some authors deny the production of properdin in birds (VON KOLLIAS, 1986).

1.1.2. Myeloid system or cellular system

1.1.2.1. Leucocytes

Heterophilic granules contain a variety of enzymes (peroxidases, proteases, hydrolases) and lactoferrin. Lysozyme, which is also found in the granules, splits the bacterial cell wall and allows the destruction and ingestion of bacteria. It is also found in secretions such as tears, saliva, plasma, secretions of the intestinal tract and albumin (NEUMANN and KALETA, 1992). Phagocytosis and destroying foreign material or damaging cells, without having to process them as antigens for presentation to the immune system, are the main functions of the heterophils (GERLACH, 1994b).

There is still rather scarce information on avian eosinophils. Birds do not respond to parasitism with an increased producion of eosinophils, but it appears that eosinophils play a role in hypersensitivity reactions (GERLACH, 1994b).

Avian basophils resemble tissue mast cells morphologically and functionally, although they have a different origin. Having only limited phagocytic abililties, they are equipped with granules containing factors, which increase the permeability of the blood vessels and so facilitate the diapedesis of the leucocytes to the affected area (GERLACH, 1994b; SANDER, 1995).

In *Anseriformes* heterophils are found in the initial phase of infection. There is no further detailed information about structure and function of heterophils, eosinophils and basophils in waterfowl so far (WANG, 1998).

1.1.2.2. Thrombocytes, macrophages and natural killer cells

Avian thrombocytes are capable of phagocytising particles as large as some bacteria (e.g staphylococci) (VON KOLLIAS, 1986). Because of their high number in the circulation system it is possible that they are responsible for most phagocytic activity (CHANG and HAMILTON, 1979, cited by SANDER, 1995). In *Anseriformes* the phagocytic activity of the thrombocytes has yet not been described (WANG, 1998).

All macrophages originate from the bone marrow. They are found throughout the body in the respiratory, gastrointestinal, haemopoietic, and nervous systems, as well as in the coelomic cavity (VON KOLLIAS, 1986). Macrophages may either be fixed in tissues or circulate in the bloodstream as so-called monocytes (SHARMA and TIZARD, 1984). They are furnished with lysosomes containing various substances which are involved in phagocytosis, inducing inflammation, processing antigen to present it to the specific immune system, stimulating an immune response and tissue healing (GERLACH, 1994b). The cells possess receptors for complement factor 3 (C3b-receptors) and antibodies or antibody-antigen complexes (Fc-receptors). Foreign particles that are coated with either antibodies or complement factors can

then be bound to these specific receptors on macrophages and be ingested (SANDER, 1995; NEUMANN and KALETA, 1992). A sub-population of macrophage expresses class II histocompatibility antigens on their surface which co-ordinate the interaction between the antigen-presenting macrophages and the antigen-recognising lymphocytes (GERLACH, 1994b). So far there is no information about the function of mast cells in waterfowl (WANG, 1998).

Cytotoxic natural killer cells (NK) destroy target cells without any obvious stimulation by antigen or the contribution of antibodies. Their task is to destroy aberrant body cells such as tumour cells or those with changed surface structures secondary to infection. They do not damage normal adult or embryonic cells (NEUMANN and KALETA, 1992; GERLACH, 1994b; SANDER, 1995). NK have been described in waterfowl (WANG, 1998).

1.2. Specific defence

The specific defence shows the characteristics of memory abilities, specificity and latency. The antigen-sensitive cells, B- and T-lymphocytes, recognise each antigenic epitope (antigenic determinant) and produce antigen-specific antibodies (humoral immune system), or to provoke cell-mediated reactions (cellular immune system). (GERLACH, 1994b). In this way the specific immune system adapts its actions to the antigen, which provokes the immune response. Depending on prior exposure, specific immune responses may be delayed for two to ten days.

An epitope or the specific site of an antigen is usually a small three-dimensional molecular structure in the antigen comprising only a few molecules. Several epitopes may exist in each antigen molecule and they are not necessarily exclusive to a given antigen. An antibody produced against one antigenic site may therefore react with an epitope of an antigen belonging to an unrelated (e.g. microbial) organism. This cross-reaction between unrelated micro-organisms sharing common antigens can create diagnostic ambiguity in some serologic tests (GERLACH, 1994b).

1.2.1. Humoral system

1.2.1.1. Immunoglobulins

In birds, so far three isotypes of immunoglobulins have been found (IgM, IgG and IgA), of which no subclasses have yet been demonstrated in birds. Immunoglobulins consist of sub-units of two heavy and two light chains (polypeptide chains) connected by disulphide links (GERLACH, 1994b; NEUMANN and KALETA, 1992). In ducks, immunoglobulins which functionally correspond to IgG, so called IgY and IgY(ΔFc) are described (HIGGENS and WARR, 1993).

IgM is the major isotype in the primary immune response. It consists of five Y-shaped sub-units joined in the shape of a star. Hence it is able to bind up to ten identical antigens with its Fab-fragments (TIZARD, 1979). Because of its large size (19S) IgM is normally confined to the peripheral blood stream and is highly effective at opsonisation, agglutination, virus neutralisation and complement activation (SANDER, 1995; GERLACH, 1994b)

In the secondary immune response IgG, in ducks so called IgY and IgY(ΔFc), which are describe in detail in **section 1.2.1.2.** (**Comparison of the avian immunoglobulins**), dominates in the blood. IgG is small with a sedimentation coefficient of 7 S and so it can penetrate tissue spaces and cross body surfaces. Due to its Y-shape it obtains just two $F(ab)_2$-fragments. Its large quantities compensate for its qualitative inferiority to IgM in agglutinating, opsonising and precipitating antigens (SANDER, 1995; TIZARD, 1979).

IgA is found in both monomeric and polymeric forms with joining chain connecting the two sub-units of the dimer. The secretory component prevents its proteolysis. Therefore, it can be secreted onto the mucosal surfaces of the respiratory, genitourinary, digestive and ocular mucosal surfaces by B-cells and plasma cells (TIZARD, 1979). In chickens, IgA is also found in the blood stream and in pigeons, there is a high concentration of IgA in the crop milk (GREUEL, 1988). Its primary role is to prevent antigens from adhering to the mucosal surfaces of the body. Inferior functions are agglutinating antigens and neutralising viruses (GERLACH, 1994b; TIZARD, 1979; NEUMANN and KALETA, 1992).

While maternally derived IgG are transmitted via the yolk, IgM and IgA are secreted by the oviduct into albumen and diffuse from here into the amniotic fluid where they are swallowed by the embryo (GERLACH, 1994b). At hatching, IgG is detected in the serum, while only IgM and IgA are found in the intestine. The avian embryo's swallowing IgA and IgM via amniotic fluid is analogous to a neonatal mammal's consuming colostrum, while the passive transfer of IgG via the yolk represents transplacental transfer of antibody in mammals (VON KOLLIAS, 1986). In ducks, studies of immunoglobulin concentrations in embryos and ducklings show that IgY, as analogue to IgG, is transmitted via the yolk (WANG, 1998).

1.2.1.2. Comparison of the avian immunoglobulins

Initial comparisons between the immune systems of chickens, ducks and geese indicate significant similarities (GERLACH, 1994b). The chicken's bursa Fabricii provides an ideal model for studying early B-cell development, but the chicken may not be a model for all avian immune systems.

Waterfowl immunology appears to resemble the immune system of reptiles and even amphibians. Waterfowl are a particularly ancient evolutionary group and unlike chickens, no distinct population of T-cells and B-cells have as yet been demonstrated amongst duck lymphocytes. Surface markers on duck lymphocytes, whilst being very different from mammalian markers, also differ from those detected on chicken lymphocytes (HIGGINS and CHUNG, 1986). The heavy chains in duck and chicken immunoglobulins show different numbers in histidine residues which are discussed to be involved in binding to protein A (HIGGINS et al., 1995).

Waterfowl immunoglobulins also share similarities with those of more ancient evolutionary groups. Ducks possess two antigenically similar and independently produced molecules, analogous to immunoglobulin G, with different sedimentation coefficients of 7.8S and 5.7S. Although some authors refer to this as IgG, these immunoglobulins are not related to human IgG, and are termed IgY and IgY(ΔFc) (HIGGINS and WARR, 1993). Following immunisation of ducks with bovine serum albumin, the initial peak of IgM gives way to a rise in IgG levels and both 7.8S and 5.7S anti-bovine serum albumin antibodies were found. Late in the immune response the 5.7S antibody predominates (ZIMMERMANN et al., 1971). The

relative quantities of these immunoglobulins in the serum vary considerably, depending on immunisation status as well as species of duck (GREY, 1967). Chickens possess an antibody with a low molecular weight similar to the mammalian IgG of 7S. The size of the duck immunoglobulin is due to an unusually long heavy chain (HIGGINS and WARR, 1993). The most striking evidence for the homology between these two immunoglobulins is represented by the results of immunodiffusion analysis. Rabbit anti-duck 7.8S immunoglobulin recognises antigenic determinants on chicken 7S immunoglobulin that are not present on the duck 5.7S protein, but are shared with determinants on the 7.8S protein. This indicates some antigenic homology between the chicken immunoglobulin and the 7.8S protein not shared by the 5.7S protein. Furthermore, some monoclonal antibodies raised to the 5.7S immunoglobulin react specifically with it and not with the larger 7.8S molecule. Yolk sac transmission of maternal antibodies to the duckling involves 7.8S IgG but not 5.7S IgG (ZIMMERMANN et al., 1971; GREY, 1967). Structurally and antigenically, the 5.7S immunoglobulin resembles a $F(ab)_2$ fragment of the 7.8S immunoglobulin, lacking the equivalent of two constant domains in the heavy chain (Fc fragment). Studies with radio-iodinated 7.8S immunoglobulin and carbohydrate analysis show, however, that the 5.7S immunoglobulin is neither a precursor nor a breakdown product of the larger immunoglobulin and that it is independently produced (HIGGINS and WARR, 1993; ZIMMERMANN et al. 1971). Some species possess either a 7.8S immunoglobulin or a 5.7S immunoglobulin, but not both, showing that these molecules can exist in evolutionary isolation. Distinct mRNAs for the two types of heavy chain are thought to be products of either the ‘two separate genes’ model or the ‘one gene alternative RNA processing’ model (HIGGINS and WARR, 1993; ZIMMERMANN et al., 1971; GREY, 1967). Whilst chickens lack this smaller immunoglobulin, it has been found in the sera of some turtles, the lungfish and groupers (ZIMMERMANN et al., 1971).

The IgM molecule found in ducks is homologous to that found in chickens, indeed in all vertebrates (HIGGINS and WARR, 1993). In serum of normal ducks, IgM is a minor component. The secretory (bile) immunoglobulin (IgX) of ducks has been characterised as physically and antigenically closely related to serum IgM. Although there may be a close antigenic and biochemical relationship between these two immunoglobulins they may, in fact be separate, secretory classes of immunoglobulins (HIGGINS and WARR, 1993). IgA has yet not been found in ducks (HÄDGE and AMBROSIUS, 1983).

During an infection or after immunisation the chronic processing of immunoglobulins is as follows: IgM > IgY > IgY(ΔFc). IgY and IgY(ΔFc), which function similarly to the IgG of mammals, represent the main component of the immunoglobulins in the serum (HIGGINS and WARR, 1993; GREY, 1967).

1.2.2. Lymphocyte activity

In evolutionary terms, birds were the first vertebrates with a clear dichotomy of lymphocytes into two major classes. These blood-borne stem cells mature and differentiate under the influence of hormones and thymopoetin, into antigen-sensitive cells (TIZARD, 1979; NEUMANN and KALETA, 1992). In a study to purify duck lymphocytes HIGGINS and CHUNG (1986) identified distinct receptors and surface immunoglobulin distributed randomly throughout the lymphoid system without the distribution of T- and B-cells expected. In an attempt to identify and characterise sub-populations of duck lymphocytes there was evidence of three different functional sub-populations, if not T- and B-cells, with different surface receptors and different response kinetics (HIGGINS, 1990).
In psittacine birds, diffuse lymphoid follicles are not common (GERLACH, 1994b). Specifically delineated-paired lymph nodes are found in waterfowl, chickens, marsh and shore birds, in the cervical *(Nodus lymphaticus cervicothoracicus*) and sacral areas (*Nodus lymphaticus lumbalis*). In the embryonic stage there are so-called lymph hearts**,** which contract to circulate lymph. These structures are found in geese and ducks, but not in poultry or pigeons. In poultry, there is also mural lymphoid tissue present in the lymph vessels (VON KOLLIAS, 1986; NEUMANN and KALETA, 1992).

1.2.2.1. B-lymphocytes

B-lymphocytes originate from the Bursa Fabricii and represent the cellular basis of humoral immunity. Around hatching time, the mature B-lymphocytes migrate into the secondary lymphatic organs (spleen, caecal tonsils, Peyer's patches, Meckel's diverticulum, lymphoid follicles in the various organs, paraocular and paranasal lymphatic tissue). The Harderian

gland embodies the main site of local production of immunoglobulins lubricating the eye surface and nasolacrimal duct (VON KOLLIAS, 1986; GERLACH, 1994b). Antigen stimulation of B-cells leads to the production of plasma cells and memory cells. For the proliferation of B-cells certain cells such as macrophages need to present an antigen and there must be a T-helper cell response to the same antigen as well (GERLACH, 1994b).

Plasma cell production reaches up to 2,000 Ig molecules per second with the same specificity as in the B-parent cell. Normally plasma cells perish after three to six days (GERLACH, 1994b). These antibody-producing cells are present in the red pulp of the spleen, the Harderian gland, ducts of the lateral nasal glands and intestinal lymphoid aggregates (VON KOLLIAS, 1986; TIZARD, 1979).
After stimulation by a specific antigen memory cells produce antigen-sensitive cells, which secret immunoglobulins in faster and more vigorous after having contact to the antigen a second time. The survival time of memory cells lasts months or even years (perhaps not strictly as individuals, but as clones) (GERLACH, 1994b).
A third sub-population of responding B-cells appears to accumulate around antigen-coated dendritic cells to form structures known as germinal centres. These are commonly found in the spleen and in other lymphoid tissue, such as the caecal tonsils (TIZARD, 1979).

1.2.2.2. T-lymphocytes

Thymus-derived lymphocytes (T-cells) can be divided into many subgroups such as effector cells, helper cells, suppressor cells and a group of T-cells serving as memory cells in a manner analogous to B-memory-cells (TIZARD, 1979; SHARMA and TIZARD, 1984). T-cells play an important role in the protection against viruses, virus-infected cells, intracellular bacteria, foreign tissue grafts, parasites, fungi and some tumour cells (GERLACH, 1994). In the thymus the different T-cell sub-populations are equipped with surface molecules (for example CD4 and CD8 molecules), which represent recognition structures for the major histocompatibility complex (MHC), which is identified as the so-called B-complex in chickens (SANDER, 1995; NEUMANN and KALETA, 1992; VON KOLLIAS, 1986). The interaction of antigen-presenting macrophage, the effector cell (carrying the CD8 antigen) and

the CD4 helper cell enables the T-cells to recognise the antigen and to initiate cell-mediated immune reaction (GERLACH, 1994b).

The co-operation between B-cells and T-cells is controlled by a sub-group of cytokines, so-called regulator factors such as interleukins. Furthermore, lymphokines can be differentiated into inflammatory mediators, macrophage activity modulators, cytotoxic factors and fibroblast stimulating factors (GERLACH, 1994b; TIZARD, 1979; VON KOLLIAS, 1986).

1.3.Influence of host and environmental factors on the immune response

Stress is thought to have a particular impact on cell-mediated immune responses. The immune response is influenced by stressors such as temperature extremes, high humidity, high stocking density, poor ventilation, high ammonium concentrations and malnutrition. Vitamin deficiencies (vitamins A, C, K and E) or lack in minerals such as zinc can impair body defence mechanisms important in mediating disease resistance; disease can increase vitamin requirements. These factors influence the immune system either directly by inhibition of plasma cells, lymphocytes, macrophages, heterophils, complement and lactoferrin or indirectly by a decrease in corticosteroid production (CHEVILLE, 1979; VON KOLLIAS, 1986).

Birds being exposed to the pathogens of a certain environment develop adaptation to those. Being moved from one environment to another leads to a lack of protection against the new group of pathogens they would encounter. Furthermore, inbreeding is known to weaken the immune system, resulting in a higher susceptibility to disease (GERLACH, 1994b).

Birds in captivity may be exposed to an increased risk of disease and may be more susceptible due to factors such as contact to a novel disease for which no evolutionary immunity is developed, low genetic heterozygosity, stress caused by overcrowding, inappropriate social groups or different and possibly inadequate diet, and the increased age which most birds reach in captivity.

2. Characterisation of mycobacteria with particular reference to *Mycobacterium avium*

2.1. Classification of mycobacteria

2.1.1. Identification and conventional typing

Mycobacteria are small (0.2 to 0.6 x 1.0 to 10.0 μm), aerobic, acid-fast, non-motile, non-spore-forming bacilli. Mycobacteria are a large group which include pathogenic, non-pathogenic and saprophytic species that are often ubiquitous in the environment. Biochemically, *Mycobacterium avium* differs from other mycobacteria in that it does not hydrolyse Tween 80. Nor does it produce niacin or reduce nitrate. It is peroxidase negative, isoniazid resistant, has a negative urease reaction and produces catalase.

The more than 50 mycobacterial species include *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Mycobacterium bovis.* Formerly the other species were classified as 'atypical mycobacteria' due to their non-classical appearance and because they are not considered as primary human pathogens. Today the terms 'mycobacteria other than tuberculous mycobacteria' (MOTT) or 'non-tuberculous mycobacteria' (NMT) are more commonly used to describe these organisms, whose epidemiology differs from that of the classical tuberculous group. They survive and multiply in an inanimate environment, infect humans only under limited opportunistic conditions and are not normally transmitted by person-to-person contact (HOOP, 1997). WAYNE and SRAMEK (1992) propose the term 'potential pathogenic environmental mycobacteria' (PPEM). The importance of environmental exposure to these mycobacteria is emphasised since there is little or no transmission of these organisms between humans (WAYNE and SRAMEK, 1992).

Non-tuberculous mycobacteria grow over a wide range of temperatures, pH, salinity, and oxygen tensions. *Mycobacterium avium* grows best between pH 5 and 5.5, microaerobically, approximately 2 to 5 mm below the surface of semisolid mycobacterial media. MAI complex strains grow equally well in water with and without salt (i.e., up to 2%). Non-tuberculous mycobacteria are relatively resistant to heavy metals and oxyanions (FALKINHAM, 1996).

Ranging in size from 0.2 to 0.6 x 1.0 to 10.0 μm, mycobacteria tend to be pleomorphic and can appear almost as cocci or as long beaded rods (ARANAZ et al., 1997). Most of them require two to eight weeks or more for growth. Growth rate is important in the classification and specialisation of mycobacteria. MOTT are classified as: fast-growing species (IV) which form colonies in less than one week; intermediate species require one to two weeks to grow; and slow growth species which take two to eight weeks to develop identifiable colonies (I-III). *Mycobacterium paratuberulosis* (*Mycobacterium avium subsp. paratuberculosis*) presents a notable exception, as it may take up to 16 weeks to develop identifiable colonies. Further criteria for classification are pigmentation and biochemical reactions (HOOP, 1997; HINES, KREEGER and HERRON, 1995). **Table 1** presents an overview of the classification of mycobacteria as mentioned above.

Table 1: Classification of mycobacteria by RUNYON (1967).

Group	Culture characteristics	Disease complex	Occurrence	Important species
A	slow-growing (3-6 weeks)	Tuberculosis	Humans, Cattle	*M. tuberculosis* *M. bovis*
B	Nonculturable slow-growing	Leprosy Paratuberculosis	Humans Ruminants	*M. leprae* *M. paratuberculosis*
C (MOTT)				
I	slow-growing (3-6 weeks) photochromogenic producing yellow pigment when exposed to light	Saprophytic Potential pathogens		*M. kansasii*
II	slow-growing (10-14 weeks) scotochromogenic producing orange yellow pigment in light and dark	Saprophytic		*M. scofulaceum* *M. gordonae*

III	Slow-growing (3-4 weeks) Non-photochromogenic	Saprophyic Birds, potential pathogenic for humans and mammals	*M. avium* *M. intracellulare* *M. genavense* *M.celatum*
IV	rapid-growing (6-7 days)	Oportunistic pathogens for humans and amimals	*M. caelonae* *M. phlei* *M. smegmatis* *M. vaccae*

2.1.2. The *Mycobacterium avium-Mycobacterium intracellulare* complex

The causative organism in mycobacterial diseases was named *Mycobacterium avium* by CHESTER (1901, cited by GRANGE et al., 1990). In 1967 RUNYON found that *Nocardia intracellulare* and a group of strains known as *Battey bacilli* were phenotypically very similar to *Mycobacterium avium.* These strains were renamed *Mycobacterium intracellulare*. Although no unanimous decision regarding nomenclature was reached, the term '*Mycobacterium avium-intracellulare* (MAI) complex was created. With minor variations this term is still used widely. Some workers, mainly in the USA, include the phenotypically similar but genetically distinct species *Mycobacterium scrofulaceum* in this group to form the MAIS complex. There is also good evidence for a relationship between *Mycobacterium avium* and *Mycobacterium paratuberculosis*. Next to *Mycobacterium paratuberculosis* there are other mycobacterial strains closely resembling *Mycobacterium avium* that have a partial or absolute growth requirement for mycobactin (MATTHEWS et al. 1977). This requirement for mycobactin for growth in primary culture may either be true dependence on mycobactin or it may be required only when small numbers of viable units are cultured. Large inocula presumably contain sufficient mycobactin associated with the organisms to permit growth (MATTHEWS et al. 1977). This trait is common to a number of isolates specific to woodpigeons (*Columba palumbus*), collectively termed 'woodpigeon bacilli'. The requirement for mycobactin can be graduated with *Mycobacterium paratuberculosis* at one end and *Mycobacterium avium* at the other, and 'woodpigeon bacilli' in an intermediate position (GRANGE et al., 1990).

2.1.3. Serotyping of mycobacteria

2.1.3.1. Agglutination serotypes

Strains of *Mycobacterium avium* and *Mycobacterium intracellulare* are divisible into many serotypes by agglutination serology. By 1984, several series of studies had identified three serotypes of *Mycobacterium avium* (1-3), and 25 of *Mycobacterium intracellulare* (4-28) (GRANGE et al., 1990). The group was at one time extended to include three additional serovars of a third species *Mycobacterium scrofulaceum*, therefore the literature may include references to the complex as the *Mycobacterium avium- Mycobacterium intracellulare* complex or the *Mycobacterium avium- Mycobacterium intracellulare- Mycobacterium scrofulaceum* intermediate complex. The inclusion of *Mycobacterium scrofulaceum* in the complex is currently in doubt (WAYNE and SRAMEK, 1992).
Serovar antigens of the MAI complex have a common lipopeptidyl-O-methyl-rhamnose linked to an oligosaccharide. In other words, serologic specificity is conferred by the specific oligosaccharide residues of the C-mycoside glycopeptidolipids (GPLs), which are integral constituents of the cell wall and envelope. This is a class of cell wall surface lipids analogous to the O-antigens of the enterobacteria. They are responsible for capsule formation, bacteriophage attachment, colony morphology and possibly, virulence (BRENNAN, 1981). The oligosaccharide represents the variable antigenic determinant, containing an internal, constant pair of sugars, L-rhamnose and deoxytalose, and an outer pair, that varies according to serotype (GRANGE et al., 1990). Strains can be now serotyped by thin-layer chromatography and enzyme-linked immunosorbent assay (ELISA) analysis of species- and type-specific glycolipids, as well as by conventional seroagglutination.

2.1.3.2. Immunodiffusion serotypes

Using immunodiffusion analysis STANFORD et al. (1974) classified soluble mycobacterial antigens into four groups: those common to all mycobacteria groups (group i); those restricted to slowly growing species (group ii); those restricted to rapidly growing species (group iii); and those unique to each species (group iv). Some strains within a species showed variation of

some group iv antigens, allowing the species to be divided into subspecies or serotypes (STANFORD and GRANGE, 1974). In further serological studies (McINTYRE and STANFORD, 1986) revealed four immunodiffusion types and grouped strains of *Mycobacterium avium* to immunodiffusion type A while *Mycobacterium intracellulare* strains were divided in immunodiffusion type B (containing the type species of *Mycobacterium brunense*) and type C, with *Mycobacterium lepraemurium* belonging to a fourth serological type. The 'woodpigeon bacilli' strains belonged to serotype A. The type strain of *Mycobacterium paratuberculosis* and indeed, most serotypes of this species isolated from cattle, were grouped to serotype B. As three species-specific antigens, so-called group iv antigens were shared by all strains, irrespective of the species, it was proposed that there should be a single species with four variants: *Mycobacterium avium avium* (type A- with agglutination serotypes 1-3), *Mycobacterium avium brunense* (type B- with agglutination serotypes 4-11 and 20/21), *Mycobacterium avium intracellulare* (type C- with agglutination serotypes 12-19 and 22-28) and ***Mycobacterium avium lepraemurium***. Though taxonomically correct, this nomenclature was not generally accepted. Based on other studies, many isolates formerly classified as *Mycobacterium intracellulare* (serotype 4, 5, 6 and 8) are currently grouped with classic *Mycobacterium avium* isolates. Serotype 7, 11-21, 23, and 25 are *Mycobacterium intracellulare*, the remaining strains are unclassified (ARANAZ et al., 1997).

DNA-DNA hybridisation studies also support that MAI complex, 'woodpigeon bacilli' and *Mycobacterium paratuberculosis* are members of a single species. Restriction fragment length polymorphism (RFLP) analysis, by using dralI and pulsed-field gel electrophoresis, showed that could be distinguished from *Mycobacterium paratuberculosis* and that *Mycobacterium avium* isolates were readily distinguished from *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum* as subspecies (INDERLIED et al., 1993; GRANGE et al., 1990; FALKINGHAM III, 1996).

Examinations of different strains of the MAI complex by multilocus enzyme electrophoretic typing correspond to the GenProbe nucleic acid hybridisation system, GenProbe DNA-rRNA hybridisation and serovar studies and to earlier DNA-DNA relatedness studies. There are two DNA-related groups that make up the *Mycobacterium avium- Mycobacterium intracellulare* (MAI) complex. Serovars 1-6, 8-11 and most likely 21 appear in the *Mycobacterium avium*

cluster, while serovars 7 and 12-28 relate to *Mycobacterium intracellulare* (INDERLIED, KEMPER and BERMUDEZ, 1993).

2.1.4. Distribution in the environment

MAI complex and most other mycobacterial species live freely in the environment, particularly in surface waters. Due to their tolerance of temperature, pH, salinity, and oxygen tension extremes they are found in marshes, ponds, lakes, rivers and estuaries from which they may enter coastal seawater. Soil is another habitat of MAI complex, particularly humid, fulvic and acidic soil that is marshy or subject to periodic flooding. It may also be present on plants, bedding material and house dust. In a study by GRUFT et al. (1979) the smaller numbers of ocean samples that yielded MAI complex, as compared with the estuaries samples, suggest that the ocean is not the primary source of the micro-organism. Strains of the MAI complex are more abundant in waters of low salinity than in marine waters.
It has been postulated that MAI complex enters aerosols generated over streams and estuaries (WENDT et al., 1980) and when present in the ocean waters, they are released into the air by bubble formation when waves break, and are carried inland by prevalent winds (GRUFT et al., 1979). Experimental aerosolisation has shown that MAI complex are readily transferred to aerosols and that bacteria-containing particles of the optimum size (0.7 to 3.3 mm) for entering the lungs and reaching the alveolar spaces are generated (PARKER et al., 1983).
MAI complex may also colonise piped water supplies (GRANGE et al., 1990). Studies of resistance of MAI complex to heavy metals showed that some isolates of *Mycobacterium avium*, *Mycobacterium intracellulare*, or *Mycobacterium scrofulaceum* were highly resistant to cadmium, mercury, silver, tellurite and relatively resistant to chlorine. That five of seven mercury-resistant isolates were isolated from water samples collected in regions of reported heavy metal pollution demonstrates that mycobacterial physiology is a determinant of their ecology and geographic distribution. Furthermore, metal metabolism and metal requirements directly influence MAI complex populations. For example *Mycobacterium avium* numbers in natural waters directly correlate with zinc levels. It may be that the persistence of MAI complex organisms in hospital water and drinking-water distribution systems is because many use galvanised (i.e. zinc coated) pipes (FALKINHAM III, 1996).

2.2. Cultivation of mycobacteria

2.2.1. Colony morphology

Strains of MAI complex produce effusive growth or discrete colonies about one mm in diameter, after two-three weeks incubation. Some strains of *Mycobacterium avium* require up to six months before colonies are identifiable (ARANAZ et al., 1997). There is often more rapid growth on media containing pyruvate. Colonies may be a pearly grey or lemon yellow colour. Most colonies emulsify easily. The temperature range of growth is extremely wide, with growth possible from 20 to 45°C. All strains grow at 25°C and the majority at 37°C. The ability of *Mycobacterium avium* to grow at 40-45°C allows it to exploit birds as a host and distinguishes it from most other mycobacteria. Mycobacteria require special handling during cultivation because of their slower growth rate and special nutrient requirements. They do not grow on standard bacteriologic media; special semi-synthetic and egg-based media that often include special additives are used. Among the common media are Middlebrook 7H9 liquid medium, Loewenstein-Jensen medium, Herrold's egg yolk medium or Petragnami medium. The specimen often requires decontamination before inoculation to prevent overgrowth of other contaminating bacteria or fungi. The most common decontamination protocols are the N-acetyl-L-custeine-NaOH, the NaOH, the Zephiran-trisodium phosphate, the oxalic acid method, the cetylpyridinium chloride-sodium chloride or the hexade-cylpyridium chloride methods. Culturing mycobacteria can be a difficult and time-consuming task (HINES et al., 1995).

2.2.2. Colony variation and virulence

A number of colony forms of MAI complex have been described and these show some variation in their virulence for experimental animals and in their susceptibility to certain anti-microbial agents. KUZE and UCHIHIRA (1984) defined four colony types: transparent, smooth, thin and flat (T); opaque, smooth and domed (O); rough (R) and a form which resembles the O-type but has a more or less dome-shaped centre with relatively large extended, thin marginal transparent lobes spreading out from the edge (IM, or intermediate). The T-type and R-type colonies were virulent in mice while the O-type and IM-type colonies

produced only minimal lesions, instead of submiliary to miliary nudules. The degree of virulence was evaluated by other methods such as average weights of lung, viable bacilli from the lung, and average weights of spleen. The T-type form appeared to give rise to O-, R- and IM-type forms and, although the genetic basis for such variation has not been fully determined, there is some indirect evidence that T-type-to-O-type variation is the result of a loss of plasmids (MIZUGUCHI et al., 1981). Other studies showed that the rate of the transparent-to-opaque transition was dependent on temperature and thus is not a consequence of mutation and that colony type mutation was not linked to mutator effects, as MAI complex is not unusually susceptible to UV-induced mutations (WOODLEY and DAVID, 1976).

On electron microscopy, the cell wall of MAI complex has three layers: an inner layer of peptidoglycan, an intermediate electron-transparent layer and an outer polysaccharide-rich layer. The outer layer is thick and continuous in cells from T-type colonies but is loosely packed and discontinuous in those from O-type colonies. It was therefore postulated that the intact outer layer conferred multiple drug resistance by a barrier effect. Further, the rough colony type lacked both polar and apolar GPLs. It was shown that the O-type colony form was significantly more susceptible to different antimycobacterial drugs than T-colony forms. The association between virulence and resistance to antimicrobial agents is not complete as the attenuated IM-type and R-type colony forms are as resistant to these agents as the T-type colony form (KUZE et UCHIHIRA, 1984; SCHAEFER et al., 1970).

The growth of MAI complex isolates, either transparent or opaque variants, occurs in three stages. During the first stage, the selected small cells undergo a lag period with regard to cell numbers, as indicated by unchanged counts of viable cells or particles. However, rapid synthesis of protein and DNA, and uptake of fatty acids leads to a considerable increase in cell mass. Cells elongate to approximately five times their original length by 26 hours of incubation. Binary fission occurs during the second stage of growth, with a generation time as short as six hours. Protein synthesis continues during the second stage of growth, but at a diminished rate, and the uptake of fatty acids decreases while intracellular pools of triglycerides, stored during the elongation phase, are catabolised to supply carbon and energy. At the termination of division, most cells are in the form of coccobacilli. During the third stage of growth, which is most analogous to the conventional stationary phase, the morphology of the cells becomes heterogeneous, and leads to a mixture of filaments, rods, and coccobacilli. Cells of both colony variants complete the cell cycle in the same sequence

and over the same time so that by the termination of fission the proportion of the two types is the same as in the original culture. The nutritional requirements of the opaque colony cells are not, however, as stringent as are those of the transparent colony form and thus the former may ultimately predominate in old cultures (McCARTHY and ASHBAUGH, 1981).

Many studies focusing on virulence factors have been performed with MAI complex organisms. Potential virulence factors include colony type as discussed above, prevention of acidification of phagocytic vesicles, prevention of phagosome-lysosome fusion, production of an electron-transparent zone around the cells, resistance to inhibitory serum constituents, delay in tumour necrosis factor secretion by infected host cells, uptake by intestinal epithelial cells, production of receptors for macrophage binding, and the ability of mycobacteria to replicate in macrophages (FALKINHAM III, 1996).

2.3. Genetic characteristics

2.3.1. DNA and plasmids

The mycobacterial genome consists of a single closed loop of DNA. Some strains also have one or more plasmids. The genome is not contained by a nuclear membrane, although the tightly packed DNA is recognisable on electron microscopy as a nuclear body. Genome sizes of MAI complex strains are within the range of 2.8-4.5 x 10^9 bp. The DNA of most mycobacteria has between 64 and 70 mol% guanine and cytosine (INDERLIED et al., 1993). Plasmids are widespread in members of the *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* groups. The plasmids are large, and some strains often have as many as three to six plasmids. In certain strains, almost 30% of the total DNA is plasmid DNA (MEISSNER and FALKINHAM III, 1986). Plasmids have been detected in 55% of human strains of MAI complex and in 75% of strains from aerosols, but in only 21% of strains from water, 7% of those from dust, less than 6% of those from sediment and 5% of those from soil. Thus, plasmids were significantly more common in aerosols than in water, and the plasmid profiles of these aerosol isolates more closely resembles those of human isolates than does the profile of any other environmental isolates. This is confirmed by studies in which isolates from Acquired Immunodeficiency Syndrome (AIDS) patients

appeared all to contain plasmids, whereas only a third of environmental isolates carried plasmids (SNIDER et al., 1987). The molecular weights of these plasmids varied from 8.8 to 160 megadaltons (MD) and there were between one and six plasmids in each strain. The heterogeneity of the plasmid content was great: strains with identical plasmids profiles were rarely encountered. Plasmids appear to be responsible for some of the characteristics of the MAI complex bacilli that harbour them. They determine susceptibility to phage lysis (MIZUGUCHI et al., 1981), resistance to salts of heavy metals (mercury and copper), and possibly colony morphology (MEISSNER and FALKINHAM III, 1986). There is indirect evidence that plasmids affect virulence (GRANGE et al., 1990).

2.3.2. Cell wall and envelope

One of the best-studied features of mycobacteria is the structure and function of the mycobacterial cell wall and envelope. The envelope is composed of a variety of soluble proteins, carbohydrates, lipids and three insoluble macromolecular components: arabinogalactan, peptidoglycan, and mycolic acid. These insoluble macromolecules constitute the mycoylarabinogalactanpeptidoglycan core of the cell wall, one of two lipopolysaccharides (LPS) common to all mycobacteria. The mycoylarabinogalactanpeptidoglycan appears as electron-dense and electron-transparent zones in thin sections of mycobacteria viewed by negative staining. Additional electron-dense layers at the surface of the cell frequently surround the core. This electron-dense layer is made up in part, of unique GPLs that are specific to the MAI complex. In addition, all mycobacteria possess a second LPS as a component of the cell envelope, a lipoarabinomannan (LAM). The LAM is not covalently linked to the mycoylarabinogalactanpeptidoglycan core but most likely is anchored on the plasma membrane of the mycobacterial cell, with the polysaccharide extending to the exterior of the cell. The mycoylarabinogalactanpeptidoglycan, lipoarabinimannan, and GPLs of the MAI complex are strongly immunogenic (INDERLIED et al., 1993). The complex array of parallel hydrocarbon chains is the most likely source of the impermeability of mycobacteria. The complex lipid-rich cell wall and thick mycolic acid layer renders the organisms acid-fast, meaning that they retain the colour of arylmethane dyes such as fuchsin after being rinsed with a dilute acid (GRANGE, 1996).

2.4. Pathogenesis

2.4.1. Mechanism of infection

Studies with the beige (C57BL/6 bg+/bg+) mouse model of oral infection demonstrate that oral exposure to *Mycobacterium avium* strains leads to intestinal colonisation and subsequent dissemination of infection. The studies show that the majority of the organisms is found in the terminal ileum and ascending colon. This is most likely because of the prevalence of Peyer's patches in these regions of the gut (BERMUDEZ et al., 1992b).

Studies have shown that *Mycobacterium avium* strains can bind and invade two epithelial cell lines, HT-29 and HE-2 cells. These results suggest that *Mycobacterium avium* can bind to entrocytes and probably M-cells, and quickly penetrate epithelial cells before translocating into the lamina propria. This interaction was more efficient when the bacterium was grown at 37°C or 40°C than at 30°C, indicating that moieties associated with binding and penetration of mucosal cells must be expressed more efficiently when the bacteria is within the host (BERMUDEZ, 1994). It is noteworthy that this optimum temperature corresponds to the body temperature of birds. The ability of *Mycobacterium avium* to penetrate epithelial cells in mice is dependent on the phase of growth of the organism. Organisms in the logarithmic phase of growth are significantly more efficient in penetrating epithelial cells *in vitro* and *in vivo* than organisms in the stationary phase of growth. Different adhesins for fibronectin have been characterised in *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium leprae,* and have been associated with the ability to bind to cells (BERMUDEZ, 1994). A series of experiments demonstrated that genomic DNA of *Mycobacterim avium* could be cloned and expressed in *Escherichia coli* K-12, which cannot normally invade cultured mammalian cells. *Escherichia coli* transformants that had acquired a 2.7kb fragment of chromosomal DNA had the ability to bind and invade HT-29 and HE-2 epithelial cells. In addition, a plasmid containing the same 2.7kb fragment was inserted into *Mycobacterium smegmatis*, creating mutants that were capable of invading HT-29 cells (BERMUDEZ, 1994). There is evidence for the presence of at least one adhesin protein in virulent strains of *Mycobacterium avium*. Specific antibody generated with a purified preparation of a 27-kDa putative adhesion protein blocked the binding of *Mycobacterium avium* strains to both intestinal and oropharyngeal mucosal cells. By extrapolation, intestinal cells may play an

active role in taking up *Mycobacterium avium*. Indeed, a 47- to 50-kDa glycoprotein present on HT-29 cells and oropharyngeal cells binds to the putative 27-kDa *Mycobacterium avium* adhesion protein and appears to be involved in the binding of several different *Mycobacterium avium* strains (INDERLIED et al., 1993).

2.4.2. Interaction with mononuclear phagocytes

Mycobacteria are facultative intracellular pathogens, which characteristically reside within mononuclear phagocytes, where they can multiply and survive in the presence of compromised host defences. It is now clear that several species of mycobacteria have receptors for fibronectin which allow them to be ingested by phagocytes by means of the fibronectin receptor. The most important mechanism used by *Mycobacterium avium* to invade macrophages may be binding to the C3/CR3 receptor (BERMUDEZ et al., 1991b) and the vitronectin receptor ($\nu\beta3$ integrin) (RAO et al., 1993). MAI complex organisms bind to serum fibronectin and the bacilli are internalised by macrophages by using the intergrin fibronection receptor. It is hypothesised that the CR3 and fibronectin receptors are more important in the presence of serum, whereas other receptors would be the preferable binding site on mucosal surfaces and in the lungs. The uptake of *Mycobacterium avium* by macrophages in the absence of serum is a slower process than that which occurs in the presence of serum, but by 24 hours the result is similar (BERMUDEZ et al., 1991b). The use of an Fc receptor-independent pathway for uptake may offer advantages for the invading microorganism, avoiding exposure to superoxide anion production and hydrogen peroxide. This pathway may also influence the structure and function of the intracellular vacuole.

Mycobacterium avium strains can remain viable within resting macrophages and resist being killed by oxidation. *Mycobacterium avium* synthesises a 23-kDa superoxide dismutase (MAYER and FALKINHAM III, 1986) that can inactivate macrophage-derived superoxide anions and other proteins such as 65-kDa protein heat shock protein, which are powerful inhibitors of superoxide anion production. The 65-kDa protein is released in large quantities in the surrounding environment in response to a variety of stressors such as high temperature, low pH and phagocytosis.

The capacity to survive within macrophages is also dependent on the ability to inhibit acidification of the phagosome as well as to prevent phagosome fusion with lysosomes, and contact with proteolytic enzymes (CROWLE et al., 1991). In the absence of phagosome fusion, the intracellular environment of the macrophage remains neutral or alkaline. This may directly influence pathogen survival and the effectiveness of certain antimicrobial therapies (INDERLIED et al., 1993). Antigens, proteins and glycoproteins, secreted by the bacterium in the milieu surrounding may interfere with signal transduction within macrophages, blocking the maturation of phagosomes and/or the migration of lysosomes. Proteins such as 33-kDa protein from *Mycobacterium avium* are capable of interfering with important pathways of signal transduction in macrophages (BERMUDEZ, 1994).

2.5. Immune response to mycobacteria

2.5.1. Cellular immunity

The little information there is about the cellular immune response to non-tuberculous mycobacteria is based on experiments with mice, the C57/BL6 bg+/bg+ beige mice model or the C57/BL6 black mice model.

In the *Mycobacterium avium* infected host, expression of class II molecules by infected macrophages results in the presentation of mycobacterial antigens to class II-restricted CD4+ T- lymphocytes of the helper/inducer type. Mice respond with the proliferation of T cells specific for mycobacterial antigens. Approximately 20% of the CD4+ T- lymphocytes that react to mycobacterial antigens recognise the mycobacterial 60-kDa heat shock protein. Depletion of the L3T4+ or LyT2+ T-cell subpopulations does not have any significant effect, but depletion of both subsets abates the immune response (COLLINS and STOKES, 1987; HUBBARD et al., 1992). *Mycobacterium avium* releases a 65-kDa protein in response to the stress of increased temperature or exposure to acid pH. This and other mycobacterial antigens, including the 71-, 38-, 33-, 30-, and 10-kDa proteins, can be released and recognised by CD4+ and CD8+ T-lymphocytes. In addition, certain glycopeptidolipid antigens from *Mycobacterium avium* can interfere with the lymphoproliferative response to different mitogens. These mycobacterial GPLs or their resulting metabolites can have an

immunomodulatory, for example, a suppressive effect (BROWNBACK and BARROW, 1988; TASSEL et al., 1992).

2.5.2. Role of lymphocytes and natural killer cells

T-cells bearing the γδ-T-cell receptor appear to have a special role in the immune defence against mycobacteria. The γδ-T-cells are localised principally on mucosal surfaces and are thought to represent a first line of defence against invading pathogens. γδ-T-cells react to mycobacterial 60-70-kDa heat shock proteins with proliferation. Observations show that γδ-T-cells can be a significant source of cytokines, which stimulate mycobacteriostatic activity in macrophages. In addition, γδ-T-cells when exposed to *Mycobacterium avium* can lyse *Mycobacterium avium*- infected macrophages (BARNES et al., 1992).
NK cells are cytotoxic in a non-restricted manner. The cytotoxicity of NK cells for infected macrophages appears to occur following binding of NK cells through the LFA-1 glycoprotein receptor (BLANCHARD et al., 1989). However, other studies showed that NK cells do not efficiently bind or lyse target cells expressing the class I major histocompatibility complex (INDERLIED et al., 1993).
Responses in chickens are thought to be similar, with γδ–T-cells in particular responding vigorously in the presence of mycobacterial antigens. This response would seem to be reliant on the presence of, and/or the soluble factors produced by CD4+ αβ-T-cells (ARSTILA, 1996, cited by CROMIE et al., 2000).

2.5.3 Role of cytokines

When exposed to *Mycobacterium avium* NK cells release large amounts of IL-6 (BLANCHARD et al., 1992). NK cells can also induce mycobacteriostatic and mycobactericidal activity in macrophages, that is secondary to the synthesis and release of cytokines such as tumour necrosis factor (TNF), interferon-gamma (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (BERMUDEZ et al, 1990; DENIS and GREGG, 1990). Macrophages stimulated in this way increase both their release of superoxide

anions and the rate of phagolysosome fusion following uptake of mycobacteria (BERMUDEZ and YOUNG, 1991a). In several experiments it has been shown that IFN-γ alone does not inhibit intracellular replication, but combinations of cytokines including IFN-γ significantly decrease the replicative capacity of the organism (HINES et. al., 1995).

Interference with cytokine-related signal transduction and transcription is probably an important mechanism of pathogenesis and contributes to the persistence of MAI complex within macrophages. The bacterium alters the host's immune response by interfering with cytokine pathways, by stimulating the production and release of suppresser molecules (e.g. transforming growth factor (TGFβ1) and interleukin-10 (IL-10)), by the induction of inflammatory factors such as IL-6 and by directly influencing the mechanism of signal transduction within macrophages. The release of inhibitory cytokines by MAI complex-infected macrophages occurs within a few days of infection. It stimulates the release of IL-6 and a suppression of macrophage function, by down-regulating the expression of TNF receptors and decreasing TNF production (BERMUDEZ et al., 1992a; DENIS and GREGG, 1990). Since TGF-β is known to impair the ability of macrophages to respond to cytokines, the release of TGFβ is likely to be responsible for the lack of response to IFN-γ by MAI complex-infected macrophages. A specific amino acid sequence within the 33-kDa protein interferes with the regulation of transcription in macrophages, which in turn influences the response of macrophages to stimulation with TNF-α (BERMUDEZ, 1993). LAM is a major component of the mycobacterial cell wall. It has been shown that LAM from a virulent strain does not stimulate murine macrophages to produce high levels of TNF-α, unlike the LAM from an attenuated strain, which was a potent trigger for TNF-α release. This suggests that TNF-α may have a beneficial effect in the host's defence against mycobacteria and that virulent strains can inhibit the release of TNF-α by macrophages (CHAMPSI et al., 1994). Secretion of TGFβ1 occurs early in the infection and appears to be dependent on the strain of MAI complex. TGFβ1 impairs the macrophage response to stimulation with TNF-α and use of anti-TGFβ1 antibody is associated with improved response to TNFα and IFNγ. These results indicate that the lack of response by MAI complex-infected macrophages to IFNγ is due to the induction of TGFβ1 by some MAI complex strains (BERMUDEZ, 1993; BERMUDEZ et al., 1992a). The inability of infected macrophages to clear MAI complex infection could be related to the generation of 'immune-suppressive' cytokines such as TGFβ,

IL-10 and IL-6. These suppress important macrophage function, such as antigen presentation, anti-tumour and anti-bacterial/parasite activity, cytokine production and response to cytokine stimulation. TGFβ and IL-10 inhibit the production of and response to TNF and IFNγ, and suppress the production of GM-CSF. IL-10 also inhibits the proliferation of T-helper cell (T-H) 1 lymphocytes and stimulates the proliferation of T-H2 lymphocytes (BERMUDEZ, 1994).

Table 2 summarises the stimulatory or hindering activity of cytokines in mycobacterial immunity in mice. The *in vitro* data derived from most studies do not always reflect *in vivo* and clinical data. It must also be considered that the concentration of cytokine production for optimal effect has not been determined; therefore the experimental data may not reflect physiological effects of cytokines (CHAMPSI et al., 1994).

Table 2: Cytokines involved in *in vitro* and *in vivo* mycobacterial immunity.

	Effect on mycobacteriostatic activity	
	Stimulates	Hinders
in vitro	TNF	IL-3
	GM-CSF	IL-1
	IFNγ	TGFβ
	IL-4	IL-10 / IL-6
in vivo	GM-CSF	
	TNF	
	IL-2	

3. Manifestation of avian tuberculosis

3.1. Epidemiological aspects

3.1.1. Importance for collections with captive birds

Avian tuberculosis is characterised by signs of unthriftiness and death. Once established within a flock or aviary it is persistent and difficult to eradicate. Signs that a bird is infected are usually only seen late in the course of the disease and death may follow relatively quickly. During the slow development of the infection, birds excrete tubercle bacilli in the litter and soil. Birds, as well as mammals, may be free of symptoms for many months and may be an undetected source of infection. Wild birds such as sparrows, pigeons, starlings, and gulls may spread avian tuberculosis from one geographic region to another. *Mycobacterium avium* is excreted in the faeces of birds, (but not those of cattle or swine) facilitating the spread of disease over long periods. Mycobacteriosis is most common where birds are present in high concentrations, such as waterways where waterfowl congregate and zoological and avicultural collections (MONTALI et al., 1976). The unnaturally high stocking densities found in captive collections and the permanent presence of birds on the same ground allow the accumulation of high levels of contamination. Once the disease becomes established in a collection it is very difficult to eradicate because of the difficulty in diagnosis and the resistant nature of the bacillus. Avian tuberculosis easily becomes a permanent problem in a collection leading to the deaths of adult birds and decreased fertility. The ease with which infection is transmitted means that birds cannot be moved between collections or reintroduced to the wild and that there is a significant risk that infection will be spread to wild birds using the same site.

3.1.2. Distribution and species susceptibility to *Mycobacterium avium*

Infection by MAI complex has been found in primates, ruminants, carnivores, other monogastric animals, rabbits and birds. *Mycobacterium avium* causes avian tuberculosis in most, probably all, species of birds although the susceptibility to disease varies widely from species to species. Species like anseriforme, falconiforme, galliforme, columbiforme, charadriiforme, gruiforme, passerine and psittacine birds are particularly susceptible. Among

psittacine birds, the most common species involved include brotoregid parakeets, budgerigars (*Melopsittacus undulatus*), ringneck and related parakeets, amazons and pionus parrots. Commonly infected passerine birds include canaries, siskins, toucans, birds of paradise and weavers (VAN DER HEYDEN, 1994; FORSTER and GERLACH, 1987; PANIGRAHY, 1983). Geese have a somewhat lower incidence of avian tuberculosis than do ducks and swans (CROMIE et al., 1993b; PAVLAS et al., 1983). This corresponds to a study in 1993 in the WWT centre at Slimbridge of tribal mortality which indicates the *Anatini*, dabbling ducks, to be relatively susceptible to the disease, and the *Anserini*, true geese and swans, to be less so (CROMIE et al., 1993a). Flamingos in captivity are believed to be relatively resistant to *Mycobacterium avium* (CROMIE, 1991). In a zoological garden in North America, Wisconsin, there had been no recorded instances of avian tuberculosis in the flamingo flock for 20 years, when in 1991 an aged flamingo was found to have tuberculous lesions on necropsy. The remaining flamingos were then laparoscoped and radiographed, and liver biopsies were collected from each bird for histopathological examination. No further evidence of avian tuberculosis was found (BEEHLER, 1990).

Excepting parrots, mycobacterial infections other than MAI complex are rare in birds. *Mycobacterium tuberculosis* and *Mycobacterium bovis* can infect parrots. They are the only birds in which natural infections with these mammalian strains of mycobacteria have been reported (ACKERMANN et al., 1974), and they are generally the result of contact with infected owners of pet psittacines. *Mycobacterium tuberculosis* infection in parrots manifests as superficial cutaneous lesions, commonly, affecting the eyelids, nasal and oral cavities (GERLACH, 1994a). This unusual pattern of infection can be explained by the inability of some mycobacteria to grow at avian body temperature, hence the human tubercle bacilli grow on the skin of the head where temperatures are lower (CROMIE, 1991). Other factors, which may affect distribution of lesions include species susceptibility, common source exposure during importation of wild-caught birds (amazons, pionus and brotoregid parakeets) and husbandry practices, particularly communal flights with solid floors (canaries and siskins) (VAN DER HEYDEN, 1997a). In a study of endemic avian tuberculosis, *Mycobacterium avium* serotypes 1, 2 and 3 were isolated from *Anatidae*, chickens, and samples of mud and soil (SCHAEFER et al., 1973). Disease is almost always caused by serotype 1, 2 or 3, or by strains of serotype 6, that exhibit the lipid chromatography patterns of one of the former three types (GRANGE et al., 1990). Previous studies of avian tuberculosis in captive collections of

birds have found serotype 1 infection to be mainly responsible for the disease, followed by serotype 2. At the WWT centre at Llanelli, however serotype 3 was isolated from two birds that were found dead (PAINTER, 1997).

Virulence in different species of birds also varies with bacterial serotype; waterfowl tend to be relatively resistant to strains of serotype 2, which are virulent in domestic fowl, whereas serotype 1 strains are frequently less virulent in fowl than in ducks. Most strains of *Mycobacterium intracellulare* are not particularly pathogenic for birds, but virulent strains are occasionally encountered (GRANGE et al., 1990). Compared with MAI complex, *Mycobacterium genavense* is less commonly isolated from pet birds. *Mycobacterium fortuitum*, *Mycobacterium tuberculosis*, *Mycobacterium gordonae*, *Mycobacterium paratuberculosis, Mycobacterium bovis* and *Mycobacterium nonchromagenicum* are only occasionally found (VAN DER HEYDEN, 1997a). In other surveys of necropsy findings in Switzerland, the increasing isolation of *Mycobacterium genavense* from pet birds was reported (HOOP et al., 1996). *Mycobacterium genavense* causes a disease in birds that is clinically and histopathologically indistinguishable from that caused by MAI complex (PORTAELS et al., 1996). It should be remembered that data on mycobacterial infections in pet birds are scarce and often incomplete. A number of factors contribute to this lack of data including the long incubation period, inadequate serological tests, difficulty in detecting infected birds, the wide spectrum of disease signs, infrequent or incomplete necropsies, and a lack of funding and research into a disease within the veterinary realm because the disease is not considered economically significant (VAN DER HEYDEN, 1997a).

Susceptibility to mycobacteria is closely tied to the immune response of the host. Mycobacteria live within macrophages in the host and they may survive, replicating at a subclinical level for years. An active infection develops when the host's immune system mounts an inappropriate response, that is predominantly humoral, if the host is immune-compromised. The immune status of the bird may play a significant role in its susceptibility to colonisation by mycobacteria and the subsequent development of disease. Other factors may include age and sex. It is rare to see mycobacterial infection in birds less than two years of age, while the disease is very common in elderly waterfowl and poultry. This is likely because of the long incubation period, accumulated risk of exposure, and compromised immune response of older birds which may be related to repeated exposure to various environmental

mycobacteria (MONTALI et al., 1976; PANIGRAHY et al., 1983; VAN DER HEYDEN, 1997a; CROMIE et al., 2000).

3.2. Clinical findings

The course and duration of avian tuberculosis depends on a number of factors, including bacterial strain, quantity of bacteria received, route of infection, and host immune response.
The clinical diagnosis of avian tuberculosis is usually possible only after the disease has reached an advanced stage. Clinical signs of mycobacteriosis are variable and non-specific, depending on the species affected, the extent and duration of disease, and the organ system involved. Mycobacteriosis is a chronic wasting disease. Most commonly the birds are weak, lethargic and fluffed for some time. Typical physical findings include weight loss with muscle atrophy (usually seen as prominent keel), anorexia, depression, diarrhoea, polyuria, anaemia, weakness, pallor, poor feathering, cachexia, abdominal distension, lameness, bumblefoot, subcutaneous and conjunctival masses, cutaneous lesions and eczema. Dyspnoea may be observed in birds with pulmonary involvement or air sacculitis (VAN DER HEYDEN, 1994). Swollen joints, particularly the tarsal joints may be common.
Weight loss may be the result of a combination of malabsorption and maldigestion, as well as inflammatory responses by the bird's immune system attempting to control the infection. Droppings are characterised by soft, poorly formed faeces and polyuria. Undigested food, mucus, and fat globules may be observed on faecal examination. Faeces may also be positive for haemoglobin, red blood cells, or white blood cells if intestinal inflammation is present. The passage of whole seeds, usually reflecting gastric involvement, is rare. Polyuria may be caused by primary renal involvement. It may also be secondary to inflammatory and mycobacterial toxins or the inability of the diseased colon to reabsorb urinary water during retroperistalsis. Biliverdinuria, which may impart a yellow or green colour to the urates, is uncommon despite massive hepatic involvement. Abdominal distension in mycobacteriosis results from organomegaly, primarily of the liver and small intestine. Ascites is rare. An enlarged liver may be palpated extending from the caudal border of the keel to as far distal as the pubic bones. Marked weight loss and thinning of the abdominal musculature may allow direct visualisation of the enlarged liver if the overlying skin is wetted down with alcohol.

Hepatic rupture and haemorrhage also can occur and may be visible. Enlarged intestines may give the abdomen a doughy feel, and the thickened intestinal loops can sometimes be picked up between the thumb and fingers or visualised through a thin abdominal wall. Marked enlargement of the spleen also may occur. Dyspnoea may be the result of pulmonary epitheloid infiltrates or secondary to air sac compression from enlarged abdominal organs or airsacculitis. Other signs of respiratory disease including coughing, sneezing, and rhinitis are rare. Lameness may occur at any stage of the disease. Skeletal and bone marrow involvement can result in osteomyelitis and pathological fractures particularly in the proximal tibia and ulna. Subcutaneous and conjuctival lesions may occur as discrete granulomas or as a diffuse thickening accompanied by xanthomatosis of the overlaying skin. The granulomas are pale and soft and may be mistaken grossly for lipomas or lymphomas. They are non-pruritic and rarely ulcerate, although some birds have feather loss in the surrounding areas. The feathers and skin of pet birds with mycobacteriosis often reflect non-specific signs of disease including failure to moult, worn feathers characterised by damaged edges and discoloration (bronzing or blackening), keratin flakes, a building of keratin around the feather shafts and feather picking in susceptible species. These changes are likely caused by the overall deterioration in health and nutritional status of the bird (VAN DER HEYDEN, 1997a).

3.3. Route of infection and localisation

In birds the route of infection is usually via the alimentary tract. Between birds transmission occurs most often through ingestion of soil and water contaminated by mycobacteria. Birds only occasionally suffer from pulmonary tuberculosis, most lesions developing in the intestinal tract, liver and spleen. Frequently bones and joints become involved, but the oral cavity and upper respiratory tract, lungs and air sacs, skin, bone marrow, gonads, heart, cloaca, gizzard, crop, serosal surfaces of the viscera, and rarely kidneys and pancreas may be affected. Organs, in which tuberculous lesions are never observed either grossly or microscopically, include the brain and the eye (MONTALI et al., 1976). However, gross lesions with evidence of avian tuberculosis in eyes were found in post mortem examinations of white-winged wood ducks (*Cairina scutulata*) in the WWT centre at Slimbridge (CROMIE, personal communication). Microscopically and by culture mycobacteria are very

frequently demonstrated in organs with negative findings on gross examination. This applies particularly to the kidney and bone marrow (PAVLAS et al., 1983). Lesions restricted to the lungs are occasionally seen and may represent primary respiratory infections acquired by inhalation rather than ingestion. Localised skin and conjunctival lesions also occur and may represent primary cutaneous infections (VAN DER HEYDEN, 1994; FORSTER and GERLACH, 1987). Tuberculous lesions are rarely found in the ovary and oviduct, so it is unlikely that egg constitutes a significant means of disease the transmission. If eggs were infected, tuberculous hatchlings would be more prevalent and this is certainly not the case (CROMIE, 1991). Some authors state that egg transmission can occur but is epornitically unimportant because *Mycobacterium avium* bacteriaemia causes an immediate cessation of egg production (GERLACH, 1994a). A study in 1981 revealed that avian tuberculosis of hens would be disseminated via infected eggs. From artificially infected eggs, by inoculating the causative agent into egg yolk or albumin tuberculous chicks were hatched (VOLNER et al., 1981). In a study by KYARI (1995) *Mycobacterium avium* was isolated from eggs of naturally infected wildfowl and moorhens (*Gallinula chloropus*) although it is not known if these eggs would have given rise to infected chicks.

It is relatively uncommon to find tubercles limited to one or more organs such as heart or bone or caecum, without gross or microscopic evidence of lesions elsewhere. This pattern would suggest that the pathogenesis of tuberculosis in birds might not always parallel that in mammals. In mammals a target organ such as the lung or intestine is first infected, regional lymph nodes are then involved, followed by involvement and either subsequent arrest or further systemic dissemination of disease. Although birds have discrete collections of lymphoid tissue, particularly in the digestive tract, and lymph node-like structures have been described only in waterfowl (PAYNE, 1971), the lack of well-developed regional lymph nodes in birds may be one reason for the relative ease with which this disease appears to become systemic (MONTALI et al., 1976).

3.4. Pathology

3.4.1. Macroscopic findings

Three mycobacterial syndromes are observed in pet birds: (1) atypical, characterised by diffuse enlargement of the affected organs, but no nodular lesions; (2) lepromatous, characterised by nodular to diffuse subcutaneous lesions; (3) tubercular, characterised by discrete, granulomatous nodules within and around internal organs, but lacking the extensive necrotic centre, central calcification, and fibrous capsule seen in mammalian tuberculosis. Atypical mycobacteriosis is the more common mycobacterial syndrome observed in pet birds, and it is usually caused by *Mycobacterium avium* or *Mycobacterium genavense*. All three syndromes may be found in the same bird or only one may be present (PANIGRAHY et al., 1983; GERLACH, 1994a). A ten year retrospective study of the disease pointed to an increasing incidence of *Mycobacterium genavense* as the cause of atypical mycobacteriosis in pet birds (HOOP et al, 1996). *Mycobacterium tuberculosis* infections frequently present with only the tubercular syndrome (ACKERMANN et al., 1974).

Most birds have marked muscular wasting, with total loss of subcutaneous and body cavity fat stores. Many organs have yellowish white nodules, both on the surface and deep within the parenchyma, in the submucosa of the intestines, subserosa of the peritoneum, air sacs, mucous membranes and dermis. Intestinal ulcers appearing as tumour-like masses on the serosa of the intestines, with caseous centres in larger tubercles and a fistulated tract entering into the lumen are common. As the tubercles in the intestinal wall commonly have this opening into the intestinal lumen, avian tuberculosis is rarely walled (GERLACH, 1994a). Mineralisation or cavitated nodules are not common. Most lesions are greyish-yellow to greyish-white nodules from 1 mm up to 4 to 5 cm in diameter. These accumulations of epithelioid cells may appear as discrete granulomas (tubercular form) or the epithelioid cells may diffusely infiltrate the organ resulting in a pale enlarged organ without obvious granuloma formation (atypical form) (VAN DER HEYDEN, 1997a; MONTALI et al., 1992). Caseating granulomas are often found in the liver of affected birds (MONTALI et al., 1976; THOEN et al., 1977a). The spleen is generally enlarged and often nodular or totally replaced by coalescing granulomas. The liver is often enlarged and tan in colour, and the intestines tubular and thickened (FORSTER and GERLACH, 1987). Infected joints are usually swollen and

often contain caseous material. In psittacine, passerine birds, columbiformes and anseriformes, however, *Mycobacterium avium* tends to cause a diffuse histiocytic enlargement of the affected organs rather than discrete granulomas (VAN DER HEYDEN, 1994; GERLACH, 1994a). Lack of softening and cavitation in the avian lesions might indicate a difference in lytic enzyme systems of heterophilic granules. Although dystrophic calcification is observed in some avian conditions, it is mostly absent in the avian tubercles (MONTALI et al., 1976).

3.4.2. Microscopic findings

In domesticated birds MAI complex infections generally cause multiple tubercle-like nodules along the intestinal tract. Early lesions consist of clusters of epithelioid macrophages with foamy cytoplasm. As foci enlarge, the centres become necrotic, eosinophilic and contain scattered nuclear debris. A ring of giant cells, a layer of epithelioid cells, and an outer fibrous capsule containing heterophils and mononuclear cells surround the necrotic centre. The giant cell can be classified mostly as the foreign body type, only rarely as the Langhans type (GERLACH, 1994a). The presence of giant cells seems to depend on the caseation. The histiocytic form is usually part of the early disease and varies from microscopic sheets of histiocytes to larger foci within parenchymatous organs and submucosal and subserosal areas. The histiocytes contain myriads of acid-fast organisms (MONTALI et al., 1973). Secondary granulomas may develop in the epithelioid cell layer, resulting in a multilobulated granuloma. In the liver especially, multinuclear giant cells are seen in a palisade arrangement at the periphery of the granuloma. Granulomas may involve all layers of intestine, and ulceration into the lumen of the intestine may occur. Organisms may be sparse in older granulomas but are usually plentiful in earlier lesions (MONTALI et al., 1976; THOEN et al., 1977a).
Mycobacterium avium can be visualised in tissue sections and imprints with acid-fast stains. The cytoplasm of *Mycobacterium avium* infected cells is grossly distended and densely packed with large numbers of acid-fast organisms. Inflammatory cells including giant cells, heterophils, lymphocytes, and foamy macrophages usually accompany the epitheliod infiltrates. Some pet birds appear to lack this inflammatory response, even with severe disseminated disease (HOOP et al., 1994; GERLACH, 1994a; PANIGRAHY et al., 1983).

This is similar to the response observed in many immune-deficient humans with atypical mycobacteriosis. The significance of this lack of response is unknown; however, it is interesting to speculate whether this may reflect the bird's immune status and if these are also the same birds that lack the haematologic response discussed below (**5.3.6. haematological analysis and blood chemistry**) (VAN DER HEYDEN, 1997a). *Mycobacterium tuberculosis* infections in parrots possess rather different histopathologic features, characterised by few mycobacteria per cell and granulomas that may appear acid-fast-negative (GERLACH, 1994a; ACKERMANN et al., 1974).

3.4.3. Amyloidosis and avian tuberculosis

Amyloidosis of the liver and spleen is seen in some cases. Grossly, these organs are enlarged and brittle and have a tannish-green translucence. The sinusoidal amyloid can be so extensive that it compresses hepatocytes, resulting in marked atrophy. Likewise, in the spleen, large 'pools' of amyloid replace red and white pulp architecture. The amyloid, confirmed by positive staining with congo red and birefringence with polarising microscopy, is also found in blood vessels, basement membranes, and parenchyma of many organs (MONTALI et al., 1976). In addition to its association with tuberculosis in birds, amyloidosis was reported as a 'primary disease syndrome', with a high incidence in waterfowl. Disease was attributed in part to stress factors. Amyloidosis was associated with marked increases and heterogeneity of serum globulin. It was suggested that deposition of amyloid was related to chronicity of infection and not to the type of immunologic deficiency (CHEVILLLE and RICHARD, 1971, cited by MONTALI et al., 1976; COWAN, 1968, cited by MONTALI et al., 1976).

3.5. Avian tuberculosis as a zoonosis

The organism is rarely a primary cause of disease in man. Most humans who are exposed to mycobacteria do not become infected, and only 3-5% of those who become infected by the human pathogenic *Mycobacterium tuberculosis* develop disease (VAN DER HEYDEN, 1994). This is because the immune system in most people is highly effective in eliminating

the bacterium. However, in a few people the immune system is unable to mount an adequate response either because of deficiencies in the system or an overwhelming inoculum of mycobacterium resulting in clinical disease. In some individuals live mycobacteria remain in the body, walled off by the host's inflammatory response, causing disease only if the immune system should fail at some point (reactivation disease) (VAN DER HEYDEN, 1997a).

Human disease caused by MAI complex reportedly occurs worldwide but is predominantly endemic in certain northern temperate geographic areas (INDERLIED et al., 1993). In general, *Mycobacterium avium* infections in birds and mammals are environmentally derived and not passed from bird to mammal and *vice versa* (WAYNE and SRAMEK, 1995). Data suggest that the environmental sources, in particular water, constitute the greatest risk of exposure of humans. Some of the serotypes isolated from humans have been isolated from other mammals and birds, but recent studies using molecular techniques show that there are considerable disparities within the serotypes (HOOP, 1997). *Mycobacterium avium* serotypes 1, 2 and 3 are primarily responsible for disease in birds. The disease in HIV (Human Immunodeficiency Virus) patients is most commonly caused by serotypes 4 and 8 (INDERLIED 1993). Disseminated infection with *Mycobacterium avium* is characteristic of disease in immune-deficient individuals, particularly those infected with HIV. There are no documented reports of *Mycobacterium avium* being directly transmitted to a human from a bird whether or not the human is immune-competent or immune-compromised. Epidemiological studies utilising DNA probes to investigate the origin of MAI complex infections in both immune-competent children and HIV infected adults, revealed that most infections were similar to the serotypes found locally in the environment (DABORN and GRANGE, 1993). Sources like tap water, soil, or food, appear to be the route by which most patients with AIDS become infected with non-tuberculous mycobacteria (BENSON and ELLNER, 1993). Studies have shown that half of the strains isolated from house dust are potentially pathogenic to humans. Arthropods can serve as mechanical vectors. Although the same mycobacterial species are isolated from animals and their droppings, the conclusion that these animals are the reservoirs for the human infection is tenuous. They may, however, contribute, irrespective of infection or carrier state, to the distribution of pathogenic mycobacteria in natural and man-made habitats (HOOP, 1997).

The prevalence of MAI complex infection in patients with AIDS is increasing. The predominance of serovars 1, 4, and 8, which are among the most virulent of the known

serotypes remains constant. Restriction-fragment-length polymorphism analysis revealed that American and European isolates of MAI complex were genetically similar to each other and distinct from African isolates (HOOP, 1997; BENSON and ELLNER, 1993; SNIDER et al., 1987). Non-tuberculous mycobacterial disease is found in patients in the developed world and rarely in patients in the developing world. This might partly be due to the fact that AIDS patients in Africa die of other causes before they reach a stage at which non-tuberculous mycobacterial disease develops (FALKINHAM III, 1996). Infection by MAI complex in AIDS patients tends to occur late in HIV disease and is most closely related to the $CD4^+$ cell count and not to age, gender, or race. Infection is rare or absent in AIDS patients whose $CD4^+$ cell counts are greater than 200 units but becomes increasingly common in AIDS patients as their $CD4^+$ cell count falls below 100 units or less. The highest frequency of disseminated MAI complex disease is in patients with $CD4^+$ cell counts below 10 (BENSON and ELLNER, 1993). Even in severely immune-compromised individuals, such as those with hairy cell leukaemia who would seem to be predisposed to MAI complex infection, the incidence of MAI complex infection is very low. MAI complex appears to have a particular predilection for infecting and disseminating within HIV-infected patients with significant morbidity and shortened survival times (AIDS patients with MAI complex infection median 7.4 months; AIDS patients without this infection median 13.3 months) (INDERLIED et al., 1993).

Four main types of disease have been described: (1) local lesions following traumatic inoculation; (2) localised primary lymphadenitis, mainly the cervical lymph nodes in children; (3) pulmonary infection, related to predisposing conditions; and (4) disseminated disease, usually associated with immunosuppression (GRANGE and YATES, 1986; SNIDER et al., 1987). Pulmonary disease due to MAI complex is indistinguishable clinically and radiographically from tuberculosis due to *Mycobacterium tuberculosis* (GRANGE and YATES, 1986).

Disseminated diseases caused by *Mycobacterium avium* infections are of special concern, since drug regimens commonly used for treating tuberculosis are not usually successful. The very small risk of transmission of *Mycobacterium avium* to children and to immune-compromised individuals can not be renounced, therefore, these individuals should not have contact with infected or potentially infected birds or their immediate environment (VAN DER HEYDEN, 1994; BENSON and ELLNER, 1993 and FALKINHAM III, 1996).

4. Treatment of avian tuberculosis

Treatment of avian mycobacteriosis is controversial due to a lack of information on zoonotic potential, the high number of bacteria excreted during therapy and the questionable effectiveness of antituberculosis drugs. The prognosis for the avian patient with opportunistic mycobacterial infection is always guarded (HOOP, 1997). Information regarding antimycobacterial therapy is limited and often extrapolated from clinical cases in humans, mammalian models for mycobacterial infections and *in vitro* data. Clinical studies have not been conducted, in part because of the reluctance of many practitioners to treat a potentially zoonotic disease with unproved drugs. Factors that may affect therapeutic outcome include the immune status and general health of the bird, the mycobacterial numbers and species present, prior drug therapy, owner compliance, drug resistance and drug sensitivity. There are two major problems when trying to apply extrapolated data to birds: the pharmacokinetics in birds for most of the antimycobacterial drugs are unknown and the mycobacterial species commonly encountered in birds are most similar to those seen in immunosuppressed patients, which complicates the comparison of therapies between humans and birds (VAN DER HEYDEN, 1997b).

4.1. Drug resistance of mycobacteria

Resistance of mycobacteria to drugs may be inherent to the mycobacterial species or acquired during therapy. Most non-tuberculous mycobacteria are naturally resistant to the anti-mycobacterial drugs isoniazid and pyrazinamide and other commonly used antibiotics, including the penicillins and cephalosporins. Drug groups with activity against most mycobacteria include the macrolides, fluoroquinolones, rifamycin, tetracyclines, and aminoglycosides as well as ethambutol, dapsone, ethionamide, cycloserine, and clofazamine (MANDELL and PETRI, 1996).

The numbers of mycobacteria present in the body at the start of therapy may be a significant factor in the development of drug resistance. Large numbers of mycobacteria are usually present at the time of initial diagnosis, increasing the risk that some of the mycobacteria will

be resistant to the chosen drug. This may lead to resistant strains during therapy resulting in treatment failure. In human tuberculosis, for example, the average mycobacterial load per cavitary lesion at the time of diagnosis in most patients is estimated to be 1×10^9. The rate of spontaneous mutation of *Mycobacterium tuberculosis* to a form resistant to either isoniazid or rifampicin is 1×10^8. It is unlikely that there are mycobacteria present that are resistant to both drugs. This is the rationale behind using multiple drugs simultaneously to treat mycobacterial infections (VAN DER HEYDEN, 1994). Similarly, the number of mycobacteria present in disseminated disease in birds is likely to be high. Drug resistance may also be a problem in birds at the onset of therapy because clinical cases are often initially misdiagnosed, and single-agent antimicrobial therapy may have already been administered, especially with frequently used drugs like enrofloxacin, doxycycline, and amikacin. Resistance may also develop as a result of cross-resistance between similar drugs, for example, rifampicin and rifabutin or isoniazid and ethionamide (MANDELL and PETRI, 1996; FALKINHAM III, 1996; BENSON and ELLNER, 1993).

Mycobacteria possess a permeability barrier and a highly hydrophobic cell surface. These factors contribute to resistance to a wide range of antibiotics. The hydrophobic nature of non-tuberculous mycobacteria is thought to restrict the activity of most hydrophilic drugs. This leads to the successful synthesis and testing of hydrophobic derivatives of drugs with borderline antimycobacterial activity. Adaptive resistance is another possible mechanism of antibiotic resistance in non-tuberculous mycobacteria. This requires continual exposure to the drug and in its absence resistance is lost. Non-tuberculous mycobacteria are also capable of producing agents, proteins, pigments, and polysaccharides that may antagonise the activity of some antibiotics (FALKINHAM, 1996).

Drug resistance may occur if the dose of the drug is inadequate. Pharmacokinetics of the drug in the patient including absorption, metabolism, and excretion may affect availability. These mechanisms may be important when treating birds because of lack of good pharmacokinetic data for most drugs in birds and difficulty in owner compliance when treating for lengthy periods (VAN DER HEYDEN, 1997b).

Susceptibility testing of the MAI complex is complicated by the variations in colony morphology, since the transparent colony type is more resistant to antimicrobial agents than the opaque colony type (WOODLEY and DAVID, 1976; KUZE and UCHIHIRA, 1984). STORMER and FALKINGHAM (1989) showed also that non-pigmented variants of *Mycobacterium avium* are significantly more resistant to a variety of antimicrobial agents than are pigmented isolates of the same strains. Since the pigmented variants grow more rapidly and are more obvious, there are speculations that in the selection of *Mycobacterium avium* colonies for susceptibility testing the less obvious non-pigmented variants, which may be analogous to the transparent colony variants, could be easily overlooked. Testing only pigmented variants could, therefore, lead to a false-susceptible result (STORMER and FALKINGHAM, 1989).

Drug selection should initially be based on the species of mycobacteria suspected, because drug sensitivities in general are species dependent. If possible, the mycobacterial species should be positively identified and drug sensitivities should be obtained. However, drug therapy should not be delayed, as results may take several weeks. Other factors that influence drug selection are solubility, availability, toxicity, ease of administration and availability of pharmacokinetic data.

In clinical practice it is unusual for susceptibility testing to be performed on more than a single isolate from a patient or specimen. Isolates of the same or different species may vary in antibiotic susceptibility. Even when two strains or species occur in the same patient, they may not both be isolated as one may overgrow the other or both may not have been present in the sample obtained. There is also evidence that *in vitro* sensitivities may not always represent true sensitivities owing to variation in the pH or nutrient content of the culture media (FALKINHAM, 1996)

4.2. Antimycobacterial drugs

As mentioned above, there is only little information about pharmacokinetics and mode of action of the different antimycobacterial drugs in birds. Therefore, most of the information is obtained from studies involving humans and/or small mammals.

Isoniazid is highly effective against *Mycobacterium tuberculosis* and *Mycobacterium bovis* but shows less *in vivo* or *in vitro* activity against MAI complex and most other non-tuberculous mycobacteria. Isoniazid and the related drugs, ethionamide and pyrazinamide, are derivatives of nicotinamide. They appear to inhibit the biosynthesis of mycolic acids in the cell wall of the mycobacterium and are bacteriostatic for 'resting bacillì', but are bactericidal for rapidly dividing microorganisms. Mycolic acids are unique in mycobacteria. This explains the selective antimicrobial activity of isoniazid. Variation in mycolic acids between mycobacterial species likely leads to variable susceptibility for these drugs. These drugs are water-soluble and excreted after acetylation in the urine (MANDELL and PETRI, 1996). As consistent resistance among *Mycobacterium avium* isolates has been reported, this drug must be used with caution (VAN DER HEYDEN, 1994).

Rifampicin is a semisynthetic derivative of the macrocyclic antibiotic rifamycin B, strictly speaking a 3,4-(methylpiperazinyl-iminome-thylidene)-rifamycin S. It inhibits DNA-dependent RNA polymerase of microorganisms, including mycobacteria but not in eukaryotes, except at high doses. It may affect mitochondrial replication by forming a stable drug-enzyme complex, leading to suppression of initiation of chain formation (but not chain elongation in RNA synthesis). The concentration of rifampicin in tissues is significantly higher than that in serum. Because of its increased lipophilia, rifabutin will penetrate more rapidly into organisms than rifampicin does. Rifambutin is a derivative of rifamycin S and has a similar mechanism of action (MANDELL and PETRI, 1996). It was much more effective than rifampicin against MAI complex phagocytosed by peritoneal macrophages, in a study with infected mice. Rifambutin inhibited intracellular growth of the organisms, even in the early phase, whereas rifampicin depressed the bacterial growth only in the later phase (SAITO and TOMIOKA, 1988). Cross-resistance with rifampicin occurs. Both drugs are bacteriocidal for intracellular and extracellular microorganisms. They are excreted primarily as active derivatives in the bile and undergo enterohepatic circulation. A small quantity is excreted unchanged in the urine. The rifamycins are potent inducers of hepatic microsomal enzymes and may, therefore, alter the pharmacokinetics of other drugs. Rifampicin may impart a red-orange colour to the faeces, urine, skin, and saliva. It is poorly soluble in aqueous solutions. A commercial preparation, Rifamate, containing both isoniazid and rifampicin, is available (MANDELL and PETRI, 1996; KEMPER et al., 1994; KEMPER et al., 1992).

Ethambutol is a dextro-2,2'-(ethylenedi-imino)-di-1-butanol-dihydrochloride that appears to interfere specifically with incorporation of mycolic acids into the mycobacterial cell wall and thereby decreasing the permeability barrier of MAI complex to other drugs. It is water-soluble and is excreted unchanged in the urine. It is tuberculostatic and may act synergistically with other antimycobacterial drugs to enhance their penetration. Resistance develops rapidly if it is used as a single agent (HOFFNER et al., 1989; KÄLLENIUS et al., 1989; MANDELL and PETRI, 1996; KEMPER et al., 1994; KEMPER et al., 1992).

Azithromycin and clarithromycin are semisynthetic derivatives of the macrolide erythromycin with marked activity against MAI complex. Clarithromycin has higher *in vitro* activity. However, azithromycin penetrates tissues more readily. Both drugs concentrate within phagocytic cells. The macrolides are bacteriostatic and exert their effect by binding to the 50S ribosomal subunit thereby inhibiting bacterial protein synthesis. They are water-soluble and are metabolised in the liver to active and inactive metabolites, and are excreted in the bile (MANDELL and PETRI, 1996). Both of these drugs have extended elimination half-lives (about 68 hours for azithromycin) that allows once daily dosing (VAN DER HEYDEN, 1994).

The fluoroquinolones include ciprofloxacin, enrofloxacin, and sparfloxacin. These drugs, which inhibit bacterial DNA gyrase, have excellent antimycobacterial properties and are widely distributed in tissues. Like the macrolides, they concentrate in macrophages. They are water-soluble and are excreted in the urine unchanged. Resistance develops when they are administrated as a single agent (MANDELL and PETRI, 1996; KEMPER et al., 1992).

Streptomycin, kanamycin, and amikacin are aminoglycosides with activity against most mycobacteria. Amikacin, a semisythetic antiobiotic derived from kanamycin is particularly effective against atypical mycobacteria, especially *Mycobacterium avium*. The aminoglycosides are bacteriocidal *in vitro* against mycobacteria, however, *in vivo* they appear to be bacteriostatic. They are water-soluble and are excreted unchanged in the urine. Chronic or high-dose administration often results in severe ototoxicity and nephrotoxicity and they have to be administered parenterally, as they are not absorbed from the gastrointestinal tract (MANDELL and PETRI, 1996).

Clofazamine is an iminophenazine red dye that binds to DNA inhibiting transcription. It appears to have better *in vitro* than *in vivo* activity against *Mycobacterium avium*. It concentrates within macrophages and enhances their respiratory burst. It is poorly water-soluble and is available only as a gel cap, which can make dosing difficult. Like the rifamycins, it may discolour the skin, urine, and tears (MANDELL and PETRI, 1996; KEMPER et al., 1992; KEMPER et al., 1994).

Doxycycline and minocycline are semisynthetic tetracycline derivatives. They have minimal activity against *Mycobacterium avium* but appear to have good efficacy against other atypical mycobacteria. They are lipophilic and accumulate in reticuloendothelial cells. They are bacteriostatic inhibiting bacterial and, to a lesser extent, host cell protein synthesis by binding to ribosomes (MANDELL and PETRI, 1996).

4.3. Recommendations for treatment of mycobacterial infections in pet birds

The apparent effectiveness of the newer macrolides, clarithromycin and azithromycin against *Mycobacterium avium* and *Mycobacterium genavense* makes them obvious choices for treating mycobacterial infections in birds as well as in humans. There are no published reports of the pharmacokinetics of either drug in any species of birds, but as these drugs are readily absorbed from the gastrointestinal tract, show wide tissue distribution, and are well tolerated by a variety of species, it is reasonable to consider their use in birds. The initial therapeutic regimen should include rifabutin, ethambutol, and either azithromycin or clarithromycin administered concurrently. Culture and sensitivity should be performed, if possible, to confirm drug sensitivity. Birds that respond poorly to therapy or that relapse should have either a fluoroquinolone or an aminoglycoside added to the regimen. An alternative or additional drug that may prove useful, especially in birds with a marked inflammatory response, would be clofazamine (VAN DER HEYDEN, 1997b).

All of the recommended drugs, except amikacin, can be administrated orally. The macrolides, fluoriquinolones, and ethambutol readily dissolve in aqueous solutions. Rifamycin and rifabutin form suspensions when mixed with aqueous solutions, whereas clofazamine will not mix with aqueous solutions. Excepting clofazamine, all the drugs can be mixed together in the appropriate ratios in an aqueous base for short periods (seven to ten days) for convenient

dosing. Alternatively, the drugs can be ground to a fine powder and diluted with a powdered sugar or dextrose base in the proper ratios. The appropriate dose of the mixture is disguised in a small amount of strong tasty food such as juice, ice cream, pudding, or spaghetti sauce to be fed to the bird daily. Clofazamine can be diluted in vegetable oil (VAN DER HEYDEN, 1997b) or lactulose for accurate administration

Duration of treatment as cited in the literature, ranges from 1 to 18 months. This is affected by the severity of the infection, sensitivity to drug therapy, compliance with drug therapy, and possibly the strain of mycobacteria involved. Since *Mycobacterium avium* lives within phagocytes (VAN DER HEYDEN, 1994), treatment should ideally be continued several months past the last positive biopsy or culture. In immune-suppressed individuals, treatment may extend for the life of the patient with the goal being suppression, rather than elimination of the organism (VAN DER HEYDEN, 1994). It should be emphasised to the client that strict compliance to the drug regimen early in treatment is critical. Resistance might otherwise develop, which may lead to a relapse or failure to respond to treatment. Lack of rapid improvement within the first month of chemotherapy suggests that diagnosis, the presence of other diseases, client compliance, dosage, or drugs should be reviewed or changed (VAN DER HEYDEN, 1997b).

Table 3 presents an overview of recommended dosages for the treatment of avian tuberculosis in pet birds.

Table 3: Published antimycobacterial drug dosages (VAN DER HEYDEN, 1997b).

Drug	Total daily dose	Reference
Isoniazid	30 mg/kg	MANDELL and PETRI (1996)
Rifampicin	10-20 mg/kg	ROSSKOPF et al. (1986), cited by VAN DER HEYDEN (1997)
	45 mg/kg	VAN DER HEYDEN (1994)
Rifabutin	15 mg/kg	VAN DER HEYDEN (1994)
	56 mg/kg	VAN DER HEYDEN (1997b)
Ethambutol	30 mg/kg	VAN DER HEYDEN (1994)

	15-25 mg/kg	ROSSKOPF et al. (1986), cited by VAN DER HEYDEN (1997)
	56-85 mg/kg	VAN DER HEYDEN (1997b)
Streptomycin	20-40 mg/kg	ROSSKOPF et al. (1986), cited by VAN DER HEYDEN (1997b)
Amikacin	30 mg/kg	VAN DER HEYDEN (1994)
Ciprofloxacin	80 mg/kg	VAN DER HEYDEN (1994)
Clofazamine	6 mg/kg	VAN DER HEYDEN (1994)
	6-12 mg/kg	VAN DER HEYDEN (1997b)
Clarithromycin	85 mg/kg	VAN DER HEYDEN (1997b)
Azithromycin	43 mg/kg	VAN DER HEYDEN (1997b)

4.4. Reported cases of treatment of avian tuberculosis

Reported by ROSSKOPF (1991):

(1) Two four year old grey-cheeked parakeets (*Brotogeris phyrrhopterus*) were diagnosed with avian tuberculosis. Treatment was initiated with the standard regimen:

- Streptomycin injectable 20-40 mg/kg divided b.i.d. for a week (0.04cc of 1:10 solution b.i.d.)
- Ethambutol 15-20 mg/kg b.i.d. (dissolve 1/4 of 100 mg tablet in 1.25cc juice and give 0.05cc orally b.i.d.), made fresh every two days
- Rifampicin 10-20 mg/kg b.i.d. (1/4 of 300 mg capsule dissolved in 5cc juice, 0.05cc given b.i.d. for a 55 g grey-cheeked parakeet)

Treatment continued for almost a year. Follow-up haemograms have been normal for four years. An apparent total remission has occurred.

(2) One 11-month-old grey-cheeked parakeet (*Brotogeris phyrrhopterus*) was only treated with rifampicin and ethambutol, as this was a very early case. After three months injectable streptomycin was added and the regimen continued for another two months. The bird has remained normal clinically and haemotologically for four years and all acid-fast tests have been negative.

(3) A seven year-old yellow-naped amazon parrot (*Amazona ochrocephala auriopalliata*) was presented with avian tuberculosis. The bird was treated with:

- Isoniazid 30 mg/kg daily (1/4 of 100 mg tablet in 1.25cc liquid,given 0.7cc daily)
- Ethambutol (1/4 of 100 mg tablet in 1.25cc liquid, given 0.7cc daily)
- Rifampicin (300 mg capsule in 10cc of liquid, given 0.7cc daily)

All were mixed together daily and given at once (2.1cc total volume)
The bird appeared normal on treatment.

(4) Other birds have been successfully treated with:

- Ciprofloxacin 20 mg/kg orally for 7 to 10 days or
  Baytril injectable i.m.15 mg/kg b.i.d. for 7 to 10 days or
  Streptomycin injectable i.m. 20-40 mg/kg/day divided b.i.d. for 7 to 10 days
- Clofazime 50 mg capsules, 1.5 mg/kg/day, one dose for 3 months to 1 year
  dissolved in acid juice or in bases
- Cycloserine 250 mg pulvules, 10 mg/kg/day, divided b.i.d. for 3 months to 1 year
  dissolved in water
- Ethambutol 100 mg tablet, 40 mg/kg/day, divided b.i.d for 3 months to 1 year
  dissolved in water

Ciprofloxacin and injectable drugs are used for advanced cases or for seriously ill birds. In most cases of early or contained disease, three oral medications are used (Clofazimine, Cycloserine, Ethambutol). Solutions are made to last a week.

VAN DER HEYDEN (1994) reported the successful treatment of 27 clinical cases of *Mycobacterium avium* in psittacines from 1985 to 1994. The treatment duration varied between 10 and 18 months; the average being 12 months. The drug combinations used to treat mycobacteriosis were:

- Isoniazid 30 mg/kg, Ethambutol 30 mg/kg, Rifampicin 45 mg/kg s.i.d. per os
- Clofazimine 6 mg/kg, Ethambutol 30 mg/kg, Rifampicin 45 mg/kg s.i.d. per os
- Ciprofloxacin 80 mg/kg, Ethambutol 30 mg/kg, Rifampicin 45 mg/kg s.i.d. per os
- Amikacin 20 mg/kg s.i.d. intramuscular, Enrofloxacin 30 mg/kg s.i.d. per os
- Enrofloxacin 30 mg/kg, Ethambutol 30 mg/kg, Rifampicin 45 mg/kg s.i.d. per os
- Enrofloxacin 30 mg/kg, Ethambutol 30 mg/kg, Rifampicin 15 mg/kg s.i.d. per os

5. Diagnosis of avian tuberculosis

The control of avian tuberculosis is notoriously difficult and usually relies on preventing the establishment of initial infection by high standards of hygiene or subsequent removal of infected individuals. These must be identified by means of a reliable diagnostic test. Diagnosis of avian tuberculosis ante mortem can be difficult because of the wide variation in disease signs, the lack of adequate serological tests, and the difficulty of culturing the organism.

5.1 Diagnosis based on genetic structures

5.1.1 Rapid culture methods

Radiometric BACTEC (Becton and Dickinson, diagnostic instrument systems, Inc, Sparks, MD, USA) culture of *Mycobacterium avium* from faecal and tissue biopsy samples offers several advantages over conventional culture methods for the isolation of mycobacteria, including its speed, sensitivity, and suitability for quantitative studies. The process involves a liquid medium that contains ^{14}C-palmitic acid, a radioactive pharmaceutical (MIDDLEBROOK et al., 1977). If mycobacteria are present, radioactive labelled $^{14}CO_2$ will accumulate above the broth. The BACTEC instrument withdraws this gas and measures the amount of radioactivity present (HOFFNER, 1988; KIRIHARA et al., 1985). In general, positive cultures are detected in 7 to 14 days by the radiometric method, and in 21 to 28 days (or longer) by the conventional method (KIRIHARA et al., 19856). BACTEC offers a very high specificity and sensitivity compared to solid culture media. The main disadvantage of BACTEC is that radiometric techniques require specialised and expensive laboratory equipment (ARANAZ et al., 1997). In the case of two birds known to be positive for avian tuberculosis, the result was false negative (VAN DER HEYDEN, 1994).

The protocol for radiometric culture of *Mycobacterium avium* from avian faecal samples is as follows. Faecal samples are first decontaminated in a 1% hexadecylpyridinium chloride solution for up to 15 minutes, then filtered through two layers of gauze to remove large particulate material, and inoculated directly into the enriched and antibiotic-supplemented

growth medium. Inoculated growth vials are incubated at 37°C under 5% CO_2 for three weeks. Growth is measured daily by a gas ionisation detector instrument called the BACTEC 460 and reported as raw growth index value. When the cumulative growth index exceeds 100, a few drops of the culture broth are plated on a blood agar plate and observed for growth for 48 hours. In addition, a drop of culture is plated on a slide and acid-fast stained. If no growth is detected on the blood agar plate and acid-fast organisms are seen on the slide, then a *Mycobacterium* species is assumed to have been isolated, and identification is attempted using a DNA probe that hybridises with *Mycobacterium avium* 16S rRNA. The protocol is the same for tissue biopsy samples, with the exception of the first step in which tissues are homogenised in saline before exposure to hexadecylpyridinium chloride solution (CLARK et al., 1995).

5.1.2 Rapid assays for identification

5.1.2.1. DNA probes

DNA-RNA probe tests are highly specific and sensitive for mycobacteria and they are the simplest, most rapid methods for identification of MAI complex isolates. At least one probe is available commercially, the Accuprobe system (GenProbe Inc, San Diego, CA). Probes can be used to test specimens directly or after enhancement by culture (VAN DER HEYDEN, 1994). Some studies show that DNA probes are not sensitive enough for direct detection of acid-fast bacilli in specimens. This method is based on the detection of high numbers of ribosomal RNA copies. Currently available probes use a chemiluminescent, acridinium ester label, producing light on exposure to peroxide and high pH MAI complex. Separate species-specific probes are available (PAO et al., 1988).

5.1.2.2. Polymerase chain reaction

The newest generation of rapid assays uses nucleic acid amplification by means of polymerase chain reaction (PCR). PCR can be used for identification by culture or for direct

detection of the organisms in clinical material, overcoming time-consuming culture and biochemical identification steps. *In vitro* amplification reactions increase the amount of a specific target sequence to a detectable level by hybridization or agarose gel electrophoresis. The technique provides rapid results (one to three days) (ANRANAZ et al., 1997). These tests can identify extremely low numbers of organisms in specimens and can accurately distinguish between the various species of mycobacteria (VAN DER HEYDEN, 1994). Specific targets for *Mycobacterium avium* have been described. An insertion sequence, the IS901, similar to IS900 from *Mycobacterium tuberculosis*, has been found in *Mycobacterium avium* strains pathogenic for birds and other animals (PAO et al., 1988). Several variations of the PCR which detect and identify mycobacteria, based on the 65kDa antigen or rRNA genes, have been described. A fragment of the gene encoding the 65kDa antigen from all mycobacteria can be amplified, and the species can be detected by hybridisation with probes that recognise variable portions within the amplified fragment (PLIKAYTIS et al., 1992). A similar, commercially available technique uses a set of primers to amplify the DNA that codes for the rRNA. Primers 246 and 264 are specific for all members of the genus *Mycobacterium;* MYCAV-R is *Mycobacterium avium* specific; and MYCINT-F is *Mycobacterium intracellulare* specific (ARANAZ et al., 1997). The product is hybridised with specific oligonucleotides to determine the species of mycobacteria. The common fragment can be digested with several restriction enzymes, which produce fragments that vary between species, due to sequence heterogeneity (PLIKAYTIS et al., 1992).

5.1.2.3. High-performance liquid chromatography

High-performance liquid chromatography (HPLC) studies the species (complex) specific mycolic acids of mycobacteria. These are unique in having very long chain lengths (60-90 carbon). HPLC analysis produces a pattern that can be used for species identification. This method has been proven to be reliable for MAI complex isolates (BUTLER et al., 1991). The HPLC method requires considerable equipment and a high level of expertise, and so, is a method best suited to large laboratories (ARANAZ et al., 1997).

5.2. Lymphocyte transformation test

The cellular immune response comprising of the interaction between lymphocytes, epithelioid cells, macrophages, cytokines and immunoglobulins, plays an important role in the immune defence against disease, including avian tuberculosis. Since the realisation that lymphocytes can be stimulated *in vitro*, the lymphocyte transformation test has grown in significance for basic immunological research and diagnostic testing. Mononuclear cells are stimulated to increased protein synthesis and to blastogenesis by antigens. The degree of stimulation is measured in the lymphocyte stimulating test and is the measure of the integrity of the immune system (KRISTENSEN et al., 1982). Cell culture/antigen combinations are usually labelled with 3H-thymidine and the uptake of 3H-thymidine is measured as counts per minute. A stimulation index is calculated by dividing mean counts per minute of stimulated cultures by mean counts per minute of unstimulated control cultures. Studies show that wildfowl lymphocytes transform in the presence of mycobacterial antigen, providing the transport and culture conditions are optimal (CROMIE et al., 1989; CROMIE et al., 2000). However, the correlation between the lymphocyte transformation response and necropsy findings has been reported as poor (CLARK et al., 1995).

5.3. Clinically relevant diagnostic techniques

5.3.1 Avian tuberculin skin test

The avian tuberculin test in domestic fowl requires that Weybridge standardised avian tuberculin (0.1 ml or 0.05 ml) is injected into a single wattle. The birds are re-examined after 72 hours for any reactive swelling. The skin reaction has a characteristically delayed onset, with the affected area becoming infiltrated by mononuclear cells, primarily lymphocytes and monocytes (HAWKEY et al., 1990). The intradermal test does not appear to work in pet birds when *Mycobacterium avium* tuberculin is used, although there are reports that it is diagnostic in poultry (MONTALI et al., 1976; VAN DER HEYDEN, 1986). The same test performed in the vent of known positive psittacine birds was uniformly negative in another study (VAN DER HEYDEN, 1994). Skin test responses in wildfowl can occasionally yield false negative

results when the bird is in an advanced state of disease (CROMIE, 1991). GERLACH (1994a) also does not recommend the use of the tuberculin test because of its frequent association with false-negative results, particularly in early and late stages of the disease. The avian tuberculin test can give false positive results due to misreading the reaction or to the presence of bruising at the injection site. Anatomical features also influence the usefulness of this test, not all species have the featherless wattles of the domestic fowl, which allows easy intradermal injection and reading of results. A further disadvantage of this test is that the birds must be re-examined after 72 hours for the result to be read. This is not always easy or advisable in the case of exotic species, which are susceptible to handling, trauma and stress, or in collections with a high number of birds (HAWKEY et al., 1990).

5.3.2. Radiology and endoscopy

5.3.2.1. Radiology

Whole body radiology is a useful diagnostic test, particularly in the tubercular form of the disease when multiple granulomas may be observed (MONTALI et al., 1976; VAN DER HEYDEN, 1989). These granulomas do not calcify like mammalian tubercles. Less specific findings include hepatosplenomegaly, intestinal enlargement accompanied by gas, miliary granulomas in the lungs, and endosteal bone densities. Bone changes involve primarily the humerus, tibia, ulna, and rarely the femur and vertebral column. They can represent bone-marrow involvement and osteomyelitis. Radiography plays an important role in localising lesions for biopsy or bone- marrow aspiration (VAN DER HEYDEN, 1997a).

5.3.2.2. Endoscopy

Endoscopy is the best method by which diagnostic material for definitive diagnosis of mycobacteriosis can be obtained. Granulomas appear white or tan, are typically round (in contrast to the more disc-like granulomas of aspergillosis), and in contrast to most bacterial granulomas incite minimal vesicular reaction around them. The granulomas are soft and

easily biopsied compared with most non-lymphoma neoplasms. Infiltrated hepatic, splenic, or renal tissue appears pale and enlarged. A biopsy of the liver in suspect birds should be obtained even if discrete granulomas or involved areas cannot be identified. Liver biopsies can also be obtained transcutaneously or via laparotomy (VAN DER HEYDEN, 1997a).

5.3.3. Direct examination by staining and microscopy

Microscopy is currently the simplest, cheapest, and most rapid procedure for detection of acid-fast bacilli in clinical specimens. Mycobacteria can only be seen if at least 5 x 10^4 mycobacteria/ml of material are present. Fluorescent dyes, such as auramine or acridine orange, offer several advantages over conventional basic fuchsin stains, because they allow the bacteria to be seen more easily and a larger area of the smear to be examined in less time (ARANAZ et al., 1997). Cytologic and histopathologic preparations can be made from biopsy material and stained with routine and acid-fast stains. With routine stains, large numbers of epithelioid cells containing non-staining (ghost) rods may be observed. Using acid-fast stains, such as Ziehl-Neelsen, large numbers of *Mycobacterium avium* can usually be seen in tissue sections and faecal smears from birds with disseminated intestinal disease. This method is less reliable in the case of other forms of mycobacteriosis (MONTALI et al., 1976; VAN DER HEYDEN, 1989). Mucus present in the faeces can interfere with test results, so samples should be processed with one of the sputum solvents used in human medicine prior to staining (GERLACH, 1994a). Microscopically the bacilli are sometimes small, appearing almost as cocci. *Mycobacterium avium* is relatively variable in shape and may also appear in long beaded rods or in short forms. Species of *Mycobacterium* can not be reliably differentiated by microscopy. Microscopy is considered to be less sensitive than culture, and a definitive diagnosis should be based on the identification by culture of the microorganism from biopsy or necropsy specimens (ARANAZ et al., 1997). It is important to realise that this test can easily give false-positive or negative results if staining conditions are not adequately controlled (VAN DER HEYDEN, 1997a).

5.3.4. Culture

Most of the specimens submitted to the laboratory also contain undesired and more rapidly growing contaminant microorganisms. These may overgrow mycobacteria in culture. The most common decontamination protocols are the N-acetyl-L-cysteine-NaOH method, the NaOH method, the Zephiran-trisodium phosphate method, the oxalic acid method, the cetylpyridinium chloride-sodium chloride method and the hexade-cylpyridium chloride method (HINES et al., 1995). The highest yield of mycobacterial cultures is expected from the mildest decontamination procedure that gives sufficient control over contaminants without interfering with the viability of the mycobacteria. All decontaminants are toxic to mycobacteria to some extent. Hexasecylpyridinium chloride method decontaminates effectively and is performed with relative speed. It is less toxic to mycobacteria, and so is considered the decontaminant of choice (CORNER et al., 1995). Following decontamination, specimens are concentrated by centrifugation. 3,000 g applied for 15 minutes or 2,000 to 2,500 g applied for 20 minutes is considered adequate.

Many different media have been used for the isolation of mycobacteria. There is little agreement as to which is best under routine diagnostic conditions. Agar based media include Middlebrook 7H10 with pyruvate and Herrolds's egg yolk. Egg-based media, such as Loewenstein-Jensen Coletsos and Stonebrink are also employed (ARANAZ et al., 1997). Some strains of *Mycobacterium avium* isolated from birds may also require the iron chelator mycobactin for growth, particularly on initial isolation. They can take from one to six months to grow (MATTHEWS et al., 1977). Recommended media are Stuart's basal medium, enriched with bovine albumin and crude mycobactin, or Watson-Reid medium. Mycobacterial strains requiring mycobactin for growth in primary culture may either be truly dependent on mycobactin or require it only when small numbers of viable units are cultured (MATTHEWS et al., 1977). Labour will be saved in the typing of difficult *Mycobacterium avium* strains by first plating on oleic acid-albumin agar medium. The selection of a transparent colony for examination will give the best chance of successful typing by agglutination or lipid analysis. The same procedure should be applied to pigmented strains in order to detect whether a mixture of strains is present; egg medium appears to be unsatisfactory for this purpose and passage may fail to separate the organism. Finally, if typing remains impossible, passage may be considered. At least two fowl should be inoculated and at least two different organs

cultured at necropsy. It may be necessary to plate the cultures for a second time, to find suitable colonies (SCHAEFER et al., 1973). Opportunistic mycobacterial infections in humans are diagnosed based on blood and stool cultures and histopathology and culture of biopsy samples (BENSON and ELLNER, 1993). Avian blood has been used successfully to detect mycobacteria in infected birds, while faecal or biopsy cultures have not (VAN DER HEYDEN, 1994). Faecal and blood cultures depend on the bird's excreting mycobacteria in the faeces or on its being mycobacteraemic. This is not always the case. Likewise, only biopsy samples positive for acid-fast bacteria should be used for cultures.

5.3.5 Agglutination test

In a 1993 study, the agglutination test was quick and easy to perform but all the data needed to be scored subjectively. For use as the agglutination antigen, a strain of *Mycobacterium avium* isolated from an avian tuberculosis-positive bird from the WWT centre at Slimbridge was used. A drop of the antigen was stirred with a drop of blood or serum on a white tile. The result was scored by an experienced researcher estimating the degree of agglutination by eye within the first 30 seconds, while gently rotating the tile in a 'see-saw' motion (CROMIE et al., 1993c; HAWKEY et al., 1990). The test carried out in the field was very sensitive although a high rate of false positives did occur. The test using serum was less sensitive, but its specificity was greater. The test using blood in EDTA was insensitive and had poor specificity (FORBES et al., 1993). The high proportion of false negatives that occurred may be due to dependence on calcium in agglutinating responses (CROMIE et al., 1993c). Incorrect results may be due to structural differences in wildfowl immunoglobulins, which render them less efficient at secondary activities such as agglutination of antigen (ZIMMERMANN et al., 1971; HIGGINS and WARR, 1993). The agglutination test has the potential advantage of requiring only one blood sample (and thus, single handling of the birds), providing an immediate result, being rapid and simple to perform, and requiring no sophisticated or expensive equipment, but the incidence of false positive and negative results demonstrates the need for standardisation of the reagents and criteria for interpreting the results as well as the need for an objective and quantifiable test (HAWKEY et al., 1990). The findings of studies by CROMIE et al. (1993c) and PAVLAS et al. (1983) suggested that the

type of sample used for haemagglutination tests may also affect the sensitivity of the test. A variable immune response, for example due to seasonal changes, of the birds tested may also influence the occurrence of false positive and false negative results.

5.3.6 Haematological analysis and blood chemistry

Clinical biochemistries are often surprisingly unremarkable in birds with mycobacterial infections. Tissue enzymes (alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, creatinine phosphokinase, gamma-glutamyl transferase, alkaline phosphatase) and hepatic function (bile acids) values may be abnormal, particularly in birds with marked hepatomegaly (VAN DER HEYDEN, 1986). Tissue enzyme levels are rarely as high as those seen with the same degree of hepatomegaly secondary to viral, chlamydial and other bacterial diseases that typically cause more hepatic necrosis than do mycobacterial infections. Albumin levels are often decreased, particularly in birds with chronic, disseminated intestinal and hepatic disease. This is likely due both to intestinal loss and decreased hepatic production. Globulin levels vary during the course of disease (FORBES et al., 1993; MONTALI et al., 1976; VAN DER HEYDEN, 1986).

Diagnostic tests usually reveal extremely high white blood cell counts (6.2-230.0 x 10^9/l) characterised by a monocytosis (1.6-82.5 x 10^9/l) and a heterophilia. Hyperfibrinogenaemia and microcytic, hypochromic anaemia are often seen (MONTALI et al., 1976; HAWKEY et al., 1990). A 1993 study of a haematological analysis detected the advanced cases, but some of the cases of early disease were missed. The total blood cell count showed a leucocytosis characterised by a monocytosis, concurrent milder heterophilia, hyperfibrinogenaemia and microcytic hyperchromic anaemia (CROMIE et al., 1993c). BUSH et al. (1978) reported a range of the white blood cell count from 3.0 to 233.3 x 10^9/l, with the highest values occurring in the more advanced cases of avian tuberculosis. A case was considered to be positive when the white blood cell count reached 18 x 10^9/l. Disease must be severe to cause leucogram changes of this degree. In the same study, many of the early cases demonstrated raised white blood cell counts (8-12 x 10^9/l). This method may be of value for the detection of individual cases, but not for the screening of large populations (CROMIE et al., 1993c).

The heterophils of affected birds can show morphological abnormalities typical of immaturity, including incomplete nuclear lobulation (left shift) and/or reduction, and shape irregularities of the cytoplasmic granules (HAWKEY et al., 1990). Polychromasia may be seen. The haematocrit is generally decreased, except in cases of pulmonary disease in which it can be dramatically elevated (VAN DER HEYDEN, 1994). Erythrocytes may exhibit mild anisocytosis and poikilocytosis. Erythroblasts (inappropriate for the degree of anaemia) may be seen as well. Extremely toxic, or more commonly, immature heterophils that persist for weeks in the circulation were noted on haematologic analysis. This contrasts with viral and chlamydial diseases in which those cells rapidly disappear with treatment (VAN DER HEYDEN, 1997a). Many pet birds with both localised and disseminated disease exhibit minimal haematologic changes, particularly in the leucogram. It is speculated that this reflects the immune response of the bird to the mycobacteria. Several studies document a significant monocytosis. Leucocytosis, heterophilia, hyperfibrinogenaemia and a hypochromic microcytic anaemia are non-specific reactions to infectious and inflammatory conditions in birds. They cannot be regarded as specific for avian tuberculosis. According to these studies the magnitude of the white cell increase seen in avian tuberculosis is greater than that found in other bacterial infections in birds, and the same degree of marked monocytosis has been identified only very occasionally in cases of aspergillosis (HAWKEY et al., 1990).

6. Diagnosis by using an enzyme-linked immunosorbent assay (ELISA)

6.1 General consideration of an ELISA

The first report of the serodiagnosis of tuberculosis was probably that of ARLOING, which was published in 1898, only 16 years after Koch's identification of the tubercle bacillus (ARLOING, 1898, cited by DANIEL and DEBANNE, 1987). In 1971 a highly sensitive and reproducible technique of enzyme-linked immunosorbent assay (ELISA) was first published by two independent groups, one in Sweden (ENGVALL and PERLMANN, 1971) and the other in Holland (VAN WEEMEN and SCHUURS, 1971). The first commercial ELISA kit became available in 1976, from Organon Teknika, and was used to detect hepatitis B surface antigen (VOLLER and BIDWELL, 1988).

Enzyme-linked immunosorbent assays (ELISA) are used widely in medical and veterinary diagnosis. The technique has been successful in detecting antibodies to *Mycobacterium avium* in experimentally infected chickens (THOEN et al., 1978). Another solid-phase, antibody-capture ELISA was developed for detection of antibody against *Mycobacterium avium* in serum collected weekly from chickens and quail challenged either orally or intravenously with *Mycobacterium avium* (CLARK et al., 1995). One study describes the inoculation of 72 guinea pigs with various atypical mycobacteria. The ELISA failed to differentiate between reactions specific to *Mycobacterium avium* and cross-reactions. Cross-reactions are seen in instances of sensitisation or infection with other mycobacterial strains. The ELISA detects anti-mycobacterial antibodies in an early stage of disease. Therefore, this test is useful to screen a population of animals (SHAHEEN et al., 1997).

CROMIE et al. (1993c) evaluated an ELISA in comparison with other diagnostic techniques, for use in wildfowl. During progressive *Mycobacterium avium* infection, serial ELISAs in wildfowl showed a marked increase in anti-mycobacterial antibodies, as much as one year prior to death from the disease (CROMIE, 1991; CROMIE et al., 2000). In a study at the WWT centre at Slimbridge in 1992, 329 feral barnacle geese (*Branta leucopsis*) were screened by ELISA, agglutination test and haematological analysis (CROMIE et al., 1993c). The study showed that the ELISA assay was very sensitive and specific for the disease, as

there were no false negative or false positive results. It was suggested that the 'borderline status' may be an indication of a bird in the early stages of infection, as the majority of the birds, which were tested positive for avian tuberculosis in the second year, were birds with borderline results in the previous year. It has also been shown that antibody levels to *Mycobacterium avium* do not increase in response to vaccination (CROMIE, 1991). The ELISA is a labour-intensive technique, taking at least 24 hours to generate results. During this time birds need to be penned or caught again, pending results (FORBES et al., 1993).
The ELISA has been used with varying degrees of success in several independent studies in the WWT centres at Slimbridge and at Llanelli (ASH and CROMIE, 1998; PAINTER, 1995; FORBES et al., 1993; CROMIE et al., 1993c).

6.2. ELISA principles

In enzyme immunoassays an enzyme is used to label either antibody or antigen such that immunological activity is retained. Labels can be: radioactive tracer label, fluorescent marker label, enzyme label, red cell label or latex label. The enzymes can be detected at very low concentrations by the addition of a substrate capable of yielding a detectable signal in the form of colour, fluorescence, radioactivity or luminescence. The antigen is immobilised in a plastic tube by simple adsorption. The coated tube is then allowed to react with the test sample, containing antibody. After capture of the antibody by the antigen, other constituents of the sample are washed away. The captured antibody is then indicated by the addition first of enzyme-labelled antiglobulin and then of enzyme substrate (VOLLER and BIDWELL, 1988).

Two basic types of immunoassay exist, 'competitive' and 'non-competitive'. The 'competitive immunoassay' relies on the competition between the antigen of interest (the analyte) and a constant amount of a similar but labelled antigen for a limited amount of specific antibody. Because the two antigens compete for the same antibody, the labelled antigen must react identically to the unlabelled.

In the ‘equilibrium assay’, the analyte and the labelled antigen are added simultaneously to the specific antibody for which they compete: the reaction then is allowed to reach equilibrium, following the law of mass action. At equilibrium, the amount of bound, labelled antigen-antibody complex is inversely proportional to the amount of the analyte of interest present in the specimen.
In the rare ‘sequential assay’, the analyte to be measured is incubated with the antibody, after which, labelled antigen is added.
The ‘non-competitive’ or immunometric immunoassay (sandwich type) uses an excess of labelled specific antibody toward the analyte of interest. Either a single-labelled antibody may be used, or two antibodies, one labelled and one unlabelled, but fixed to a solid support. Following completion of the antigen-antibody reaction, the amount of bound-labelled antibody is directly proportional to the amount of analyte present in the specimen
In competitive immunoassays, the analyte is labelled, while in immunometric immunoassays, the reagent is labelled (SLAGLE and GHOSN, 1996).

After antigen-antibody reaction, the bound and unbound-labelled fractions must be distinguished. To achieve this, heterogeneous immunoassays require a physical separation step. Such assays rely on physical, chemical, or immunologic differences such as size, charge, and adsorption to solid surface for the separation. This may occur in either the liquid or solid phase. Homogeneous immunoassays do not require a separation step. They rely instead on changes in label characteristics as a result of binding. Such changes include conformational change or inhibition of an enzyme, or decrease in rotational movement of a fluorophore. These changes are usually due to a size difference between the (small) unbound antigen and the (large) antigen-antibody complex (SLAGLE and GHOSN, 1996).

6.3. Components of an ELISA

Several compounds, such as cellulose, glass or plastic may be used as a solid phase. Plastics are particularly useful, as antibodies and viral proteins are easily coated on to surfaces like polyvinyl or polystyrene. The physical forms available include beads, tubes and microtitre plates.

The enzymes most commonly suggested for use as labels are horseradish peroxidase (HRP), alkaline phosphatase and glucose oxidase. The most important criteria for the selection of an enzyme are the sensitivity, ease and speed with which it is detected, as well as its stability, availability and cost. It is essential that in linking the enzyme to antibodies or antigens neither antibody/antigen or enzyme activity is lost.

Several chromogenic substrates may be used with horseradish peroxidase such as alph-napthol, toluidine, 5-aminosalicylic, azido-benzo-thiazolidine-sulphate, o-phenylenediamine (OPD) and 3,3',5,5'-tetramethylbenzidine (TMB). Of these, the two most common are OPD and TMB. The end colour of OPD, which is read at a wavelength of 492 nm, is easily visible to the naked eye as orange or brown and does not change after the reaction is stopped with sulphuric acid. However, OPD is affected by metal ions, light and it is mutagenic. The TMB reaction, in which a charge transfer complex of TMB and its diamine oxidation product are formed, yields a blue compound. Under acidic conditions, when the stop solution is added, the stable yellow colour is formed which is detected photometrically at 450 nm. TMB has the added advantage that it is neither mutagenic nor light sensitive. For both OPD and TMB, urea peroxide is used as the oxygen source for the substrate reaction (SLAGLE and GHOSN, 1996).

6.4. Interpretation of the ELISA results

6.4.1. Determination of the cut-off point

Results from the test sera are expressed as the percentage of difference between positive and negative controls, after subtraction of background responses (responses from those wells containing coating buffer, but no antigen). Results are divided into categories 'avian tuberculosis negative', 'borderline' and 'avian tuberculosis posistive'.

There are four ways to calculate a cut-off value:

1) 0.5 (N+P)
2) N + 0.150

   With N as the mean value of the negative controls, and P as the mean value of the positive controls.
3) Statistically: The cut-off value is determined by obtaining a logarithmic mean of all negative samples' absorbencies. A standard deviation is calculated and a cut-off is established by using a number of standards deviations above the mean for control values.
4) When a screening test is applied to a collection, a diagnostic accuracy is considered acceptable if it attains a sensitivity and specificity of approximately 70% (CROMIE, personal communication and SPANGLER et al., 1992).

The method of improving sensitivity by using more than one antigen has been outlined in several earlier studies (SUGDEN et al., 1997 and LYASHCHENKO et al., 1998).

6.4.2. Sensitivity and specificity

Sensitivity represents the fraction of subjects with the disease having positive test results, while specificity represents the fraction of subjects without disease having negative test results. Generally the interaction between sensitivity and specificity is such that one of them can be increased only at the expense of the other. Sensitivity can be influenced by selecting antibodies with an extremely high affinity for the antigens or by reducing the background reaction and so making low concentrations of analyte more readily detectable. The ideal specificity of any assay depends on the rationale for testing. Screening requirements may be best served by a broad-spectrum polyclonal assay, whereas highly specific monoclonal assays may be preferable for confirmation of disease in an individual, prognosis of a disease or for monitoring drug therapy. The accuracy of positive and negative prediction (the predictive values of the test) is crucial to interpretation. Predictive values vary with the prevalence of the condition being tested for in the population under study. For fixed sensitivity and specificity, lower prevalence rates lead to fewer false negative test results and hence, better negative predictive accuracy. Similarly, higher prevalence rates lead to more true positive test results and hence, better positive predictive accuracy (DANIEL and DEBANNE, 1987).

The accuracy of positive prediction is the fraction of true positive test results among all positive test results obtained. When a test is applied to a population in which the disease in question is present in a minority of individuals, then the accuracy of positive prediction is influenced by the specificity of the test. Higher specificity reduces the number of false positives, and so reduces the denominator of the positive predictive fraction accuracy (DANIEL and DEBANNE, 1987).
It should be remembered that using a complex (polyclonal) antigen means that strongly positive sera will contain some antibodies which will cross react with common mycobacterial antigens. This is demonstrable at low dilutions (VOLLER and BIDWELL, 1988). Mycobacterial antigens have been classified into four groups, of which one (group i) is common to all species of mycobacteria (GRANGE et al., 1980). Therefore, antibodies to other mycobacteria may cross react with *Mycobacterium avium* antigens used in the ELISA. It is speculated that positive results caused by non-specific binding of antibodies may indicate birds in early stages of infection.

The accuracy of negative prediction is the fraction of true negative tests among all negative test results obtained. When disease prevalence is low, the accuracy of negative prediction is not influenced by test specificity. This is because false negative results, regardless of the extent of misclassification of those with disease, can only be a small fraction of all of the negative test results obtained (DANIEL and DEBANNE, 1987).
False negative results are possibly indicative of non-binding of the secondary antibody to the test antibodies. Differences in the structure of the antibodies of the various tribes of *Anatidae* are unknown, but may account for false negative results. A state of anergy in the bird may also yield false negative results due to the reduced humoral immune response to the mycobacterial antigens (NEVILLE and CROMIE, 1998; CROMIE et al. 2000).

The antigens that are used in the ELISA influence specificity and sensitivity. Secreted antigens are produced by actively proliferating mycobacteria and are therefore potentially diagnostic of active infection. They may also be more species specific than sonicated antigens, which are produced by a breakdown of whole organisms (PAINTER, 1995). To prevent the enzyme-mediated colour change from progressing too quickly in those wells with sonicated antigens, relative to the other wells, PAINTER (1995) halved the concentration of

conjugated antibody. The importance of choosing the correct antigen is confirmed by a study in 1990 in which zoo and wild animals were screened with an ELISA. Antigens containing mainly cell wall proteins were shown to yield more specific results than, for example, sonicated antigens containing cytoplasmic antigens, which have a low specificity (HAAGSMA and EGER, 1990).

6.5. Preliminary use of the ELISA

In PAINTER's study of avian tuberculosis in a collection of wildfowl (1995) 183 blood samples were tested, approximately 15% of the collection. Five mycobacterial antigens were used: two strains of *Mycobacterium avium* originally isolated from tuberculous birds from the WWT centre at Slimbridge, *Mycobacterium fortuitum* a common environmental species, *Mycobacterium vaccae* and BCG (attenuated *Mycobacterium tuberculosis* strain used as a human vaccine). *Mycobacterium vaccae* and BCG carry many antigenetic determinants common to all mycobacteria. Dr. John Stanford of University College Medical School supplied the antigens from stocks previously prepared by Dr. R.L. Cromie. The ELISA identified positive birds, but false positive results were obtained and negative sera yielded in some cases higher absorbency values than expected. Significant differences were found in the results from the different groups tested.

In a study by CROMIE et al. (2000) this ELISA technique was used to screen serum against five sonicated mycobacterial antigens: *Mycobacterium avium* serotype 1 (two isolates), *Mycobacterium fortuitum*, *Mycobacterium vaccae* and BCG. Diagnosis was based on an assessment of the responses to the five antigens. The results showed a good correlation with post-mortem data. This association decreased with time after testing (overall false negative results after seven years, compared to the first year false negative results). This is likely to be due in part to birds developing avian tuberculosis after testing was conducted. In this study the ELISA was neither 100% sensitive nor specific, and refinement of the assay is required for definitive diagnosis. It is also clear that test reliability varies markedly between the different tribes (CROMIE et al., 2000).

In 1993, 329 feral barnacle geese were screened with the ELISA developed by CROMIE (1991) (CROMIE et al., 1993a). The ELISA was specific and sensitive for the disease, as there were no false positive or false negative results. The procedure was carried out using whole blood, either fresh or taken into EDTA, and serum. The antigens were prepared by the method of STANFORD et al. (1975) using the same antigens as mentioned above, and a secreted antigen of *Mycobacterium avium* from culture filtrate. No anti-wildfowl conjugated antibodies suitable for anti-mycobacterial work were available commercially, so a polyclonal anti-duck antibody was prepared by Dr. D.A. Higgins. From preliminary investigations in which known tuberculous birds were tested, a cut-off point of 50% was chosen. The ELISA detected all of the tuberculous birds and yielded no false negative results in the non-tuberculous group. Whilst antibody levels to the sonicated *Mycobacterium avium* antigens gave the best correlation between ELISA results and the presence of disease, the levels of all the sonicated antigens were generally raised in the tuberculous birds. Response to the secreted antigen was less conclusive (CROMIE et al., 1993c). The difficulty lies in interpretation of borderline results. Borderline status may be indicative of a bird in the early stages of infection. The bird may or may not develop progressive disease although the work of FORBES et al. (1993) suggest that approximately half the birds in this category may go on to develop the disease. Even those that do not become diseased may retain 'persistent' bacilli, i.e. a low-level latent infection, which may reactivate, particularly if the bird experiences physiological stress. One bird with high non-specific antibody levels was found to be tuberculous. This polyclonal B-cell activation is a recognised phenomenon in cases of *Mycobacterium tuberculosis* infection in humans and in other diseases in which there is a marked increase of auto-antibody production (FORBES et al., 1993). This ELISA uses an anti-duck antibody, which renders it unsuitable for other avian genera. Even within wildfowl, which are a diverse and primitive group of birds, there have been a number of anomalies, which may be ascribed to low binding to the anti-duck antibody (FORBES et al., 1993).

Another solid-phase, antibody-capture ELISA was developed for the detection of antibody against *Mycobacterium avium*, in serum from chickens and quail. Assays are performed in polystyrene 96-well microtitre plates coated with a whole cell homogenate of a field isolate of *Mycobacterium avium*. Diluted serum samples are added to the antigen-coated wells and antigen-bound antibody is detected using a commercially available goat anti-chicken

immunoglobulin G horseradish peroxidase conjugate, in the case of chicken serum, and a goat anti-turkey immunoglobulin G horseradish peroxidase conjugate, in the case of quail serum. The substrate for the enzyme conjugates is 3,3',5,5'-tetramethylbenzidine (TMB). The extent of cross-reactivity among immunoglobulins G of diverse avian species will determine the usefulness of ELISAs for diagnosis of *Mycobacterium avium* infection in birds (CLARK et al., 1995).

6.6. Comparison of ELISAs for detecting antibodies to infectious diseases in birds

Indirect ELISAs, using anti-species conjugates such as rabbit anti-porcine IgG, rabbit anti-chicken IgG, labelled with horseradish peroxidase, have been used for detecting mycobacterial antibodies in tuberculous chicken (THOEN et al., 1978) and swine (THOEN et al., 1979). Because of the high specificity THOEN et al. (1978) consider the ELISA to be of practical value in detecting infected birds before they secrete tubercle bacilli.
Protein A, produced by certain strains of *Staphylococcus aureus*, binds the Fc portion of immunoglobulins, chiefly immunoglobulin G, of several mammalian species. The production of anti-species conjugated immunoglobulins is labour intensive and time-consuming, therefore, studies were conducted to explore the use of protein A conjugated with horseradish peroxidase in ELISAs involving sera from different species of animals. The conclusions support the use of enzyme-labelled protein A as a general-purpose reagent capable of detecting antibodies in tuberculous exotic animals (THOEN et al., 1980).
Since zoo animal anti-species immunoglobulins G were not available, protein A and protein G were used in a 1990 study. A screening programme for tuberculosis using this modified ELISA was considered successful when applied to several zoo and wild animal species (HAAGSMA and EGER, 1990).

The main object of another project (AIKEN, 1999) was to determine whether protein A reacts with the non-variable region of immunoglobulins of a variety of bird species. Protein A has been shown to bind to immunoglobulin G in humans and other mammals by a non-immune mechanism, i.e. by Fc-region reactivity. The author tested an indirect ELISA using rabbit anti-duck antibodies conjugated to horseradish peroxidase, rabbit anti-duck and rabbit anti-chicken monoclonal antibodies to compare them to an ELISA performed with protein A, also

conjugated to peroxidase. It was shown that protein A is unlikely to be a satisfactory conjugate in the development of an avian tuberculosis test due to its weak binding to immunoglobulins from all bird species tested. ELISAs using anti-sera are approximately 75% accurate, and it was shown in this study on comparative binding capacities that a particular anti-serum might be used for birds within the order *Anatini*, and possibly in other closely related orders. But there were differences in the accuracy of the ELISA between tribes with a poor binding for example within the *Dendrocygnini* (AIKEN, 1999).
It is also reported, however, that duck IgY binds to protein A efficiently, but poorly to protein G. IgM binds both to protein A and to protein G with low efficiency. It is assumed that efficiency parallels affinity, but the results could reflect subpopulations with different binding affinities within each Ig class. Protein A is considered a useful tool for the purification of duck 5.7S immunoglobulin and 7.8S immunoglobulin (HIGGINS et al., 1995).

Multiple studies support the transferability of the tests detecting antibodies from one avian species to another. An indirect antigen-capture ELISA was used for the diagnosis of type C botulism in wild birds. The presence of antibody was confirmed by membrane immunoassay, with enzyme-labelled goat anti-chicken IgG. The test was found to be sensitive and specific in 236 individuals from 50 avian species, representing nine orders (ROCKE et al., 1998).
Although IgG belonging to different avian species cross-react very occasionally, serological diagnosis of infectious disease in birds using direct assays, such as conventional ELISAs, requires antibody directed specifically at the epitopes of the IgG molecule of each avian species. Given the number and variety of avian species, production of these antibodies is difficult and time consuming.
TORO et al. (1996) demonstrated the successful binding of human C1q to antigen-antibody complexes of two avian species using a sandwich immunoflourescence complement fixation test (virus in cells + decomplemented avian antiserum + human-C1q + goat anti-human-C1q + fluorescein isothiocyanate rabbit anti-goat-IgG). Since both avian species tested belonged to different, and phylogenetically distant avian orders (Galliformes and Falconiformes), they believe that antibodies of other avian species will also bind to human C1q. Consequently, the sandwich immunoflourescence complement fixation test may be a promising method for serological surveys in non-captive birds (TORO et al., 1996).

Disadvantages of the indirect ELISA include that it requires a relatively pure antigen and a species-specific anti-enzyme-conjugated antibody to produce the colourimetric reaction. ZHOU et al. (1998) suggest a competitive ELISA for the detection of avian influenza virus. The competative ELISA format uses a type-specific nucleoprotein antigen and its corresponding monoclonal antibody, and may represent the basis for a universal screening test for avian influenza virus (ZHOU et al., 1998).

A study involving a monoclonal antibody blocking enzyme-linked immunosorbent assay to detect paramyxo-virus 1 antibodies, offers advantages over an indirect ELISA: standardisation of the test is relatively easy, antigen purification is less critical than in the case of the indirect ELISA and the dispersion of the target epitope in the plate is easily controlled. Furthermore, the origin and isotype of blocking antibodies are irrelevant; sera from any species (including exotic or wild birds as well as mammals) can be tested (CZIFRA et al., 1996).

7. Summary and assignment

Due to long latency periods, difficulties in diagnosis, no effective treatment and a pronounced tenacity in the environment, avian tuberculosis may pose a threat to populations of captive and wild birds. In the WWT centre at Llanelli infections by *Mycobacterium avium* are found responsible for approximately 20% of annual mortality by gross post mortem examinations (PAINTER, 1995).

The diseased birds spread the bacteria via faeces long before they show clinical signs and cause a high level of contamination in the environment. Recognising those infected birds *ante mortem* and eradicating them is considered as the mean of choice to control the disease. Clinically relevant diagnostic techniques involve avian tuberculin skin test, radiology, endoscopy, direct examination by staining and microscopy of tissue sections and faecal smears, culture, agglutination test, haematological analysis and blood chemistry and ELISA. All diagnostic techniques do no prove sufficiently reliable and show significant disadvantages. The ELISA tends to be more specific and sensitive than agglutination test and haematological analysis (CROMIE et al., 19993a) and represents a useful and practicable diagnostic tool.

To assess the diagnostic accuracy of the modified ELISA used in the current study, blood samples were taken from a percentage of the birds in the WWT centre at Llanelli and their antibody level for mycobacteria was measured in serial tests over two years. On a number of the birds tested gross post mortem examinations were performed to validate the ELISA results. Post mortem diagnosis was completed by histopathological examination, including Ziehl-Neelsen staining of liver and spleen samples, and by PCR.
With the help of data from birds that died in the years from 1989 to 1999 the distribution of avian tuberculosis in the WWT centre at Llanelli was analysed according to tribes, pens of origin, sex, age, seasons and annual incidence. The findings of this epizootiological study contributed to develop guidelines to manage and control avian tuberculosis in collections of captive and wild birds such as the WWT centre at Llanelli.

C. Materials and Methods

1. Description of the birds used in the experiments

The taxonomy of wildfowl has been the subject of much debate. DELACOUR and MAYR (1945), first emphasised similarities within tribes according to evolutionary relationships, rather than division of species into an enormous number of subfamilies, as earlier taxonomists had done. JOHNSGARD (1978) has since modified the classification slightly. Although the taxonomy of SIBLEY and MONROE (1990 and 1993) is now used widely, the classification of JOHNSGARD will be described here as this allows comparison with previous avian tuberculosis research of wildfowl (e.g. CROMIE et al., 1993a).

The order *Anseriformes* comprises two families. The family *Anhimidae* contains three species, these are the Screamers of South America. The family *Anatidae* is large, comprising 148 species (although some may now be extinct), grouped into 43 genera and 13 tribes. The family *Anatidae* is divided into three subfamilies. The *Anatidae* are distinct from other families in that they are water birds with relatively short legs and they all have webbing between their toes. The bill is variable in shape, adapted to different feeding strategies, and possesses characteristic rows of lamellae, arranged at the edges of the mandibles, for grasping food. They share certain skeletal and musculature features and the feathers are thick and waterproof with an additional underlying layer of down. These down feathers, as well as providing heat insulation, may also be plucked from the female's breast to line the nest. Beyond these similarities, the *Anatidae* show enormous amount of variation in size, shape and plumage coloration. **Table 4** presents an overview of the classification of wildfowl by subfamily and tribe, as used in this study. As shown in **Table 5** each tribe is coded by a figure.

Table 4: Classification of wildfowl by subfamily and tribe (JOHNSGARD, 1978)

Family *Anatidae*

Subfamily *Anseranatinae*

Tribe *Anseranatini* (the Magpie Goose)

This species (*Anseranas semipalmata*), from Australia and New Guinea has several peculiarities including only semi-webbed feet. It may represent a link between the *Anhimidae* and the *Anatidae*.

Subfamily *Anserinae*

Tribe *Dendrocygnini* (the Whistling Ducks)

All nine species are called whistling ducks, due to their very distinctive multisyllabic whistle.

Tribe *Anserini* (the Swans and True Geese)

All of these twenty species are very large with relatively little sexual dimorphism. Most of them are found in cooler areas of the Northern Hemisphere and are strongly migratory.

Tribe *Cereopsini* (the Cape Barren Goose)

This goose, (*Cereopsis novae-hollandiae*) from the islands of South Australia, may be a survivor of a transitional group between true geese and sheldgeese.

Tribe *Stictonettini* (The Freckled Duck)

Despite its typical duck-like appearance, this species (*Stictonetta noevosa*) from Southern Australia and Tasmania, shares more anatomical traits with geese and swans and is a unique contemporary form.

Subfamily *Anatinae*

Tribe *Tadornini* (the Sheldgeese and Shelducks)

This tribe comprises 14 species. The sheldgeese are goose-like and occupy a similar niche, feeding by grazing. The shelducks are dabblers feeding on aquatic invertebrates.

Tribe *Tachyerini* (the Steamer Ducks)

Two of the three species are flightless. They are found off the coasts of South America where they feed on marine invertebrates.

Tribe *Cairinini* (the Perching Ducks)

These 13 species have long claws to enable them to perch. They tend to nest in cavities and they share some anatomical features and behaviour patterns with the dabbling ducks.

Tribe *Merganettini* (the Torrent Duck)

This highly adapted species lives in the fast running streams of the Andean Mountains. Its specialisation has obscured many similarities to the perching and dabbling ducks, to which it appears to be most closely related.

Tribe *Anatini* (the Dabbling Ducks)

All 39 species surface-feed, dabble or tip up for food. Most species are temperate breeders, adapted to marshy habitats.

Tribe *Aythyini* (the Pochards)

All 16 species of these primarily fresh-water diving ducks have large feet and widely placed legs that are situated relatively caudally. This makes the birds somewhat ungainly on land but enhances their diving abilities. Their relatively small wing surface area means that they must run over the surface of the water before becoming airborne.

Tribe *Mergini* (the Sea Ducks)

Some authors classify the eiders in their own tribe, the *Somateriini* (DELACOUR and MAYR, 1945; CROMIE, 1991*).* All 20 species of the sea ducks are excellent divers and are found in both fresh and marine environments.

Tribe *Oxyurini* (the Stiff-Tailed Ducks)

The 9 species are characterised by long, stiff tail feathers that act like rudders when they are diving. Like the pochards, they have large feet on legs situated well back on the body and so find it difficult to walk on land.

Table 5: The following codes are used in this study to describe the different tribes.

Code	Tribe
1	*Anseranatini* (Magpie Goose)
2	*Dendrocygnini* (Whistling Ducks)
3	*Anserini* (True Geese and Swans)
4	*Tadornini* (Sheldgeese and Shelducks)
5	*Tachyerini* (Steamer Ducks)
6	*Anatini* (Dabbling Ducks)
7	*Stictonettini* (Freckled Duck)
8	*Somateriini* (Eiders)
9	*Aythini* (Diving Ducks)
10	*Cairinini* (Perching Ducks)
11	*Mergini* (Sea Ducks)
12	*Oxyurini* (Stifftails)
13	*Phoenicopteridae* (Flamingos)

2. Blood sampling

Blood samples were taken from a total of 418 birds of the WWT centre in Llanelli to perform an ELISA. This number represents approximately 35% of the total population. 215 birds of those birds were tested once and 203 birds of those birds were tested a second time, seven months later. Detailed information about each individual bird tested including origin, tribe, identification and ELISA results is listed in **Appendix 3**. The birds were caught in February 1999, before the breeding season (299 birds) and after the breeding season in November 1999 (322 birds).

In September 2000, from 19 of the birds considered positive for avian tuberculosis by ELISA a third blood sample was taken. Those birds were culled and a gross post mortem examination including histopathology was performed. Detailed information about the gross post mortem and histopathology findings are presented in section **D.3.1.** (**Results, Post mortem and**

histopathological examination) and in **Appendix 4**, including the ELISA results. 13 of the samples which were prepared for histopathological examination were also examined by PCR to detect DNA fragments of *Mycobacterium avium*.
Details of the birds are shown in **Table 6** and **Figure 1a to 1i**. Only one bird of the tribe *Cereopsisi*, which was a female with seven years of age, was involved in this study. 198 (47.4%) of the birds tested by ELISA were female and 220 (52.6%) were male. Gross post mortem and histopathology were performed on ten female birds (52.6%) and nine male birds (47.4%). Eight female birds (61.5%) and nine male birds (38.5%) were also examined by PCR.

The pens selected for this study had previously shown evidence of avian tuberculosis on the basis of post mortem findings. Other criteria were the ease with which birds could be caught. At the time of the first catch the pens were covered in ice and snow, because of this it was not possible to catch the smaller ducks, as the risk of frostbite to the feet following a prolonged time on the frozen ground and snow covered ground was too great. The birds were corralled in a small, fenced area and then caught in hand held nets.

Directly after catching, blood samples were obtained and the birds were then released into their pens. The blood samples were taken from the metatarsal vein or the basilic vein using a 25 gauge 1`` needle and a 2 ml syringe. The samples were held in labelled tubes containing four small uncoated glass beads, which prevent the formation of a serum clot.
All blood sample tubes were labelled with continuous numbers. Details of ring number, sex, age and species were recorded for each individual. The samples were kept at room temperature for not longer than five hours and then refrigerated overnight at 4°C to encourage clot reaction. Erythrocytes and fibrin threads if present will affect the test performance. The following day all samples were centrifuged at 13,000 rpm for 10 minutes. The serum was separated by pipetting, then labelled and frozen.

Table 6: Details of the birds tested by ELISA, examined by post mortem, histopathology and PCR, according to tribe, pen and age.

Groups		Number of birds tested by ELISA	Number of birds examined by necropsy and histopathology	Number of birds examined by PCR
Tribe	*Dendrocygini*	65 (15.6%)	4 (21.1%)	3 (23.1%)
	Anserini	97 (23.2%)	6 (31.6%)	4 (30.8%)
	Tadornini	23 (5.5%)	2 (10.5%)	2 (15.4%)
	Cereopsisi	1 (0.2%)	0 (0%)	0 (0%)
	Anatini	129 (30.9%)	5 (26.3%)	3 (23.1%)
	Somateriini	9 (2.2%)	2 (10.5%)	1(7.7%)
	Aythini	44 (10.5%)	0 (0%)	0 (0%)
	Cairinini	21 (5.0%)	0 (0%)	0 (0%)
	Mergini	22 (5.3%)	0 (0%)	0 (0%)
	Oxyurini	7 (1.7%)	0 (0%)	0 (0%)
Pen	Asian pen	73 (17.5%)	1 (5.3%)	0 (0%)
	Australian pen	6 (1.4%)	1 (5.3%)	0 (0%)
	N. American pen	54 (12.9%)	0 (0%)	0 (0%)
	S. American pen	97 (23.2%)	6 (31.6%)	4 (30.8%)
	Side pen B	2 (0.5%)	0 (0%)	0 (0%)
	Side pen C	5 (1.2%)	0 (0%)	0 (0%)
	Side pen E	7 (1.7%)	0 (0%)	0 (0%)
	Smew pen	6 (1.4%)	0 (0%)	0 (0%)
	Top pond	168 (40.2%)	11 (57.9%)	9 (69.2%)
Age in years	1	23 (5.5%)	0 (0%)	0 (0%)
	2	46 (11.0%)	1 (5.9%)	1 (7.7%)
	3	17 (4.1%)	0 (0%)	0 (0%)
	4	21 (5.5%)	1 (5.9%)	1 (7.7%)
	5	14 (3.3%)	0 (0%)	0 (0%)
	6	31 (7.4%)	0 (0%)	0 (0%)
	7	44 (10.5%)	1 (5.9%)	2 (15.4%)
	8	29 (6.9%)	2 (11.8%)	1 (7.7%)
	9	60 (14.4%)	1 (5.9%)	1 (7.7%)
	10	123 (29.4%)	8 (47.1%)	4 (30.8%)
	11	10 (2.4%)	3 (17.6%)	3 (23.1%)

Figure 1a: Determination of the number of birds in percentage which were tested by ELISA, according to age in the tribe *Dendrocynini* (n=64).

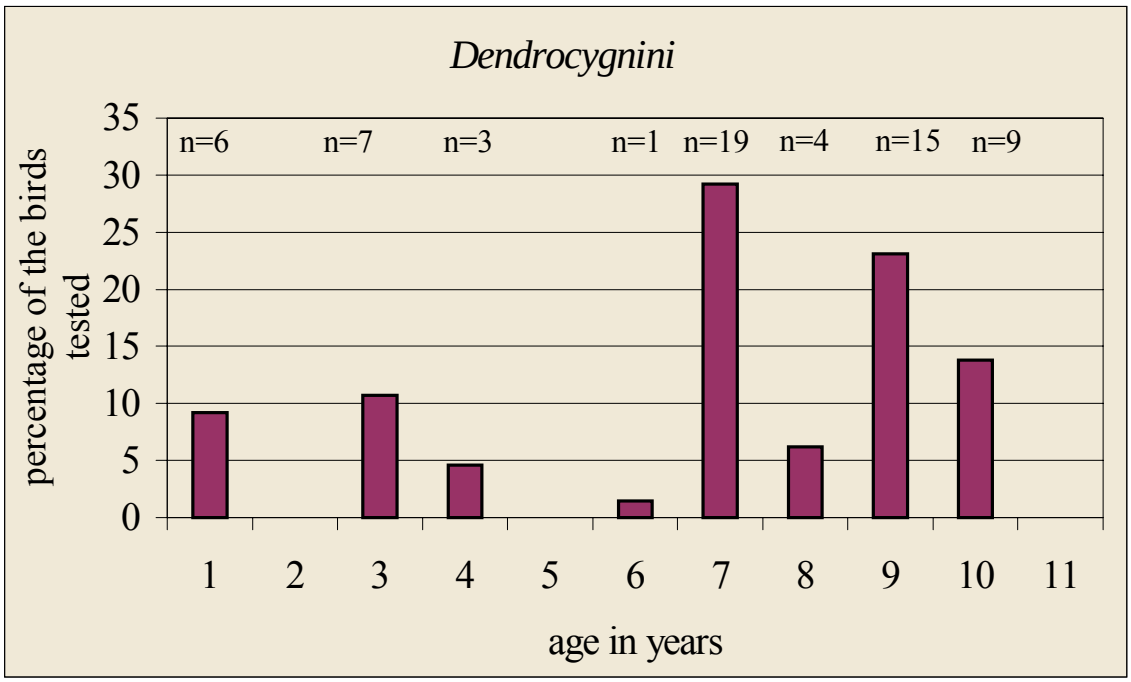


Figure 1b: Determination of the number of birds in percentage which were tested by ELISA, according to age in the tribe *Anserini* (n=97).

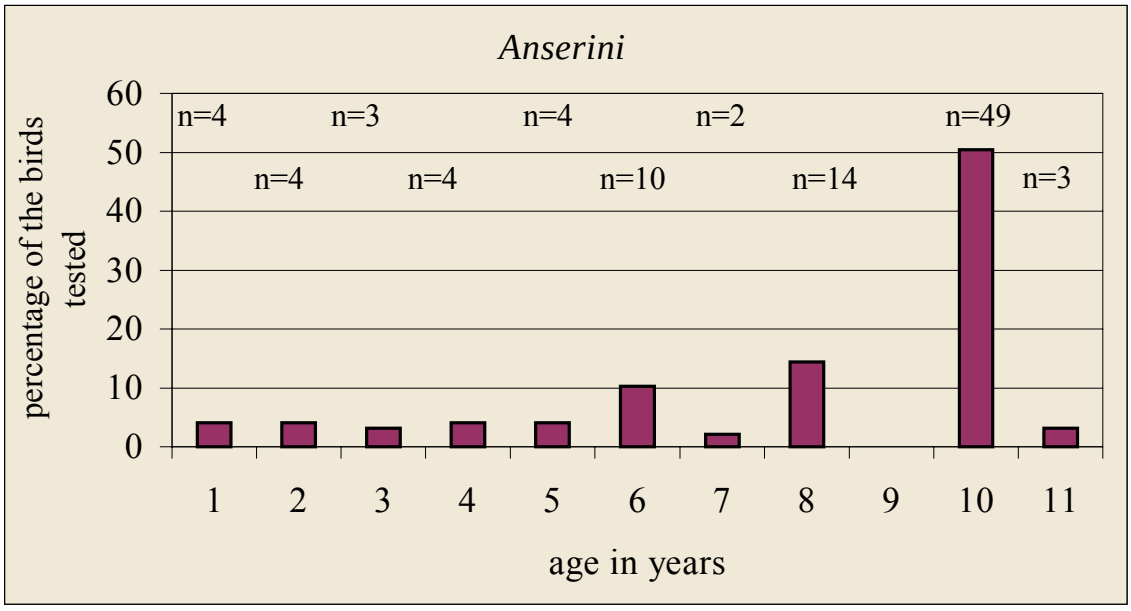


Figure 1c: Determination of the number of birds in percentage which were tested by ELISA, according to age in the tribe *Tadornini* (n=23).

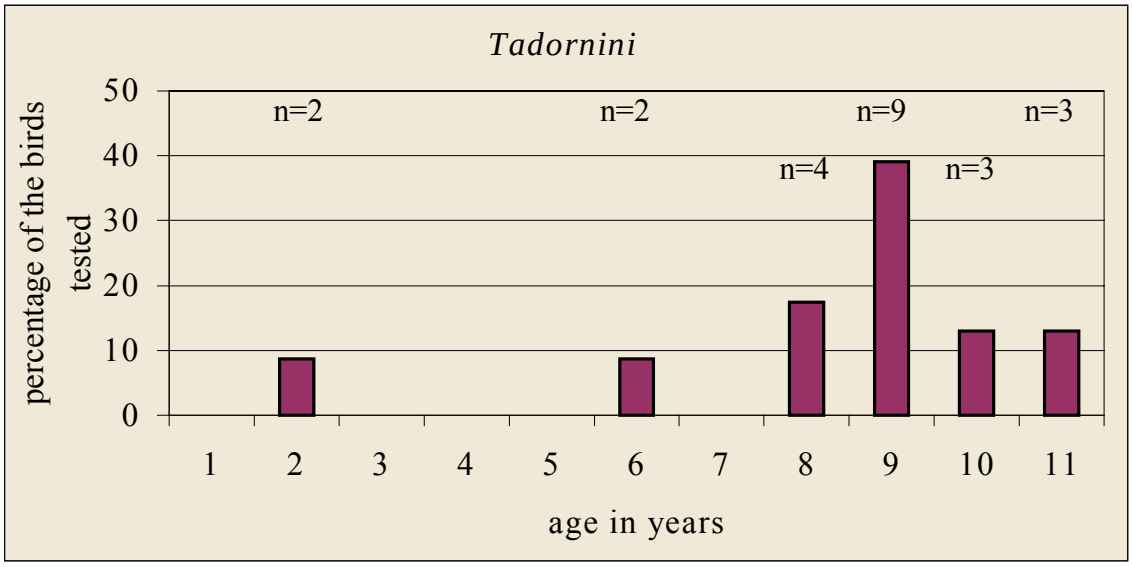


Figure 1d: Determination of the number of birds in percentage which were tested by ELISA, according to age in the tribe *Anatini* (n=129).

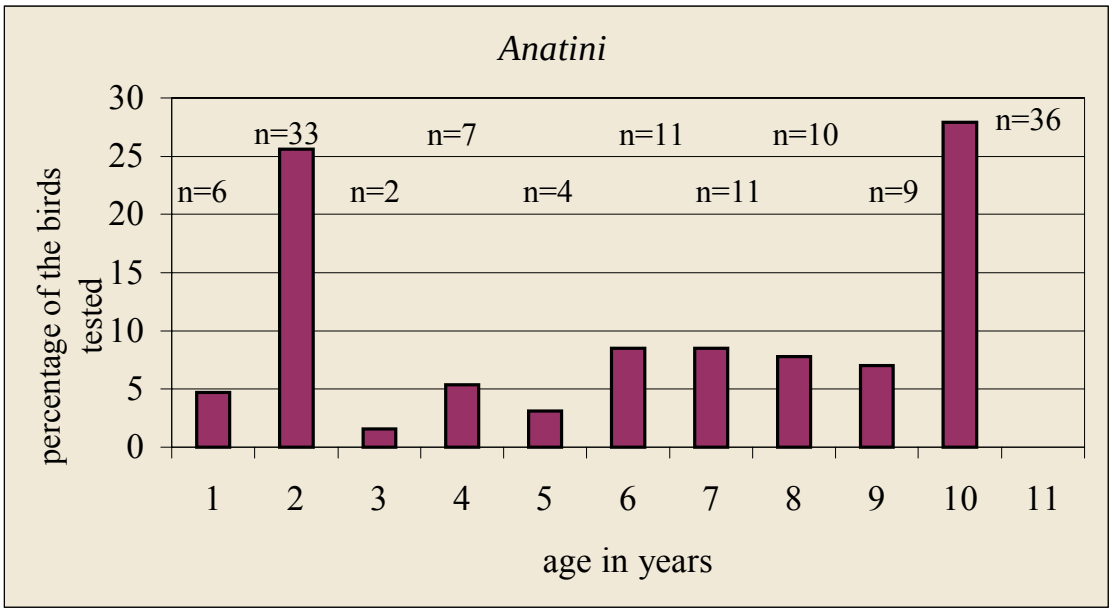


Figure 1e: Determination of the number of birds in percentage which were tested by ELISA, according to age in the tribe *Somateriini* (n=9).

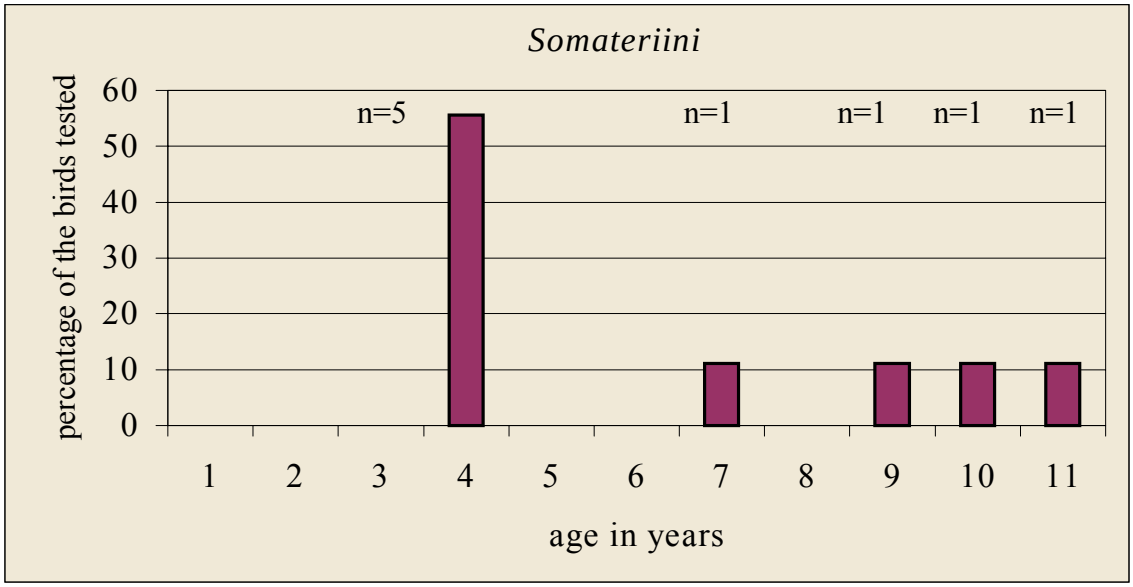


Figure 1f: Determination of the number of birds in percentage which were tested by ELISA, according to age in the tribe *Aythini* (n=44).

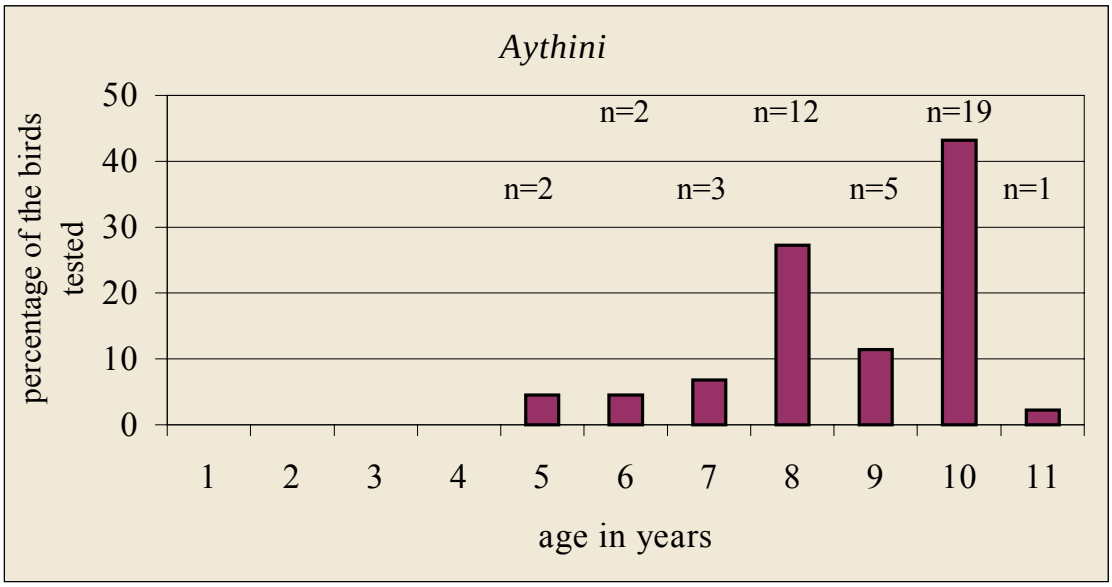


Figure 1g: Determination of the number of birds in percentage which were tested by ELISA, according to age in the tribe *Cairinini* (n=21).

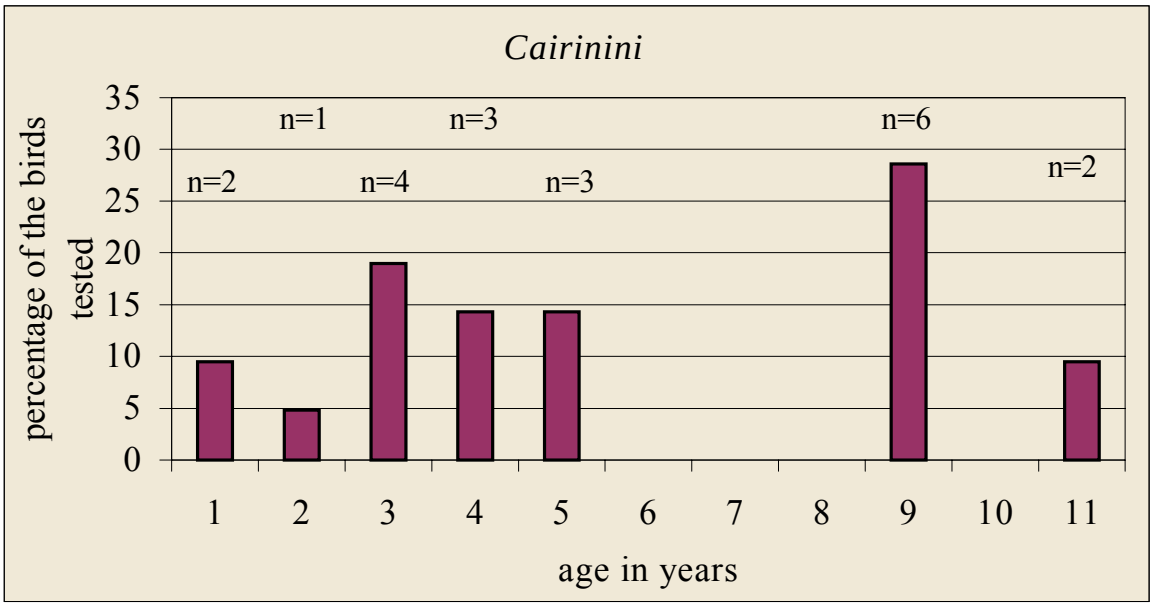


Figure 1h: Determination of the number of birds in percentage which were tested by ELISA, according to age in the tribe *Mergini* (n=22).

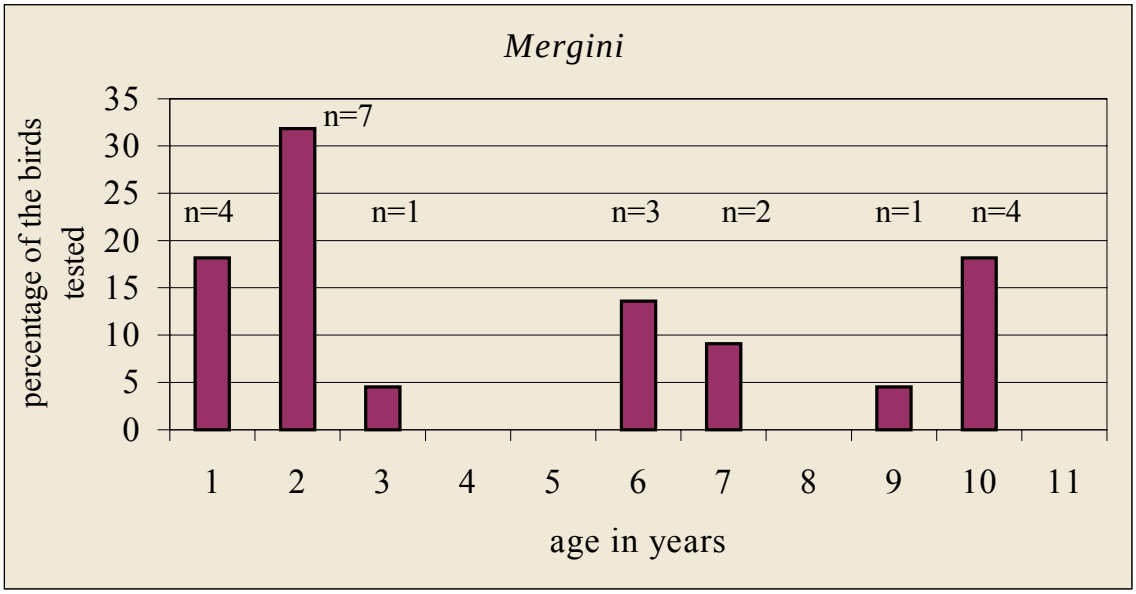
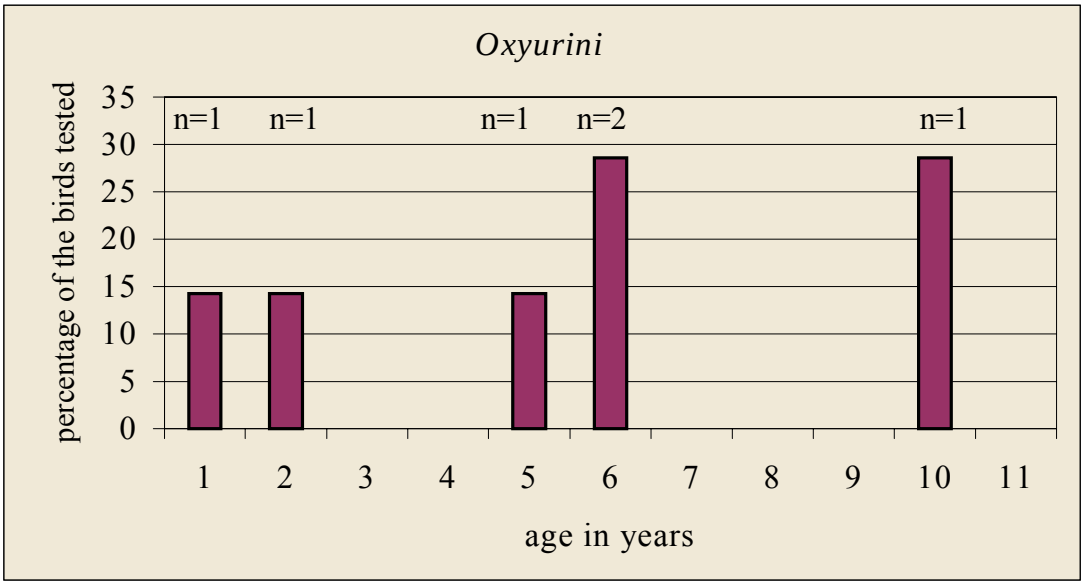


Figure 1i: Determination of the number of birds in percentage which were tested by ELISA, according to age in the tribe *Oxyurini* (n=6).



3. Identification of the birds' enclosures

The majority of birds in the WWT centre at Llanelli are grouped in pens according to their continent of origin, and the pens are named accordingly. Some pens simply contain groups of birds of the same species or tribe. In most pens there are some birds, which follow neither of these basic classifications, but have been housed in particular pens for other avicultural reasons. Stiles and gates separate the pens to allow access by the public.
Each pen is identified with a code as shown in **Table 7**.

Table 7: Codes identifying pens in the WWT centre at Llanelli.

Code	Pen
AS	Asian pen
AUS	Australian pen
EUR	European pen
EUS	European side-pen
FLM	Flamingo pen
GE	Goldeneye pond
HOS	Hospital pen
ISL	Island pen
LAG	Lagoon
NA	North American pen
SA	South American pen
SAS	South American side-pen
SMW	Smew pen
SPA, B, C	Side-pens A, B, C
ST	Stifftail pen
TOP	Top pond

4. Principles of the enzyme-linked immunosorbent assay used in this study

All samples taken in the WWT centre at Llanelli were examined for antibodies to *Mycobacterium avium* with an enzyme-linked immunosorbent assay (ELISA) in the laboratory at the WWT centre at Slimbridge. The ELISA is modified by using different antigens and a different conjugated antibody than in previous ELISAs.

The ELISA uses antibody/antigen interaction to demonstrate quantitatively the presence of specific antibodies in a serum sample. Serum samples are added to plastic microwells, which are coated with mycobacterial antigen. Any antibodies to mycobacteria in the sample will

attach to the antigen coating the well. The presence of these antibodies (immunoglobulins) is revealed by adding anti-duck antibody, which is conjugated to the enzyme horseradish peroxidase. Affinity purified anti-duck antibody, raised in a rabbit with complete Freund's adjuvant, which was provided from stocks previously prepared by Dr. D.A. Higgins, Queen Mary Hospital, Hong Kong, was considered to be too old and a renewed production too expensive. Therefore, commercially available horseradish peroxidase conjugated rabbit anti-duck antibody was used in this study (Nordic, Immunological Laboratories, RADu/IgG(H+L)/Po). The secondary antibody attaches to the duck immunoglobulin in the well and causes a colour change to green when a suitable substrate (2,2 azino-di-(3 ethyl benthiazoline sulphonic acid) (ABTS, Sigma A1888)) is added. The intensity of the colour change is proportional to the amount of antibody in the original sample and is measured colorimetrically using a Dynatech MR7000 ELISA reader.

Manual and automated photometric readers have made it easier to generate ELISA results. These readers use a principle of directing a light beam through the microtitre wells, the signals are converted to digital information and then converted to absorbency values by a microprocessor. The operator can then obtain a hard copy of results in a very short time, and results in relation to positive and negative controls can be automatically calculated, and any positive results flagged. Computerisation of the process permits more complicated calculations, standardises sample identification, and reduces data transcription error.

The goal of this project is to provide a test, which will permit the control or eradication of avian tuberculosis within captive waterfowl collections. Central to this is the evaluation of the ELISA as a practical diagnostic tool. Diagnostic techniques such as Becton-Dickenson Diagnostic instrument system, DNA-RNA probe test, polymerase chain reaction or immunofluorescence test are effective, but expensive and complex. In the present study, a practice orientated enzyme-linked immunosorbent assay is used with a commercially available conjugated antibody.

4.1. ELISA method

The method employed in this study was performed as described by CROMIE et al. (1993c).

(1) The antigens are diluted in coating buffer (0.05 M carbonate / bicarbonate buffer, pH 9.6) to a concentration of 10 µl/ml.
(2) The wells of 96-well microtitre plates (Nunc) are each coated with 100 µl of antigen solution.
(3) The plates are incubated overnight at 4º C. All incubations are carried out in a damp box to prevent drying of the plates.
(4) The plates are washed thoroughly (three washes lasting three minutes each) in washing / incubation buffer (1 M phosphate-buffered saline pH 7.4 with 0.05% Tween 20 (Sigma P-1379)).
(5) To reduce non-specific binding, the plates are coated with 1% bovine serum albumin (BSA) (Sigma A-9647, FractionV) in washing / incubation buffer for one hour, at room temperature.
(6) The wells are washed out as above.
(7 100 µl of test serum, diluted 1:200 in washing / incubation buffer with 1% BSA is added to each well. Each serum / antigen combination is duplicated.
(8) The plates are incubated for two hours at room temperature.
(9) The wells are washed out as above.
(10) 100 µl of horseradish peroxidase-conjugated anti-duck antibody, diluted 1:2500 in washing / incubation buffer, is added to each well.
(11) The plates are incubated at room temperature, for three hours.
(12) The wells are washed out as above.
(13) 100 µl of substrate 2,2 azino-di-(3 ethyl benthiazoline sulphonic acid) (ABTS, Sigma A1888), diluted in citrate phosphate buffer (0.1 M, pH 4.3), is added to each well.
(14) The plates are incubated in the dark, at room temperature, for between 30 and 50 minutes to allow the colour to develop.
(15) The optical density of each well is measured using a Dynatech MR7000 ELISA reader at 630 nm. A well in which test sera has been substituted by washing / incubation buffer alone, serves as a blank.

Positive and negative controls, which have been evaluated in previous studies (CROMIE, et al., 1993c), are included in each assay. The positive control is serum from a duck from the WWT centre at Slimbridge with advanced avian tuberculosis. The negative control is serum from a healthy 10-day-old duckling (PAINTER, 1995).

The assays are run in batches of six plates, with eight samples per plate. **Figure 2** demonstrates the template for optimal antigen/test sera combinations on the ELISA plate as used in this study.

Figure 2: Template for optimal antigen/test sera combinations on the ELISA plate.

	Background		M. avium 1238		RBG		Goosander		YBD		Mallard	
Negative control												
Positive control												
Serum 1												
Serum 2												
Serum 3												
Serum 4												
Serum 5												
Serum 6												

Background= microtitre wells with no antigen but only coating buffer

M. avium 1238= microtitre wells with antigen *Mycobacterium avium* serotype 3 from one bird

RBG, Goosander, YBD, Mallard= microtitre wells with anitgen *Mycobacterium avium* serotype 1 from four different birds: Red Breasted Goose (RBG), Goosander, Yellow Billed Duck (YBD) and Mallard

Serum samples from six individual birds

Positive and negative controls, which were evaluated in previous studies (CROMIE, et al., 1993c; PAINTER, 1995), are included in each assay on the first plate of each batch. The positive control is serum from a Mallard from the WWT centre at Slimbridge with advanced avian tuberculosis; the negative control is serum from a healthy ten-day-old duckling.
Recipes for the buffers and substrates used in the ELISA are given in **Appendix 2**.

4.2. Determination of conjugated antibody dilution

As a novel conjugated antibody was used, an optimal dilution was determined by the following titration. Two serum samples with known low and high antibody levels were tested against conjugate dilutions of 1:1000, 1:2500, 1:5000 and 1:10,000. A positive and a negative control were included in the assay. For each dilution a separate microtitre plate was used. The four microtitre plates of the ELISA were processed simultaneously in any given assay. The same antigens and sera from the same known avian tuberculosis positive or known avian tuberculosis negative bird were used on each microtire plate. The only variable was the conjugated antibody dilution.
100 µl of horseradish peroxidase-conjugated anti-duck antibody, diluted in washing / incubation buffer was added to each well of the microtitre plates.

Non-specific binding of the conjugated antibody must be taken into account when conducting assays. To quantify primary binding of the conjugated antibodies to the mycobacterial antigens an ELISA was run with four microtitre plates to which no sera were added apart from the positive and negative control.

4.3. Antigens used in the ELISA

The ELISA used antigens produced from pure cultures of mycobacteria to identify the presence of mycobacterial antibodies in the serum of sampled birds. The antigens may be either sonicated (produced by ultrasonic disruption of whole mycobacteria) or secreted (secreted by growing mycobacteria into the culture medium, from where they are harvested).

Secreted antigens were used in PAINTER's study (1995). *Mycobacterium avium* serotype 3 isolates from a northern pintail (*Anas acuta*) from the WWT centre at Llanelli, and a serotype 1 isolate from a yellow-billed duck (*Anas undulata*) from the WWT centre at Slimbridge were cultured in Sauton's medium. Once good growth was obtained, the bacterial colonies were scraped off the culture medium and stored, in sterile universal containers at -20°C for sonication. The culture medium for each isolate was then cut into small pieces and an equal volume of borate buffer (0.1 M, pH 7.4) added. The containers were shaken and allowed to stand overnight at 4°C. The following day, the buffer was decanted off the culture medium into sterile universal containers and serially filtered through 0.45 nm and 0.2 nm membrane filters to remove all mycobacteria. The antigen solutions were divided into small aliquots and transferred into plastic bijou bottles. The protein concentration of each of the antigens was determined by spectrophotometry, based on absorption of ultra-violet light at 260 and 280 nm. The solutions were stored at -20°C.

Sonicated antigens from *Mycobacterium avium* serotype 1, isolated from birds in the WWT centre at Slimbridge, *Mycobacterium fortuitum*, a common environmental organism, BCG and *Mycobacterium vaccae* were also used in earlier work (PAINTER, 1995 and CROMIE et al., 1993c). The suspension was treated for 15 minutes in a MSE 100 Watt ultrasonic disintegrator with an amplitude of 6-8 μm. The resultant mixture of residual whole organisms, broken cell walls and cytoplasm was then centrifuged at 3000 rpm for 20 minutes. The supernatant was passed through a 0.45 μm and two 0.2 μm membrane filters.

In this survey only sonicated antigens were made up anew and were included in the ELISA to allow comparison with previous tests. The mycobacterial cultures were kept and provided by Prof. J.L. Stanford of University College Medical School.

- *Mycobacterium avium* serotype 3: reference strains M. avium 1238 and M. avium 1339, isolated from birds at the WWT centre at Llanelli
- *Mycobacterium avium* serotype 1: reference strains Red breasted goose (RBG), Mallard, Yellow-billed duck (YBD), Goosander and Barnacle goosse, isolated from birds from the WWT centre at Slimbridge
- Glaxo BCG, an attenuated *Mycobacterium bovis* strain used as a human vaccine

- *Mycobacterium vaccae* (R877R)
- *Mycobacterium fortuitum*, a common environmental organism

Ten different antigen solutions of 10 µl/ml were used to titrate the conjugate and to determine the cut-off point. The five most suitable antigens (M. avium 1238, RBG, Goosander, YBD and Mallard) were chosen by virtue of their cut-off points being closely grouped. By using five antigens, eight sera could be tested on one microtitre plate.

4.4. Interpretation of the ELISA results

The ELISA was initially performed using previously tested sera from birds of known disease status, in order to determine the optimum concentration of conjugate, and to assess the consistency of the results to those of previous studies (CROMIE et al., 1993c; FORBES et al., 1993).
The results of serum testings were expressed as percentages of the difference between the positive and negative controls, after subtraction of background responses (responses from those wells containing coating buffer, but no antigen). The results were divided as follows:

Avian tuberculosis negative:	values < 20% of the adjusted positive control
Borderline:	values of 21-50% of the adjusted positive control
Avian tuberculosis positive:	values >50% of the adjusted positive control

The interpretation 'avian tuberculosis positive' was made from an assessment of the antibody responses to the mycobacterial antigens, taking special note of the *Mycobacterium avium* antigens. The term 'avian tuberculosis positive' was used for birds which showed a high antibody titre for mycobacterial antigens and were therefore considered as diseased by avian tuberculosis. Consequently a diagnosis of non-tuberculous was made if birds showed a low antibody titre ('avian tuberculosis negative').

The cut-off value for the results of an assay determines whether a given sample yields a positive, borderline or a negative result. A cut-off value should be chosen such that it permits

the greatest sensitivity with the fewest false positive results. The comparison of results of assays performed at different times, and possibly under different temperature and humidity conditions or incubation times, is considered valid because positive and negative control samples were included in each group.

The cut-off point in this study has been established on the basis of consideration according to the fact that diagnostic accuracy is considered acceptable if it attains a sensitivity and specificity of approximately 70% (CROMIE, personal communication and SPANGLER et al., 1992).

5. Post mortem and histopathological examination and PCR of birds testing positive for avian tuberculosis

16 birds considered positive for avian tuberculosis following ELISA testing of blood samples and three other birds due to avicultural reasons were culled during the course of this study and submitted for gross post mortem examination. The decision to cull a bird was not based solely on the ELISA result. Each case was considered individually and a decision taken in light of species susceptibility, physical examination findings and its value for the collection. Prior to euthanasia a blood sample was taken from the chosen birds to perform an ELISA for the third time.

In order to minimise variation in technique and interpretation, all gross post mortem examinations were performed under the supervision of M. J. Brown at the WWT centre at Slimbridge as a standard necropsy examination (as described in RITCHIE, HARRISON and HARRISON by LATIMER and RAKICH, 1994). A 1mm x 1mm sample of liver and spleen tissue was taken from each bird and preserved in formal saline. The samples were sent to the Clinic for Poultry Disease of the School of Veterinary Medicine Hanover. Histopathological examination of the samples was performed under the supervision of Prof. Dr. U. Neumann. The tissues were preserved in 10% formal saline. They were processed routinely and embedded in paraffin-wax. Sections were cut at five microns and stained using the Ziehl-Neelsen technique. Mycobacteria have a lipid-rich cell wall, which is capable of taking up strong phenol-dye solutions in such a way that they retain the dye upon subsequent differentiation in acid or alcohol, i.e. they are acid and alcohol fast. Most other organisms lose

the dye and take up the counterstain. The staining was carried out as follows: (1) Take sections to water. (2) Filter on carbol fuchsin and heat three times until the 'steam rises' (over a period of ten minutes). (3) Wash well in water, differentiate in 1% acid-alcohol for 10 minutes. Wash in water for five to ten minutes. (4) Counterstain with the methylene blue solution for 30 seconds. Wash in water. (5) Differentiate and dehydrate in alcohol (until the sections are a weak blue). Clear and mount in a DPX-type mountant. The slides were examined under the microscope with x100, x400 and x600 magnification (Standard 16 microscope, Carl Zeiss, Göttingen, Germany). Mycobacteria show up as red bacilli (COOK, 1974).

The histopathological examination was followed up by performing a PCR on 13 of the formalin-fixated and paraffin-embedded tissue samples which did not show clear findings on histology and exhibited high *Mycobacterium avium* antibody titres. The PCR was carried out by Dr. Ruesch-Gerdes and Dr. Richter of the National Reference Centre for Mycobacteria, Research Centre Borstel, Germany. The following procedure was used to determine mycobacterial DNA: To deparaffinise the tissue samples, 15µm sections were incubated twice in xylol (1ml) in a rotator for ten minutes with subsequent centrifugation at 13.000 xg for five minutes and residual xylol was removed by being washed and centrifuged similarly in ethanol (1ml). DNA isolation was performed by using the QIAmp tissue kit (QIAGEN, Beckmann Instruments, München, Germany) according to the manufacturer's protocol with two additional temperature treatments before and after the proteinase-K-incubation step. Tris-HCl (pH 8.4), KCl, MgCl2, dNTP (Promega), and Taq-Polymerase were used for digestion of the tissue and DNA extraction. Concentration of the DNA has been measured spectrophotometrically and undesired degradation of DNA was detected by gel-electrophoresis. A oligonucleotide primer for a 590 bp genus-specific fragment of mycobacterial 16S rDNA, was used for amplification. After proteinase treatment, samples were subjected to a series of heat/cold shocks to lyse residual mycobacterial cells walls. Negative and positive controls were included in each PCR (GOLDMANN et al., 1998; RICHTER et al., 1995).

The post mortem data were analysed in respect of correlation with the findings of the ELISA. In this way specificity and sensitivity were determined.

6. Epidemiological considerations

The birds used in this study are kept under conditions, which are more or less artificial. These factors included stocking density, water flow, species and age structure of the flock, and contact with wild birds. Personal communication with members of staff (R. EDWARDS and G. WALTERS, WWT centre at Llanelli) and direct observation of the site provide information about the physical environments of the pens and husbandry details. Stocking density is calculated by measuring the ground area covered by each pen from a site map, and by performing an actual count of the birds in each pen of the collection Therefore, the epidemiology of disease is affected by avicultural practices and research findings must be interpreted in light of various factors.
Post-mortem examination is routinely carried out for several of the WWT centres by M.J. Brown as part of the programme to monitor and improve the health and husbandry of captive waterfowl. Post-mortem data are, therefore, available for all the birds that died in the WWT centre at Llanelli over the last ten years. According to age standardisation procedures in the WWT centre at Slimbridge, an adult bird is classified as one surviving to its first January or the equivalent for those Southern Hemisphere birds that breed earlier. Diagnosis is made on macroscopic appearance indicative for tuberculous lesions in the diseased bird. Birds with lesions suspicious for avian tuberculosis even if it was not the primary cause of death at gross post-mortem examination are considered tuberculous, and are combined in the analysis.

The data of 330 birds from the WWT centre at Llanelli were analysed with respect to several considerations, such as distribution of tuberculous birds in different tribes, pens of origin, sex, age, seasons and the incidence of the disease over the last ten years. The seasons were classified as spring (March to May), summer (June to August), autumn (September to November) and winter (December to February). **Appendix 5** gives detailed information about each individual bird.

7. Statistical analysis

The ELISA study:

Kruskal Wallis test was used to demonstrate differences between positive and negative values of the ten antigens initially used in the ELISA, in order to determine the five most suitable antigens.

The chi-square test was used to compare the specificity and sensitivity of the ELISA with the gross post mortem and histopathology results obtained during this study. This test was also performed to analyse the results of the first and the second ELISA.

The sign rank tests were carried out to elucidate changes in the results in the repeated ELISAs from the first bleeding in February to the second in November.

The post mortem data study:

Chi-square tests or, if the sample size is smaller than five, Fisher's exact tests (two tailed) were performed to investigate differences in the gross post mortem results obtained from deaths that occurred in the WWT centre at Llanelli over the last ten years in birds of different tribes, sexes or pens of origin.

The Pearson Correlation test was used to describe the correlation between stocking density in different pens and the incidence of avian tuberculosis in these pens.

Logistic regression was used to calculate the likelihood of an individual of a given age, from a given tribe being diagnosed positive for avian tuberculosis on gross post mortem. Student-t test was carried out to compare the average age of the birds dying from avian tuberculosis in the different tribes.

Statistical analysis was performed by using SAS programme (SAS 6.12, Statistisches Analyse System, SAS Institute Inc., SAS Campus Drive, Cary, NC 27513, USA) in the Department for Statistics of the School of Veterinary Medicine Hannover in Germany.

D. Results

1. Preliminary investigations

The optimal dilution of conjugated antibody in washing / incubation buffer was obtained by titration. Dilutions of 1:1000, 1:2500, 1:5000 and 1:10,000 were tested. As shown in **Figure 3 and 4** a dilution of 1:2500 could be considered optimal, as it provided consistent results with all ten antigens used in this assay, for birds of known avian tuberculosis status. The amplitudes of the absorbency values were similar to those obtained with a dilution of 1:1000. Practical considerations, such as conservation of antibody were taken into account in choosing the higher dilution.

Figure 3: Absorbency values in percentages of the antigens initially used in the ELISA, which were obtained by titration with different dilutions of conjugated antibody for birds with known negative tuberculous status.

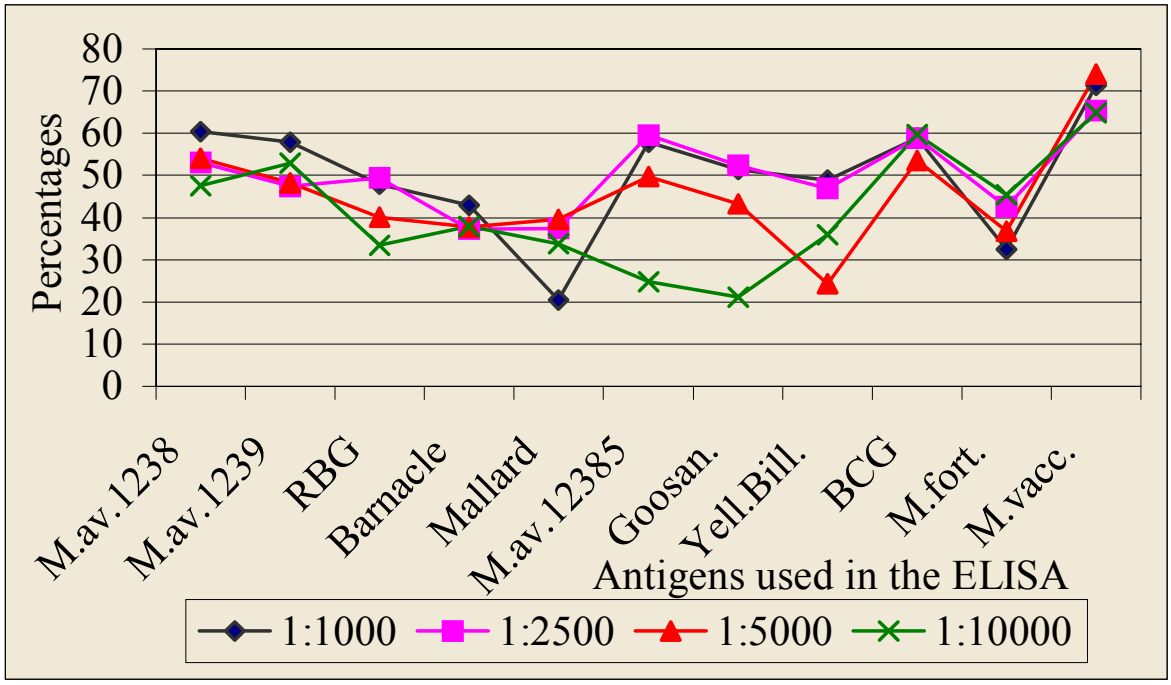
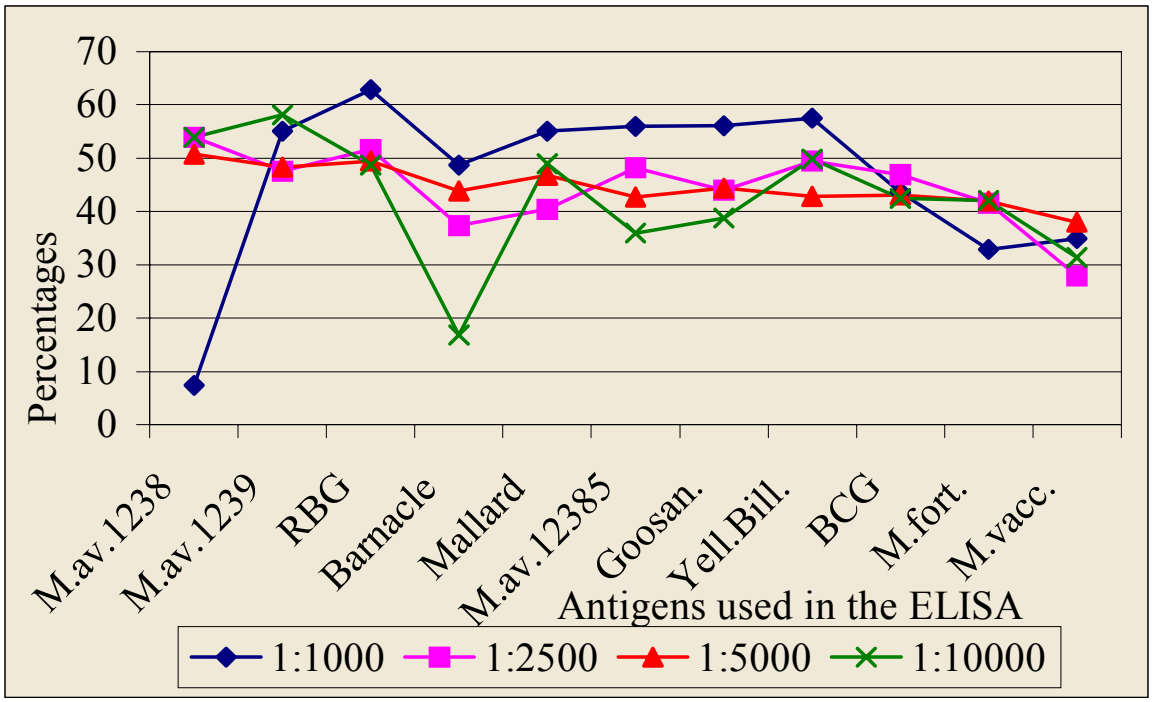


Figure 4: Absorbency values in percentages of the antigens initially used in the ELISA, which were obtained by titration with different dilutions of conjugated antibody for birds with known positive tuberculous status.



Antigens used in the experiment were either isolated from bacterial cultures by Dr. R. Cromie, WWT centre at Slimbridge (serotype 1 and 3), or taken from NTCC collection (*Mycobacterium fortuitum* and *vaccae*, BCG):
Mycobacterium avium serotype 3: reference strains M.av. 1238 and M.av. 1239
Mycobacterium avium serotype 1: reference strains Red Breasted Goose (RBG), Barnacle Goose, Mallard, Goosander and Yellow-billed Duck (Yell.Bill.)
Mycobacterium fortuitum (M.fort.)
Mycobacterium vaccae (M.vacc.)
BCG

The Kruskal Wallis test demonstrated that all ten antigens show a significant difference between positive and negative values.

In this study the five most suitable antigens were chosen by virtue of their cut-off points being closely grouped:

M*ycobacterium avium* serotype 1: M.av. 1238

Mycobacterium avium serotype 3: Red Breasted Goose (RBG), Mallard, Goosander and Yellow-billed Duck (YBD)

The antigens selected are all highly pathogenic for waterfowl. These points are key to interpretation of the ELISA results. In a indirect sandwich ELISA, as performed in this study, samples which have an absorbency value above the cut-off point are considered positive.

A bird was considered to be sereopositive for avian tuberculosis in the reaction if its serum to one of the five antigens showed high antibody titres, as all the antigens were prepared from wildfowl pathogenic strain of *Mycobacterium avium*.

The cut-off point for determining positive or negative results was based on diagnostic accuracy being considered acceptable if it attains a sensitivity and specificity of approximately 70% (CROMIE, personal communication; SPANGLER et al., 1992). The obtained cut-off point corresponded to the cut-off value when calculated with the formula 0.5(N+P).

To quantify non-specific binding of the conjugated antibody to the mycobacterial antigens and to the plastic plate, an ELISA was run with four microtitre plates to which no sera are added, apart from the positive and negative control. Non-specific binding of 3-5% occurred in the ELISA. Therefore, the cut-off point was raised by 5% to reduce the risk of false positive results due to the interpretation of a high absorbency value.

As lined out in **chapter 4.4.** (**Methods and Materials, Interpretation of the ELISA results**), the cut-off points, determined in the preliminary experiments, were as follows:

Avian tuberculosis negative:	values < 20% of the adjusted positive control
Borderline:	values of 21-50% of the adjusted positive control
Avian tuberculosis positive:	values > 50% of the adjusted positive control

2. ELISA results

As explained in **chapter C.2.** (**Methods and Materials, Blood sampling**), a total of 418 birds were tested. Detailed information of all the birds tested by ELISA is shown in **Appendix 3**.

A bird was considered to be seropositive for avian tuberculosis with a conspicuous high antibody level if the absorbency value of its serum to one of the five antigens was above the cut-off point. As the absorbency values were expressed in percentages, the results could be continuously grouped: group 1 representing negative results (<20%), group 2 representing borderline results (20-50%), and group 3 representing positive results (>50%). The sign rank test demonstrated that the results of the second ELISA in total ($p<0.0001$) and in comparison of each single group to each other (group 1 – $p<0.0001$; group 2 – $p<0.0001$; group 3 – $p<0.0002$) were higher than the results of the first ELISA. The chi-square test showed that the number of birds in each group in the first ELISA was significantly different from the number in each group as determined by the second ELISA ($p<5.109E-10$). This was explained by the serum samples tested in the second ELISA producing a stronger positive reaction than those tested in the first ELISA.

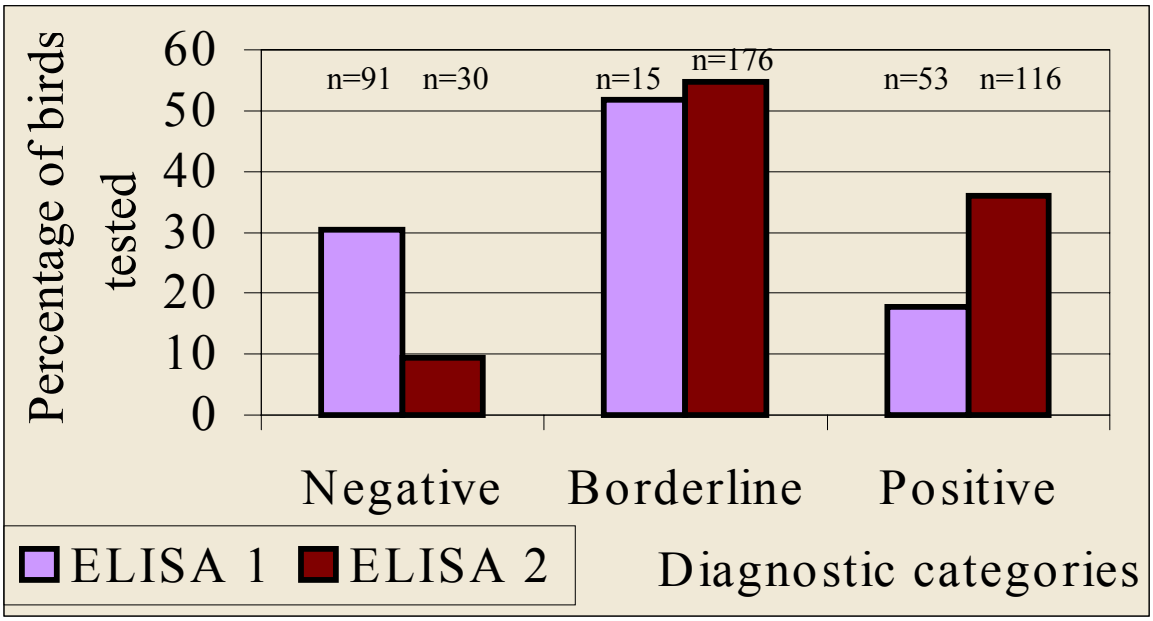
For birds that were tested twice the results of the first and second ELISAs were combined such that the 'stronger positive' result of both was taken into account. As seen in **Table 8**, 37.6% of the birds tested in total produced positive reactions, 51.4% were classified as borderline reactions and 11.0% were negative.

Table 8: Results of ELISA 1 added to ELISA 2, expressed as a proportion of the total number of birds tested, in the different diagnostic categories.

Negative/number tested (%)	Borderline/number tested (%)	Positive/number tested (%)
46/418 (11.0%)	215/418 (51.4%)	157/418 (37.6%)

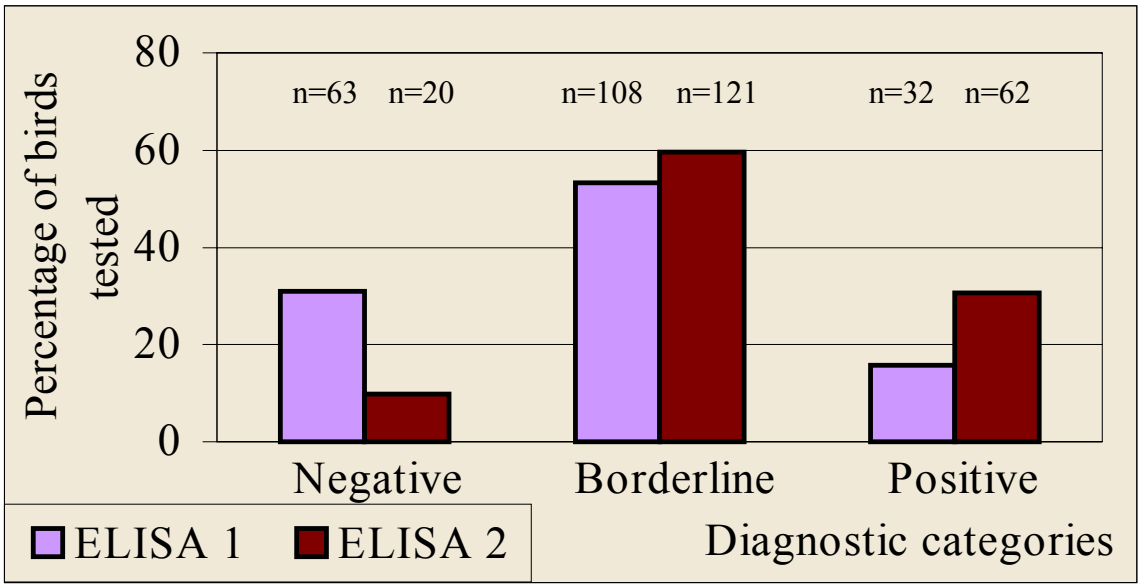
When both ELISAs were analysed separately, there were 17.7% of the birds reacting positive in the first ELISA compared to 36.0% in the second ELISA. The percentage of birds being classified as negative in the first ELISA was 30.4% while there were 9.3% in the second ELISA. As seen in **Figure 5** (and in detail in **Table I in Appendix 1**), there was a reversal of the valence of the number of birds in the single diagnostic categories from the first ELISA to the second ELISA.

Figure 5: Number of birds tested by ELISA 1 (n=299) compared to the number of birds tested by ELISA 2 (n=322), as a proportion of the total number of birds tested, in the different diagnostic categories.



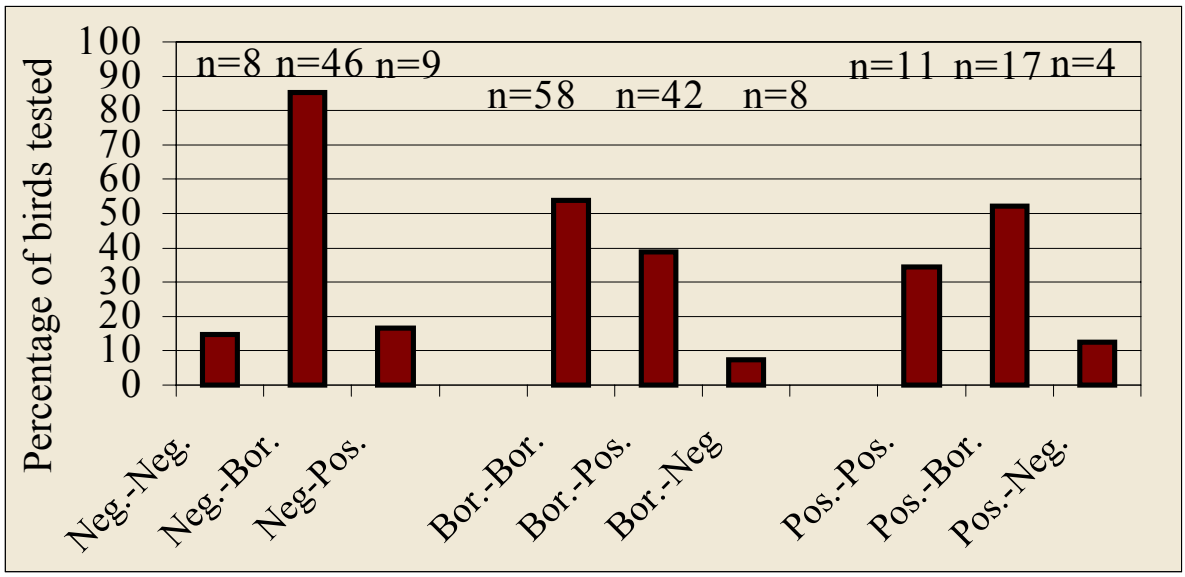
Similar findings were seen when only the birds that have been repeatedly tested by the first ELISA and the second ELISA were taken into account. As seen in **Figure 6** (and in detail in **Table II in Appendix 1**), 15.8% of those birds are considered to be positive by the first ELISA and 30.5% by the second ELISA. 31.0% of the birds were classified as negative in the first ELISA, while 9.9% of the birds showed a low antibody titre of less than 20% in the second ELISA.

Figure 6: Number of birds tested by both ELISA 1 and ELISA 2, as a proportion of the total number of birds tested (n=203), in the different diagnostic categories.



Using just those birds tested twice, a comparison of the results of the first and second ELISA showed a tendency towards more strongly positive results in the second ELISA. As seen in **Figure 7** (and in detail in **Table III in Appendix 1**), 27.1% of the negative results became borderline results. 28.6% of the borderline results remained borderline and 20.7% of the borderline results became positive results.

Figure 7: The shift of diagnostic categories (initially negative results n=63, initially borderline results n=108, initially positive results n=32) from ELISA 1 to ELISA 2, as a proportion of the total number of individual birds tested (n=203).



Neg.-Neg.=	Negative results remain negative
Neg.-Bor.=	Negative results become borderline
Neg.-Pos.=	Negative results become positive
Bor.-Bor.=	Borderline results remain borderline
Bor.-Pos.=	Borderline results become positive
Bor.-Neg.=	Borderline results become negative
Pos.-Pos.=	Positive results remain positive
Pos.-Bor.=	Positive results become borderline
Pos.-Neg,=	Positive results become negative

Statistical analysis of these results in respect of constant, increased or decreased values, by chi-square test showed significant differences in total ($p<1.633E-26$) and in each diagnostic category (negative – $p<1.887E-09$, borderline – $p<0.002$ and positive – $p<6.76E-17$). Most negative values increased, mainly into the borderline category (27.1%). As seen in **Table 9**, the borderline values showed mainly either constant results (28.6%) or a shift to the positive diagnostic category (20.7%). Ten percent of the positive values decreased in the second ELISA.

Table 9: Number of birds tested by ELISA 1 and ELISA 2 with constant, increased and decreased shift in diagnostic categories.

Diagnostic Category	No shift/ no. tested (%)	Increased shift/ no. tested (%)	Decreased shift/ no. tested (%)
Negative	8/203 (3.9%)	55/203 (27.1%)	0/203 (0%)
Borderline	58/203 (28.6%)	42/203 (20.7%)	8/203 (3.9%)
Positive	11/203 (5.4%)	0/203 (0%)	21/203 (10.3%)

Based on the results of the first ELISA, it was decided to cull a number of birds in order to evaluate the sensitivity and specificity of the ELISA in light of post mortem data. A blood sample was taken from each of the selected birds to be used in a third ELISA. The blood samples for the first ELISA were taken in February 1999 and the last sampling and culling took place one and a half years later in September 2000. As seen in **Table 10**, there is a distinct discrepancy between the results of the first and the third ELISA.

The sensitivity and specificity of the first ELISA, the second ELISA, the third ELISA, and the pooled results of the first and the second ELISA are discussed below, in **section 3** of this chapter.

Table 10: Number of birds tested by ELISA 1 and ELISA 3, as a proportion of the total number of birds tested.

ELISA	Negative/ no. tested (%)	Borderline/ no. tested (%)	Positive/ no. tested (%)
1	1/17 (5.9%)	0/17 (0%)	16/17 (94.1%)
3	6/17 (35.3%)	7/17 (47.1%)	3/17 (17.6%)

3. Post mortem examination and comparison of post mortem findings with ELISA results

Post mortem data were available from 32 birds which were sampled and which died during the period of this study. Detailed information of each individual bird including ELISA results, post mortem, histopathology and PCR results are given in **Appendix 4**. These data were used to preliminarily assess the sensitivity and specificity of the ELISA. A chi-square test was performed on the results of the first ELISA and second ELISAs and on the pooled results of the first ELISA and the second ELISA (considering all diagnostic categories, negative and borderline results grouped together against positive results and the 'positive category'). Significant differences were found only in the 'positive category' ($p<0.03$) and in the comparison of negative and borderline results against positive results ($p<0.0004$) of the first ELISA. As shown in **Tables 11a to 11d**, a comparison of the sensitivity and specificity of the first ELISA, the second ELISA and the combination of the two revealed that only the first ELISA offers acceptable results, with a sensitivity of 62.5% and a specificity of 93.3%. Analysis of the ELISA results showed that there is a significant increase in the strength of a positive reaction in the second ELISA compared to the first. A considerable percentage of false negative (62.5%) and false positive results (55.6%) occurred in the second ELISA. The first ELISA was shown to have yielded more reliable results than the second. For this reason, in the next stage of the study, birds were selected for culling from those tested positive in the first ELISA.

Table 11a: Determination of sensitivity (X) and specificity (*) of ELISA 1, using post mortem data as reference criteria.

Diagnostic category	ATb deaths$^{\Delta}$	Percentage	Non ATb. Deaths$^{\Delta}$	Percentage
Negative/Borderline	3	37.5	14	93.3 (*)
Positive	5	62.5 (X)	1	6.7

Table 11b: Determination of sensitivity (X) and specificity (*) of ELISA 1 combined with ELISA 2, using post mortem data as reference criteria.

Diagnostic Category	ATb deaths$^{\Delta}$	Percentage	Non ATb. deaths$^{\Delta}$	Percentage
Negative/Borderline	5	38.46	13	68.4 (*)
Positive	8	61.5 (X)	6	31.58

Table 11c: Determination of sensitivity (X) and specificity (*) of ELISA 2, using post mortem data as reference criteria

Diagnostic Category	ATb deaths$^{\Delta}$	Percentage	Non ATb. deaths$^{\Delta}$	Percentage
Negative/Borderline	5	62.5	4	44.4 (*)
Positive	3	37.5 (X)	5	55.6

Table 11d: Determination of sensitivity (X) and specificity (*) of ELISA 3, using post mortem data as reference criteria

Diagnostic Category	ATb deaths$^{\Delta}$	Percentage	Non ATb. deaths$^{\Delta}$	Percentage
Negative/Borderline	4	66.7	11	84.6 (*)
Positive	2	33.3 (X)	2	15.4

Δ 'ATb deaths' stands for the number of birds which showed evidence of avian tuberculosis on gross post mortem
'Non-ATb deaths' represents the number of birds which showed no finding suspicious for avian tuberculosis on necropsy.

3.1. Post mortem and histopathological examination and PCR

53 out off 299 birds showed absorbency values above the cut-off point of 50% in the first ELISA. It was agreed with Wildfowl and Wetland Trust to consider each bird individually, with species susceptibility, physical examination findings and its value to the collection taken into account. 23 birds were selected to be culled, but seven could not be located. Most likely the birds had died and could not be found in densely planted pens. While the causes of these birds' deaths cannot be confirmed, it is known that birds suffering from chronic progressive diseases, such as avian tuberculosis, seek cover. Three other birds were euthanased because of poor body condition and other poor prognostic signs. All 19 birds were subjected to gross post mortem examination. Samples of liver, spleen and any other macroscopical suspicious organs were collected for histopathological examination and Ziehl-Neelsen staining. Detailed information of each individual bird including ELISA results, post mortem, histopathology and PCR results are presented in **Appendix 4.**

Body condition score was assigned by assessing development of the pectoral muscles, subcutaneous fat and intra-abdominal fat stores. Body condition was expressed on a scale from 1 to 4, with 1 being the thinnest. A subjective assessment of body condition if performed consistently by the same person was considered adequate.

3.1.1. Individual gross post mortem findings

Birds, which have not been classified as positive for avian tuberculosis by ELISA 1:

Bird 1 (no ring)
Ringed Teal (*Anas leucophrys*), adult, male, body condition score 2.
The left foot was missing below the tarsal joint, with signs of healing by second intention. No gross findings suspicious of avian tuberculosis were seen.

Bird 2 (ring number SB3965)
Sharp Winged Teal (*Anas flavirostris flavirostris*), adult, female, body condition score 2.

There was a slight red discolouration and thickening of the wall of the ileojejunum. No gross finding suspicious of avian tuberculosis were seen.

Bird 3 (ring number LC0231)
Chinese Spotbill (*Anas poecilorhyncha zonorhyncha*), adult, male, body condition score 1.
The suborbital sinuses and nasal cavity were filled by yellowish caseous material protruding from the nostrils. There was severe splenomegaly with an attached 0.9 x 1.2 cm nodular whitish firm mass. Multiple miliary white lesions were distributed diffusely in the hepatic parenchyma. Three 0.2 x 0.2 cm whitish-grey firm and prominent nodules were present on the intestinal wall and the adjacent mesentery of the ileojejunum. Both adrenal glands were severely enlarged. The findings were suspicious for avian tuberculosis.

Birds, which have been classified as positive for avian tuberculosis by ELISA 1:

Bird 4 (ring number SD2181)
Lesser White Fronted Goose (*Anser erythropus*), 10 years old, male, body condition score 3.
There was severe splenomegaly with a generalised black discoloration. No gross findings suspicious of avian tuberculosis were seen.

Bird 5 (ring number SD2145)
European Eider (*Somateria mollissima mollissima*), 11 years old, male, body condition score 1.
The left kidney was mottled, and moderately enlarged, with the cranial lobe being severely hypertrophic. No gross findings suspicious of avian tuberculosis were seen. Unilateral renal atrophy is an occasional post mortem finding in captive, but not wild eiders (BROWN, personal communication).

Bird 6 (ring number S2106)
Bar-headed Goose (*Anser indicus*), 10 years old, male, body condition score 2.
The spleen was congested and moderately enlarged. Lesions consistent with ischaemia on the liver were pale, up to 2 cm diameter extended into the parenchyma and infiltrated areas along

the edges. Cranial to the thoracic inlet five round to oval structures were located bilaterally. They were 0.5 mm to 1 cm, reddish-white structures with a smooth surface and an internal vascular system. These structures were specifically delineated paired lymph nodes, found in several waterfowl species, chickens, marsh and shore birds *(Nodus lymphaticus cervicothoracicus*) (VON KOLLIAS, 1986; NEUMANN and KALETA, 1992). No gross findings suspicious of avian tuberculosis were seen.

Bird 7 (ring number LC0240)
Eyton´s Whistling Duck (*Dendrocygna eytoni*), 8 years old, female, body condition score 2.
The liver was severely mottled, with centrolobular discolouration and peripheral brightening (ischaemia). No gross findings suspicious of avian tuberculosis were seen.

Bird 8 (ring number LB0466)
Sharp Wing Teal (*Anas flavirostris flavirostris*), 4 years old, male, body condition score 3.
A 2.1 x 1.7 cm, reddish-white firm area was present on the edge of the left liver lobe, infiltrating the surrounding parenchyma. The spleen and heart were mottled, the heart also showing pinpoint white lesions in great number. White pinpoint spots were also found in the caudal lungs and on the diaphragmatic side of the liver. No gross findings suspicious of avian tuberculosis were seen.

Bird 9 (ring number LB0525)
Argentine Red Shoveler (*Anas platalea*), 2 old, female, body condition score 1.
The heart showed pinpoint spots in great number. The edge of the left ventral liver lobe was pale (ischaemic) and firm. No gross findings suspicious of avian tuberculosis were seen.

Bird 10 (ring number SC3198)
Red Breasted Goose (*Branta ruficollis*), 10 years old, male, body condition score 2.
No abnormal findings were seen.

Bird 11 (ring number SC3197)
Red Breasted Goose (*Branta ruficollis*), 10 years old, male, body condition score 2.
No abnormal findings were seen.

Bird 12 (ring number SC3193)
Red Breasted Goose (*Branta ruficollis*), 10 years old, female, body condition score 2.
No abnormal findings were seen.

Bird 13 (ring number SD2128)
Common Shelduck (*Tadorna tadorna*), 11 years old, female, body condition score 2.
Two 0.3 x 0.3 cm whitish circumscribed nodules were present on the ventral edge of the right liver lobe. No gross findings suspicious of avian tuberculosis were seen.

Bird 14 (ring number SD2134)
Common Shelduck (*Tadorna tadorna*), 11 years old, male, body condition score 2.
The spleen is slightly enlarged and mottled. The hepatic parenchyma is mottled, with a 0.2 x 0.2 cm black, slightly depressed lesion and 1 x 1.3 cm whitish area of discolouration on the edge. No gross findings suspicious of avian tuberculosis are seen.

Bird 15 (ring number LC0230)
Fulvous Whistling Duck (*Dendrocygna bicolor*), 8 years old, male, body condition score 2.
The liver showed multiple irregular circumscribed whitish lesions, extending into the parenchyma, from 0.2 to 0.4 cm in diameter. The spleen was mottled and moderately enlarged. In the left caudal lung there was a 0.2 x 0.5 cm, hard, black, round mass, surrounded by minor haemorrhages. The findings were suspicious for avian tuberculosis.

Bird 16 (ring number SD2186)
Lesser White Fronted Goose (*Anser erythropus*), 10 years old, male, body condition score 4.
The spleen was severely enlarged, with three 0.2 x 0.4 cm circumscribed whitish nodules. The spleen was mottled. The findings were suspicious for avian tuberculosis.

Bird 17 (ring number SD2169)
European Eider (*Somateria mollissima mollissima*), 11 years old, female, body condition score 1.
There were three to four white miliary nodules, on the surface and within the parenchyma of the ventral aspect of the left and right liver lobes. The left cranial kidney lobe was severely

enlarged. A firm white caseous mass was presented ventral to the left lobe, and is firmly attached to the sacrum. Both kidneys were moderately enlarged and mottled. The findings were suspicious for avian tuberculosis.

Bird 18 (ring number LC0136)
White-faced Whistling Duck (*Dendrocygna viduata*), 9 years old, male, body condition score 2.
A 1.2 x 1.7 cm poorly circumscribed whitish nodule was present on the left liver lobe. Two similar nodules in the right liver lobe were 0.5 cm in diameter. The spleen was mottled and slightly enlarged. A class IV bumblefoot lesion (REMPLE, 1993) affected the main pad of the right foot. The findings were suspicious for avian tuberculosis.

Bird 19 (ring number LC0316)
White-faced Whistling Duck (*Dendrocygna viduata*), 7 years old, female, body condition score 1.
Multiple whitish nodular lesions from less than 1 mm to 2 x 2.4 cm were present diffusely on the surface of the liver. The entire liver appeared pale. There were pinpoint white spots on the heart in great number and the spleen was moderately enlarged. Firmly attached, caseous nodules, up to 0.5 cm in diameter were present diffusely on the entire mesentry. These findings were common in White-faced Whistling Ducks with avian tuberculosis (BROWN, personal communication).

Findings on post mortem examination do not support a correlation between poor body condition and gross post mortem evidence of avian tuberculosis.

Of the 16 birds originally selected for culling, five were found to have lesions suggestive of avian tuberculosis. In addition to those birds considered initially as likely to be tuberculous, three birds were culled for other avicultural reasons. Of these birds, which acted as a control group, one was found to have the pathological appearance of avian tuberculosis at necropsy.

3.1.2. Findings of histopathological examination and PCR

The histopatological examination, including Ziehl-Neelsen staining, confirmed the presence of acid-fast mycobacteria in one bird (bird number 19, ring number LC0316), but not in the remaining 15 birds which were considered to be tuberculous by ELISA and not in the three birds which were culled for other avicultural reasons (see **Tables 12 and 19**). There is no significant difference in the overall number of birds diagnosed positive or negative for avian tuberculosis by post mortem and histopathological examination (Chi-square test, $p<0.13$). Referring the gross post mortem to the histopathological examination as gold standard there were false positive results obtained in 27.8% of cases, but no false negative results (see **Table 12**). The analysis of the data of all the birds, on which ELISA 1, ELISA 3, post mortem examination and histopathology were performed showed consistency in being diagnosed positive or negative for avian tuberculosis only for the birds being tested by ELISA 3 and the gross post mortem data, as is seen in **Tables 13 and 14**.

Determining a final diagnosis based on histopathological findings still contains the risk of missing tuberculous birds due to false negative results. Reasons for mycobacteria not showing up as acid-fast bacilli in Ziehl-Neelsen staining are elucidated in **section E.1.3.** (**Discussion, 1.3., Determination of the cut-off point**).

PCR did not confirm the presence of mycobacterial DNA fragments in any of the 13 specimens submitted (**Table 15**). Sequencing of PCR products was not performed as there were no specimens showing positive reactions for DNA fragments of *Mycobacterium avium*.

Table 12: Number of birds diagnosed by post mortem (PM) and by histopathological examination as positive (ATb) or negative for avian tuberculosis (Non-ATb), as a proportion of the total number of culled birds.

	Histopath. diagnosis ATb	Percent-age	Histopath. diagnosis Non-ATb	Percent-age	Total PM diagnosis
PM diagnosis Negative	0	0	13	72.2	13
PM diagnosis Positive	1	100	5	27.8	6
Total Histopathol. diagnosis	1		18		19

Table 13: Proportion of birds diagnosed by ELISA 1, by ELISA 3, by post mortem (PM) and by histopathological examination as positive for avian. Each single diagnostic category is put into relation to the other diagnostic categories.

	ELISA 1 positive	**ELISA 3** positive	**PM** positive
ELISA 3 positive	3/17 (17.6%)		
ELISA 3 negative	13/17 (76.5%)		
PM positive	5/17 (29.42%)	2/19 (10.5%)	
PM negative	11/17 (64.7%)	2/19 (10.5%)	
Histopathology positive	1/17 (5.9%)	0/19 (0%)	1/19 (5.3%)
Histopathology negative	15/17 (88.2%)	4/19 (21.5%)	5/19 (26.3%)

As mentioned in **chapter D.3.** (**Results, Post mortem examination and comparison of post mortem findings with ELISA results**), ELISA 2 was not considered in choosing the birds for culling and post mortem examination.

Table 14: Number of birds diagnosed by ELISA 1, by ELISA 3, by post mortem (PM) and by histopathological examination as positive or negative for avian tuberculosis, as a proportion of the total number of culled birds.

Diagnosis	ELISA 1	ELISA 3	Post mortem	Histopath.
Positive	16 (94.1%)	4 (21.1%)	6 (31.6)	1 (5.2%)
Negative	1 (5.9%)	15 (79.0%)	13 (68.4)	18 (94.7%)
Total	17	19	19	19

Table 15: Summary of findings of the birds diagnosed by post mortem and by histopathological examination.

Birds considered negative for avian tuberculosis by ELISA:

Identification	Condition score*	Post mortem results	Histopatholog. results	Final Diagnosis
No ring	2	No abnormalities	Negative	Non ATb
SB3965	2	No abnormalities	Negative	Non ATb
LC0231	1	Abnormalities	Negative	Non ATb

Birds considered positive for avian tuberculosis by ELISA:

Identification	Condition score*	Post mortem results	Histopatholog. results	Final Diagnosis
SD2181	3	No abnormalities	Negative	Non ATb
SD2145	1	No abnormalities	Negative	Non ATb
S2106	2	No abnormalities	Negative	Non ATb
LC0240	2	No abnormalities	Negative	Non ATb
LB0466	3	No abnormalities	Negative	Non ATb
LB0525	1	No abnormalities	Negative	Non ATb
SC3198	2	No abnormalities	Negative	Non ATb
SC3197	2	No abnormalities	Negative	Non ATb
SC3193	2	No abnormalities	Negative	Non ATb
SD2128	2	No abnormalities	Negative	Non ATb
SD2134	2	No abnormalities	Negative	Non ATb
LC0230	2	Abnormalities	Negative	Non ATb
SD2186	4	Abnormalities	Negative	Non ATb
SD2169	1	Abnormalities	Negative	Non ATb
LC0136	2	Abnormalities	Negative	Non ATb
LC0316	1	Abnormalities	Positive	Questionable

* As described in section **3.1.** (**Results, Post mortem and histopathological examination and PCR**), the body condition score established on the development of pectoral muscles and fat storages and expressed on a scale from 1 to 4, with 1 being the thinnest.

3.2. Validation of sensitivity and specificity of the ELISA

The specificity and sensitivity of the ELISA were assessed by analysing ELISA 1 and post mortem results. To remain consistent the histopathological findings were not taken into account, as there were only gross post mortem data available from the birds that died prior to culling during this study. Data were available from 23 birds that were bled and died in the period of this study, from 16 birds that were classed as positive for avian tuberculosis by the first ELISA results and were culled, and from one bird which was culled for avicultural reasons and which was tested negative for avian tuberculosis by the first ELISA. **Tables 16 and 17** and **Figure 8** present those pooled number of birds diagnosed by ELISA and post mortem examination.

A chi-square test comparing the overall number of birds showing signs of infection with avian tuberculosis diagnosed by ELISA and post mortem examination showed no significant difference.

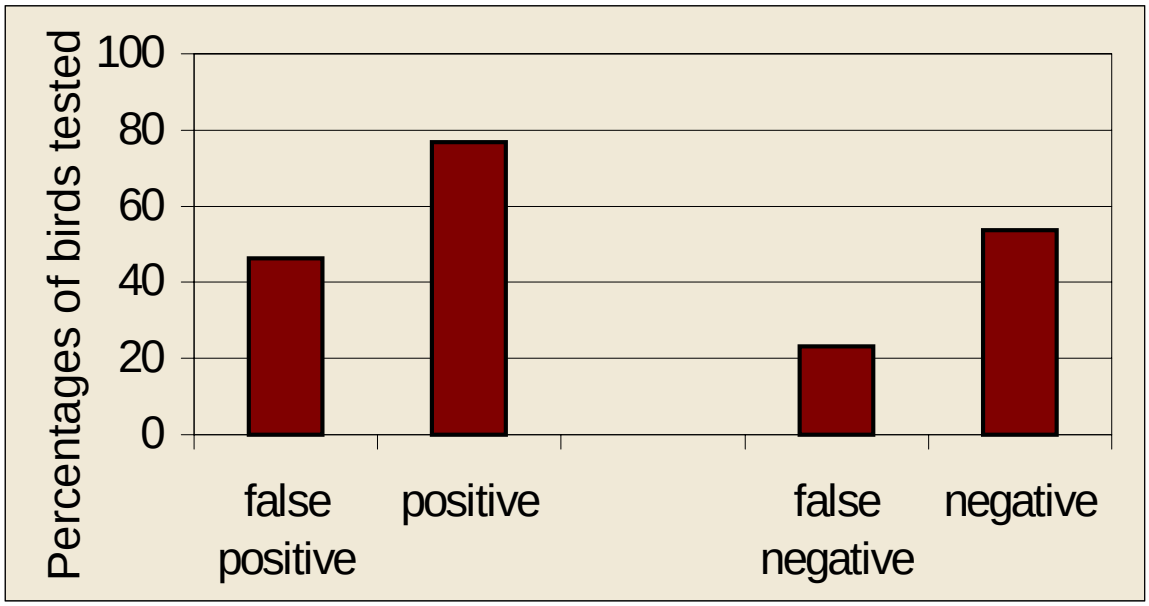
Table 16: Number of birds diagnosed by ELISA and by post mortem examination (PM) as positive (ATb) or negative for avian tuberculosis (Non-ATb), as a proportion of the total number of birds in each ELISA diagnostic category (n=40).

ELISA diagnostic category	PM diagnosis ATb	Percent-age	PM diagnosis Non-ATb	Percent-age	Total ELISA diagnosis
Negative	0	0	4	14.8	4
Borderline	3	23.1	11	40.7	14
Positive	10	76.9	12	44.4	22
Total PM diagnosis	13		27		40

Table 17: Number of birds diagnosed by ELISA and by post mortem examination (PM) as positive (ATb) or negative for avian tuberculosis (Non-ATb), as a proportion of the total number of birds after grouping negative and borderline results against positive results, with determination of sensitivity (X) and specificity (*) (n=40).

ELISA diagnostic category	PM diagnosis ATb	Percent-age	PM diagnosis Non-ATb	Percent-age	Total ELISA diagnosis
Negative/borderline	3	23.1	15	55.6 (*)	18
Positive	10	76.9 (X)	12	44.4	22
Total PM diagnosis	13		27		40

Figure 8: Determination of the number of birds in percentage which were diagnosed as positive or negative and false positive or negative by ELISA, referring the results to post mortem examination as gold standard (n=40).



Twelve false positive results were recorded from the 27 birds diagnosed positive for avian tuberculosis (44.4%). False negative results are obtained in 23.1% of cases. Sensitivity is 76.9% and specificity reaches 55.6%. If the results are analysed for each tribe, no tribe showed a significant difference between the numbers of birds diagnosed positive or negative for avian tuberculosis by ELISA and post morten examination. **Table 18** demonstrates the number of birds in percentage, which were diagnosed as false or true positive or negative for avian tuberculosis by ELISA.

Table 18: Number of birds in each tribe diagnosed by ELISA as positive or negative (negative and borderline results combined) for avian tuberculosis, as a proportion of the total number of birds in each group.

Tribe	ELISA diagnosis positive				ELISA diagnosis negative			
	False positive		Positive		False negative		Negatives	
	No.	%	No.	%	No.	%	No.	%
Dendrocygnini	2	50	3	100	0	0	2	50
Anserini	5	71.4	2	50	2	50	2	28.6
Tadornini	2	100	1	100	0	0	0	0
Anatini	2	25	0	0	1	100	6	75
Somateriini	1	50	1	50	0	0	0	0
Cairinini	0	0	2	100	0	0	1	100
Oxyurini	0	0	0	0	1	50	1	50
Total (n=40)	12	44.4	10	76.9	3	23.1	15	55.6

4. Physical environment and husbandry details

To analyse the epidemiology of mycobacterial infections it is necessary to assess husbandry details and physical environment of the enclosures of the birds. The codes identifying the different pens are explained in **section C.2. (Materials and methods, Identification of the birds' enclosures)**.

Description of pens

Flamingo pen	no trees; dense vegetation with red hot poker and single shrubs
Top pond	wide open space; bank with a few trees; hedge line which is very dense in summer
Asian pen	open space with three very dense hedges
Australian pen	open space with one very dense hedge
European pen	little open space excepting the pond itself; one long hedge; overgrown banks; many trees
North American pen	open space, one dense hedge, heavily treed
South American pen	little open space, very dense vegetation with a lot of pampas grass
South American side pen	little open space, some trees
European side pen	little open space, many trees, very dense vegetation
Side pen A, B, C	little open space, dense vegetation and shrubs
Lagoon	open land, large pond with several banks
Island pen	large open space, partly dense vegetation
Hospital pen	open space, some shrubs
Duckery	open space
Stiff-tail pen	open space, a few shrubs on island
Goldeneye pen	little open space, small pond surrounded by trees
Smew pen	little open space, very dense vegetation

Pens with more water surface than land were Smew pen, the Goldeneye pen, and the Flamingo pen. A pen with more land than water surface was the Island pen. The remaining pens had approximately equal ratios of land area to water surface. Pens with poor drainage after heavy raining were the North American pen, the South American pen, the Australian pen, the Goldeneye pen and the Flamingo pen. It is not possible to prevent entirely mixing of birds between pens. Gates are on occasion left open by visitors and several birds despite pinioning can fly short distances. A high degree of mixing occurred between the Australian pen and the South American pen, and between the Asian pen and the North American pen. Mixing between the Island pen and the Asian pen occurred to a lesser extent. The birds of the collection had ongoing contact with wild birds. Moorhens were especially common in European pen, the South American pen, the Goldeneye pen and the Australian pen. Mallards and gulls were most common in the Top pond, while Gadwalls and Tufted Ducks frequent the European pen and the Smew pen. Few pigeons were found in the grounds.

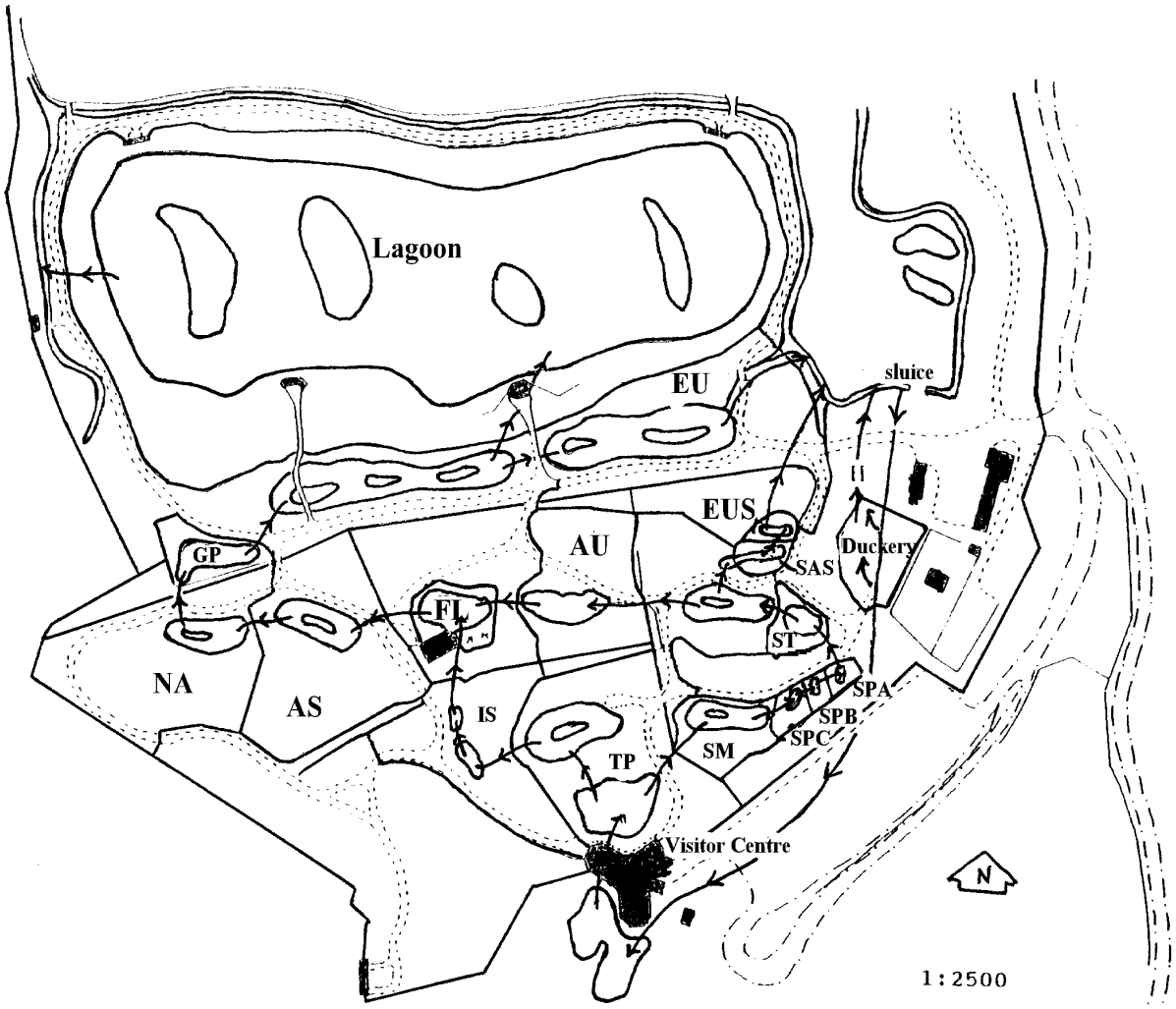
Figure 9 shows the watercourse through the grounds. The water supply to the collection originates from the river Loughor. Water is pumped through a sluice at the lower end of the grounds to a header tank near the car park, and then flows to a pool south of the Visitor Centre. Water runs through a ditch from the river to the tank. Reed beds, which harbour a great number of wild birds, line the ditch on both sides. The tank is small and open, but wild birds have not been seen using it to a great extent. From the tank, water passes under the Centre into the Top Pool, where the flow divides into two branches. Each branch supplies several ponds, in series, as illustrated. Drainage back to the sluice is from the European Side pen, the European pen and the Lagoon. The Duckery receives water directly from the main supply, before it reaches the header tank. The water then passes the hospital pen and drains into the sluice. The Duckery is, therefore, the only area with an isolated water supply. There is no filter system, apart from the reed beds next to the ditch through which the water runs before it reaches the pens.

The following observations on the physical environment of the pens were made:

- all ponds in the grounds, except the Lagoon, have anti-erosion concrete edges;
- all paths through the collection pens are tarmac;
- all paths are fully exposed to direct sunlight;
- a steel factory allows waste water to drain into the river Loughor and a rubbish dump, closed in 1960, is located close to the sluice, on the river bank;
- the birds are fed twice a day, mainly whole wheat and pelleted supplement (breeder or maintenance diet, depending on the season); sea ducks, such as Eiders (*Somateria mollissima*), Bufflehead (*Bucephala albeola*), Goosander (*Mergus mer*ganser), Smew (*Mergus albellus*) or Hooded Merganser (*Mergus cucullatus*) receive floating pellets; flamingos are also fed a species appropriate diet; although there are also some wooden troughs in the pens, most birds are fed directly onto the ground, by the pond edges.

Figure 9: Wildfowl and Wetlands Trust Llanelli

Map of grounds showing watercourse



Pen Codes:

AS	Asian pen	IS	Island pen
AU	Australian pen	NA	North American pen
EU	European pen	SA	South American pen
EUS	European side-pen	SAS	South American side-pen
FL	Flamingo pen	SM	Smew pen
GP	Goldeneye pond	SPA, B, C	Side-pens A, B, C
H	Hospital pen	ST	Stifftail pen
TP	Top pond		

Pools 

Watercourse 

Buildings 

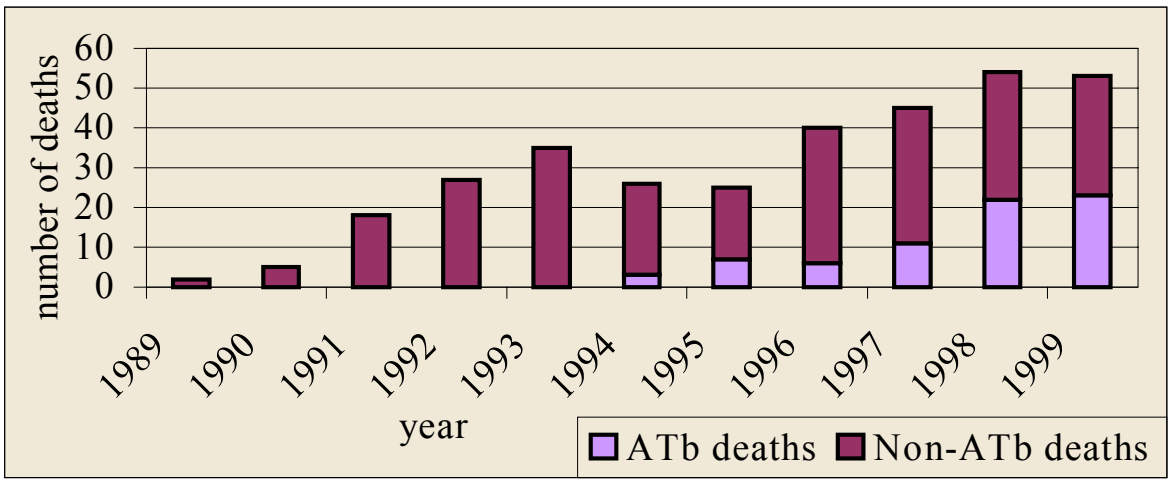
5. Analysis of post mortem data 1989 to 1999

A total of 330 birds died in the WWT centre in Llanelli from 1989 to 1999 and post mortem examinations were performed on them. The data from those 330 birds are summarised by tribe, pen, sex and annual incidence of avian tuberculosis. Detailed information of those birds is listed in **Appendix 5**.

5.1. Incidence of avian tuberculosis over a ten year study period (1989 to 1999)

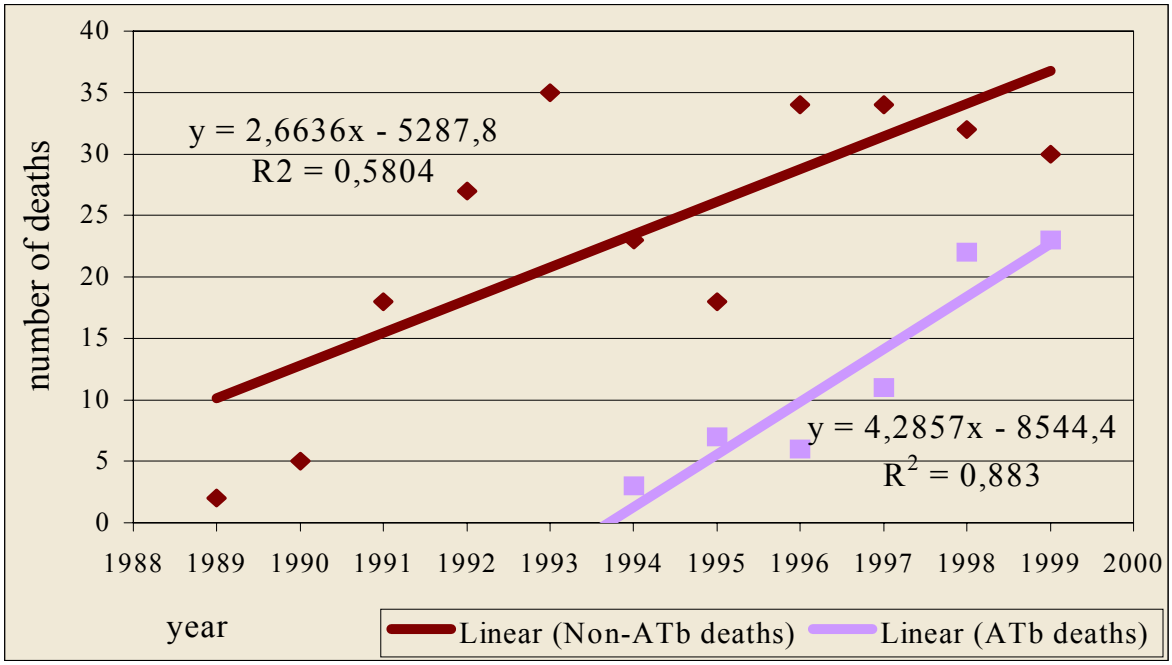
Post mortem data were available for all the birds that died in the WWT centre in Llanelli from 1989 to 1999. As shown in **Figure 10** (and in detail in **Table IV in Appendix 1**), from 1989 to 1999 no cases of avian tuberculosis were identified. In 1994 three birds died showing post mortem findings consistent with avian tuberculosis. Since then, mortality due to avian tuberculosis has risen to 40.7% in 1998 and 43.4% in 1999. The chi-square test revealed significant differences in the total annual mortality rate ($p<0.001$). In 1998 the incidence of avian tuberculosis was higher than in 1994 ($p<6.66E-3$) and in 1996 ($p<0.007$). In 1999 the highest mortality was found compared to 1994 ($p<5.02E-3$), 1996 ($p<0.003$) and 1997 ($p<0.05$).

Figure 10: Number of birds with post mortem evidence of avian tuberculosis (ATb) from 1989 to 1999, as a proportion of the total number of birds in each group (n=330).



By scattering the results (see **Figure 11**), the increase in mortality from 1989 to 1999 can be shown. The regression coefficient of the number of birds which died from avian tuberculosis (linear ATb deaths) (R^2=0.58), is noticeable higher than the one of the number of birds dying of other causes (linear Non-ATb deaths) (R^2=0.88).

Figure 11: Number of birds dying with post mortem evidence of avian tuberculosis (ATb) from 1989 to 1999



Data from 1994 to 1999 were divided into 1994 to 1997 and 1998 and 1999. This division has been chosen because of the notable increase in incidence of avian tuberculosis in 1998 and 1999. As shown in **Table 19**, incidence in the remaining tribes rises from the first to the second period. For some tribes the results reached statistical significance. In the *Cairinini* (tribe 2), there was a significant rise in the incidence of diseases, from 13.3% to 40% (p<2E-05). The *Anserini* (tribe 3) showed a significant increase in incidence from 25% to 38.9% (p<0.035). For the *Anatini* (tribe 6) the incidence of avian tuberculosis in the first period was significantly lower than in the second (12.5% to 29.4%; p<0.003). In the *Mergini* (tribe 11)

the incidence was doubled from 36.4% to 68.8%. This tribe showed the greatest increase in incidence of avian tuberculosis ($p<4.5E-06$). These results need to be interpreted in conjunction with the analysis of the results according to tribe. The overall incidence of avian tuberculosis in the two periods increased significantly from 21.3% to 42.4% ($p<0.001$).
There was a rise in the number of deaths in the second period. From 1994 to 1999 there were 221 deaths. 122 birds (55.3%) died in the first four years and 99 birds (44.8%) died in the remaining two years. Approximately half of the deaths from 1994 until 1999 occurred during the last two years of this period.

Table 19: Number of birds dying with post mortem evidence of avian tuberculosis (ATb), from 1994 to 1997 and 1998 to 1999, according to tribe, as a proportion of the total number of birds in each group.

1994-1997

Tribe	Total deaths	Non-ATb deaths	Percentage	ATb deaths	Percentage
Dendrocygnini	30	26	86.7	4	13.3
Anserini	8	6	75	2	25
Anatini	48	42	87.5	6	12.5
Aythini	11	6	54.5	5	45.5
Cairinini	14	9	64.3	5	35.7
Mergini	11	7	63.6	4	36.4
Total	122	96	78.7	26	21.3

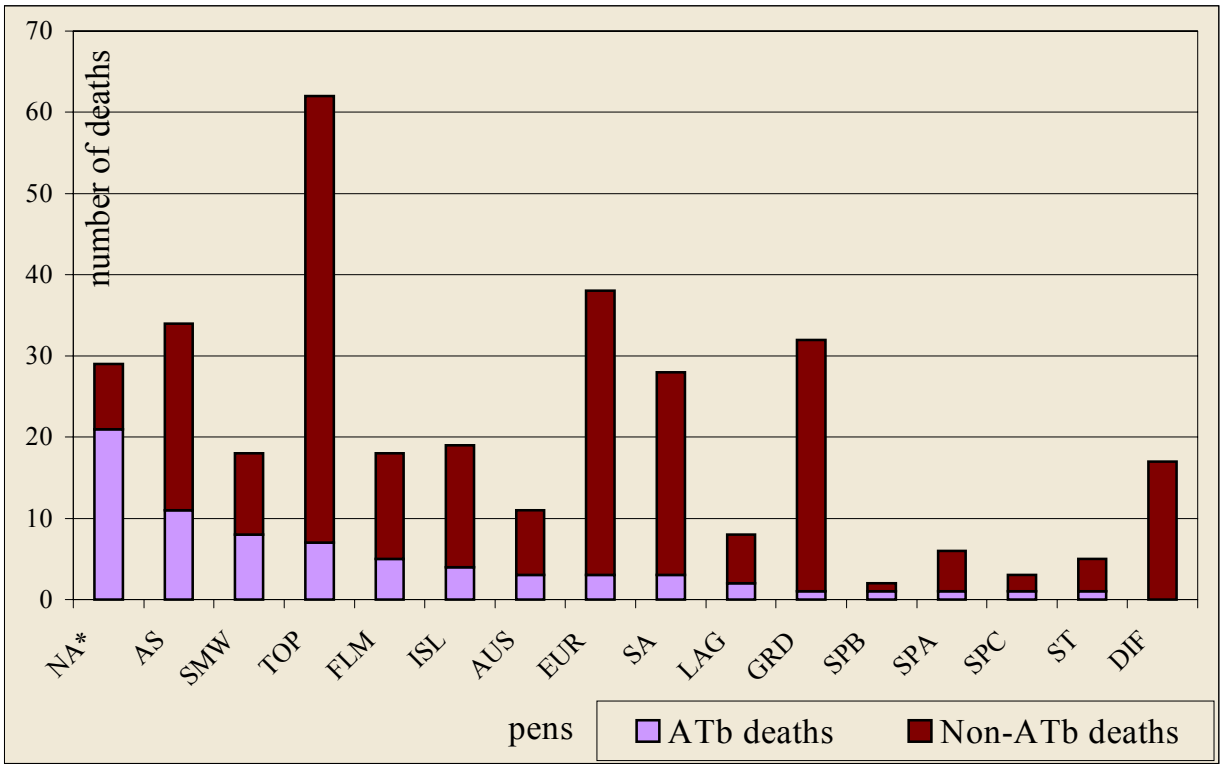
1998-1999

Tribe	Total deaths	Non-ATb deaths	Percentage	ATb deaths	Percentage
Dendrocygnini	10	6	60	4	40
Anserini	18	11	61.1	7	38.9
Anatini	34	24	70.6	10	29.4
Aythini	9	4	44.6	5	55.6
Cairinini	12	7	58.3	5	41.7
Mergini	16	5	31.3	11	68.8
Total	99	57	57.6	42	42.4

5.2. Analysis of the data by pens

There are significant differences in tuberculous mortality between the overall results from all the pens ($p<0.001$) and between the results of some of the pens, when compared to each other. The highest incidence of avian tuberculosis is found in the North American pen, in comparison to the Asian pen ($p<0.002$), the Australian pen ($p<0.014$), the European pen ($p<0.48E-8$), the Flamingo pen ($p<0.003$), the Island pen ($p<9.27E-4$), the South American pen ($p<2.55E-6$), Side pen A ($p<0.019$), the Stifftail pen ($p<0.042$), the Top pond ($p<0.042$), and the Lagoon ($p<0.035$). As seen in **Figure 12** (and in detail in **Table V in Appendix 1**), the incidence of avian tuberculosis is higher in the Asian pen than in the European pen ($p<0.015$) or in the Top Pond ($p<0.011$). The Smew pen shows a higher incidence of avian tuberculosis compared to the European pen ($p<2.72E-3$), the South American pen ($p<0.014$) and the Top pond ($p<0.002$).

Figure 12: Number of birds with post mortem lesions indicative for avian tuberculosis (ATb) summarised by pen, as a proportion of the total number of birds in each group.



*Abbriviations see next page

NA=	North American pen
AS=	Asian pen
SMW=	Smew pen
TOP=	Top pond
FLM=	Flamingo pen
ISL=	Island pen
AUS=	Australian pen
EUR=	European pen
SA=	South American pen
LAG=	Lagoon
GRD=	Grounds, where birds couldn't be assigned to a certain pen
SPA, B, C=	Side pen A, B, C
ST=	Stiff-tail pen
DIF=	Different pens where no avian tuberculosis was diagnosed (European side pen, South American side pen, Hospital pen and Outdoor duckery)

There was no significant correlation between stocking density and the incidence of avian tuberculosis in each pen (Pearson correlation coefficient $p<0.185$). **Table 20** shows the relation between incidence of avian tuberculosis and stocking density, with m^2 representing the total area of the pens.

Table 20: Results of the post mortem data, with special consideration to stocking density

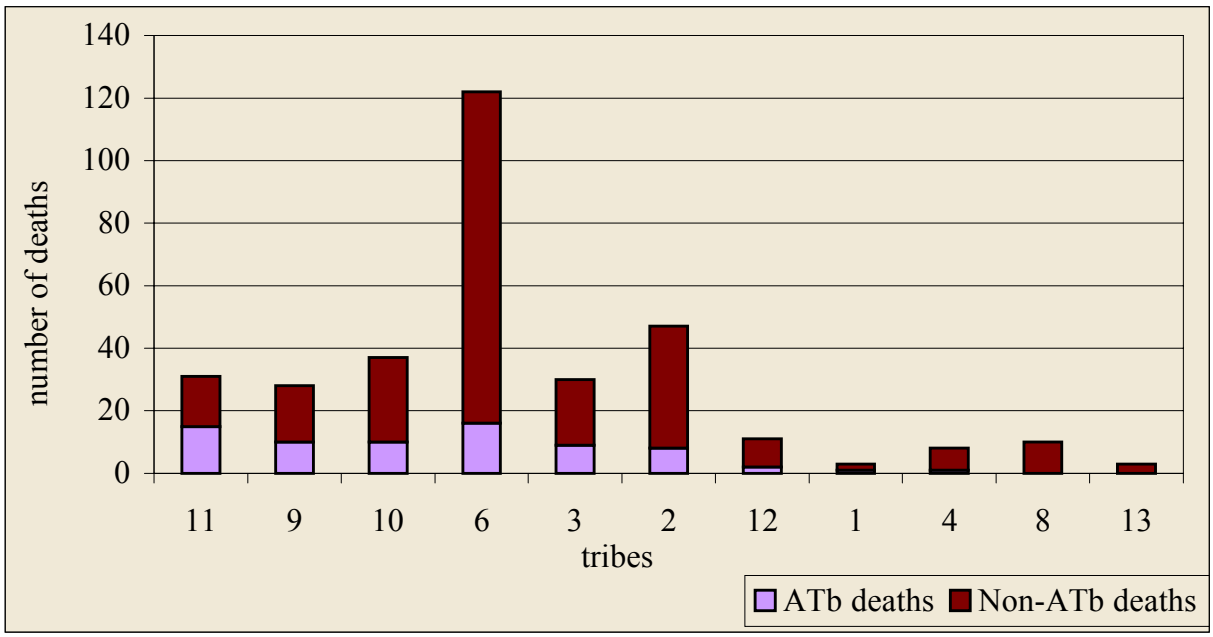
Pen	Number of birds	m^2	bird/m^2	m^2/bird	% deaths due to ATb in pens
NA	64	4605	0.014	71.95	68
SMP	37	1045	0.035	28.24	53.3
SPB	2	119	0.017	59.5	50
SPC	6	163	0.037	27.17	33.3
AS	53	4630	0.011	87.36	29.0
FLM	56	950	0.059	16.96	27.8
AUS	54	2820	0.019	52.22	27.3
IS	97	3175	0.031	32.73	21.1
ST	23	550	0.042	23.91	20
SPA	3	225	0.013	75	16.7
TP	132	5245	0.025	39.73	11.3
EU	110	5445	0.02	49.5	9.1
SA	52	2570	0.02	49.42	7.7
EUS	4	190	0.021	47.5	0
GE	22	750	0.029	34.09	0

Examination of differences in tribes in different pens was carried out by chi-square test. For the three pens with a significantly higher incidence of avian tuberculosis (Asian pen, North American pen and Smew pen) the individual tribes in each pen were also tested against each other. The results did not reach statistical significance in these examinations. An analysis of individual species in different pens has not been performed due to insufficient sample sizes.

5.3.Analysis of the data according to different tribes

A significant difference was demonstrated by chi-square test between the results from different tribes in total ($p<0.002$) and between the results of some tribes when compared to each other. The incidence of avian tuberculosis according to taxonomic tribe varied, ranging from 0% (0/10) in the *Somateiini* (tribe 8, eiders), to 48.4% (15/31) in the *Mergini* (tribe 11, sea ducks). As seen in **Figure 13** (and in detail in **Table VI in Appendix 1**), the incidence of avian tuberculosis in the *Anatini* (tribe 6) was higher than in the *Anserini* (tribe 3) ($p<0.025$). The *Aythini* (tribe 9) showed a higher incidence than the *Anatini* (tribe 6) ($p<0.004$). In the tribe *Cairinini* (tribe 10) also, there was a higher incidence than in the *Anatini* (tribe 6) ($p<0.045$). The incidence in the *Mergini* (tribe 11) was significantly higher than in the *Dendrocygnini* (tribe 2) ($p<0.003$) and the *Anatini* (tribe 6) ($p<0.001$).

Figure 13: Number of birds showing post mortem evidence of avian tuberculosis (ATb) according to tribe, as a proportion of the total number of birds in each group.



11=	*Merginini*	2=	*Dendrocygnini*
9=	*Aythini*	1=	*Anseronatini*
10=	*Cairinini*	4=	*Tadronini*
6=	*Anatini*	8=	*Somateriini*
3=	*Anserini*	13=	*Phoenicopteridae*

Examination of differences in species within a tribe was also carried out by using chi-square test. There were no significant differences apart from the results in the *Cairinini* (tribe 10) and the *Mergini* (tribe 11). As seen in **Table 21**, in tribe 10, 58.3% of White-winged Wood Duck (*Cairina scutulata*) deaths were due to avian tuberculosis, a higher mortality of avian tuberculosis than the Carolina ($p<0.069$). In tribe 11 the mortality of avian tuberculosis, at 78.6%, was higher among the Hooded Merganser (*Mergus cucullatus*) than among the Bufflehead (*Bucephala albeola*) ($p<9.55E-3$) and the Goosander (*Mergus mer*ganser) ($p<1.69E-3$). Smews (*Mergus albellus*) had also a high incidence of avian tuberculosis (66.7 %).

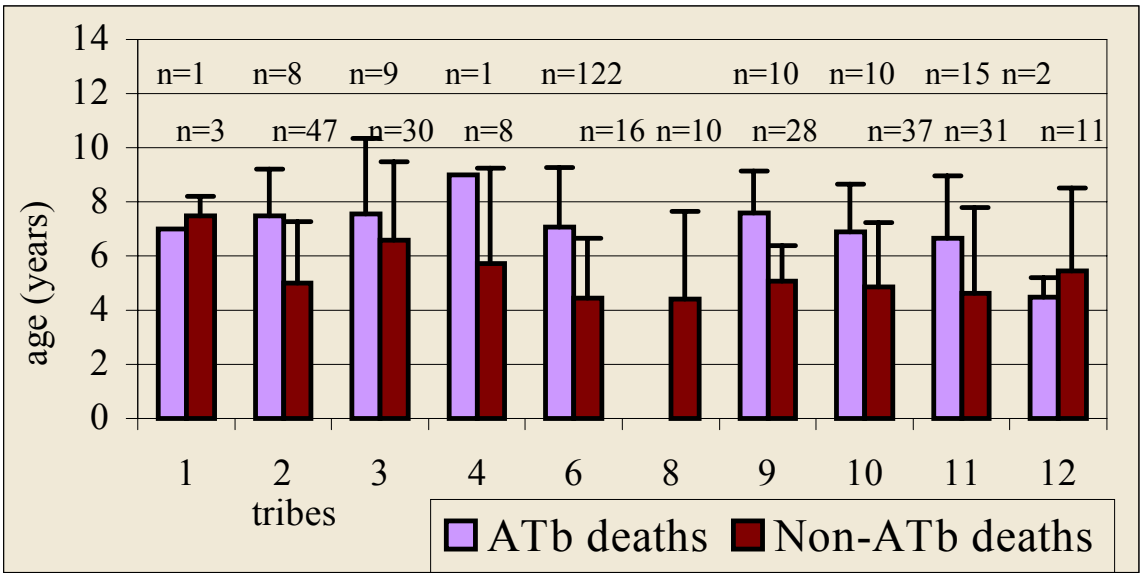
Table 21: Number of birds dying showing post mortem evidence of avian tuberculosis (ATb) according to species, as a proportion of the total number of birds in each group, in the tribes *Cairinini* (10) and *Mergini* (11).

Tribe	Species	Non-ATb deaths	Percentage	ATb deaths	Percentage
Cairinini	Carolina	8	80	2	20
	White-winged wood Duck	5	41.7	7	58.3
Mergini	Bufflehead	5	71.4	2	28.6
	Smew	1	33.3	2	66.7
	Hooded merganser	3	21.4	11	78.6
	Goosander	6	85.7	1	14.3

In analysing the age of the birds that died due to avian tuberculosis it must be remembered that the birds being compared are from different tribes. Accurate analysis is only possible within a tribe, because of the range in life expectancy between tribes. The average life span of some ducks is only six years, while swans can live for over 20 years.

Within some tribes, but not in others the student-t test showed significant differences in the average age of birds dying due to avian tuberculosis and those dying due to non-tuberculous diseases. As seen in **Figure 14** (and in detail in **Table VII in Appendix 1**), the average age of birds dying from avian tuberculosis was significantly higher in the *Dendrocygnini* (tribe 2) ($p>0.0035$), in the *Anatini* (tribe 6) ($p>0.0003$), in the *Aythini* (tribe 9) ($p>0.0006$) and in the *Cairinini* (tribe 10) ($p>0.0321$).

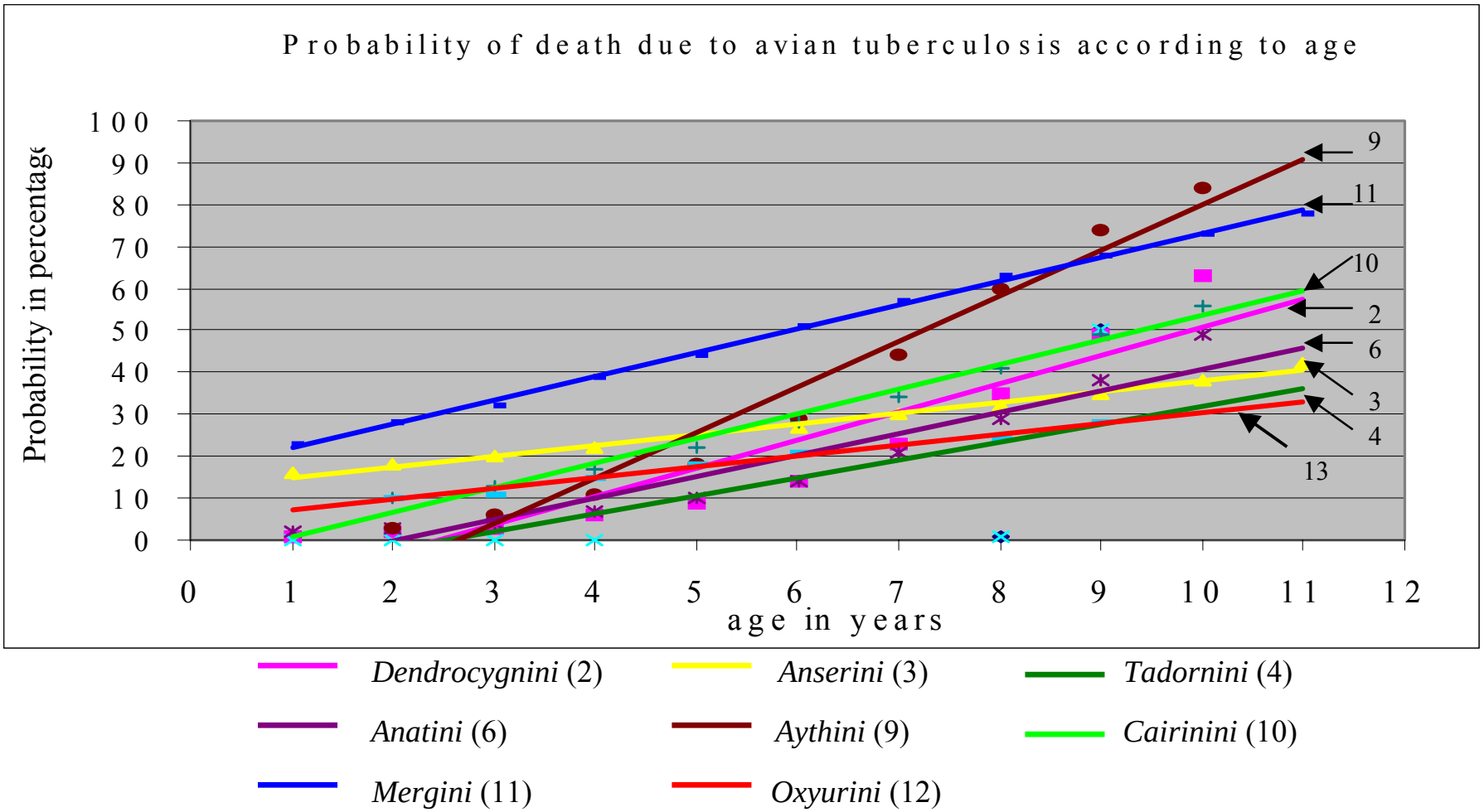
Figure 14: Average age of the birds dying from avian tuberculosis (ATb) or other non-tuberculous diseases (Non-ATb), summarised by tribe.



1= *Anseronatini*
2= *Dendrocygnini*
3= *Anserini*
4= *Tadornini*
6= *Anatini*
8= *Somateriini*
9= *Aythini*
10= *Cairinini*
11= *Mergini*
12= *Oxyrurini*
13= *Phoenicopteridae*

Logistic regression was used to describe the likelihood of dying from avian tuberculosis in a given tribe according to age. Scattering the results gives the likelihood, in percent, of birds in a given tribe dying of avian tuberculosis according to age (**Figure 15**) For example, there is a 15% chance that an eight year old bird, of tribe 4 (*Tadornini*) will die from avian tuberculosis, and a 65% chance that an eight year old bird of tribe 11 (*Mergini*) will die from the disease. Equally, a given percentage can be related to the age of a bird and the tribe to which it belongs. The regression coefficient of the number of birds, which died from avian tuberculosis in the different tribes, represents the rise of the trendline for each tribe. Those tribes with a higher incidence of avian tuberculosis are characterised by a higher regression coefficient than the others (*Tadornini* with R^2=0.472, *Dendrocygnini* with R^2=0.867, *Anatini* with R^2=0.901, *Aythini* with R^2=0.961, *Cairinini* with R^2=0.983 and *Mergini* with R^2=0.998).

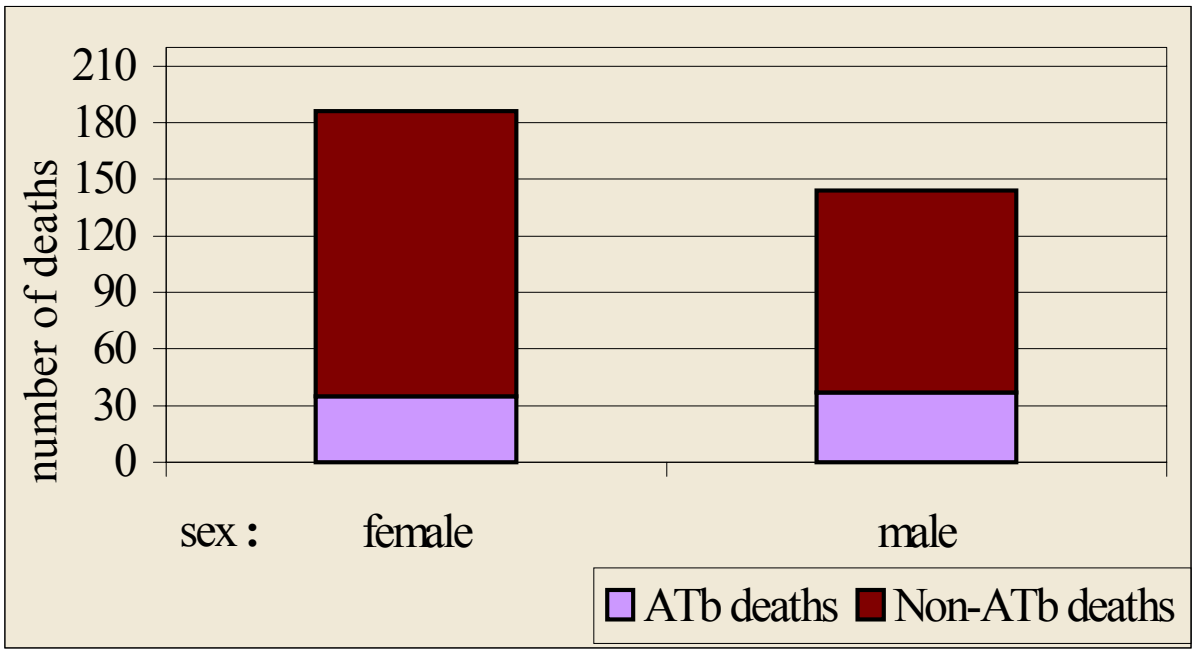
Fig. 15: The probability that birds of a given tribe are dying from avian tuberculosis at a given age is expressed in percent.
Each trendline represents a tribe (2; 3; 4; 6; 9; 10; 11; 12). The steeper the trendline (and the higher the regression coefficient; see text, **chapter D.5.3., Results, Analysis of the data according to different tribes**), the higher is the likelihood of birds of a given tribe dying of avian tuberculosis at a given age.



5.4. Analysis of the data according to sex and season

As **Figure 16** (and in detail **Table VIII in Appendix 1**) shows, at 25.7% the incidence of avian tuberculosis in males appears to be higher than in females (18.8%). However, the chi-square test comparing the overall number of deaths from avian tuberculosis in male and female birds showed no significant difference ($p<0.134$), i.e. there was no sex predilection for avian tuberculosis.

Figure 16: Number of birds dying with post mortem evidence of avian tuberculosis (ATb) according to sex as a proportion of the total number of birds in each group (n=330).



As shown in **Table 22**, there were also no significant differences if the sex was classified by tribes.

Table 22: Number of birds dying with post mortem evidence of avian tuberculosis (ATb) classed by sex and tribe, as a proportion of the total number of birds in each group (n=330).

Tribe	Male deaths		Female deaths	
	ATb deaths/Total deaths	Percentage	ATb deaths/Total deaths	Percentage
Anseranatini	0/1	0	1/2	50
Dendrocygnini	5/23	23.8	3/26	11.5
Anserini	3/14	21.4	6/16	37.5
Tadornini	1/3	33.3	0/5	0
Anatini	7/59	11.9	9/63	14.3
Somateriini	0/4	0	0/6	0
Aythini	6/11	54.6	3/16	18.8
Cairinini	5/11	45.5	5/26	19.2
Mergini	9/18	50	6/13	46.2
Oxyurini	1/1	100	1/10	10
Phoenicopteridae	0/0	0	0/3	0

Avian tuberculosis mortality was analysed by chi-square test according to sex and season during which death occurred. There was no significant difference in mortality rates according to season of death in total ($p<0.466$), or in the results when seasons are compared to each other (see **Figure 17** and in detail **Table IX in Appendix 1**). If the seasonal mortality is broken down into numbers of males and females, as shown in **Figure 18** (and in detail in **Table X in Appendix 1**), male mortality was highest in winter with 31.3%. There appeared to be a higher incidence of avian tuberculosis in females during spring and winter (23.9% and 23.5%). Results were not, however, statistically significant, neither when grouped ($p<0.195$), nor between single groupings. There were also no significant differences when deaths of male or female birds in one season are compared to those in another.

Figure 17: Number of birds dying with post mortem evidence of avian tuberculosis, according to season of death, as a proportion of the total number of birds in each group (n=330).

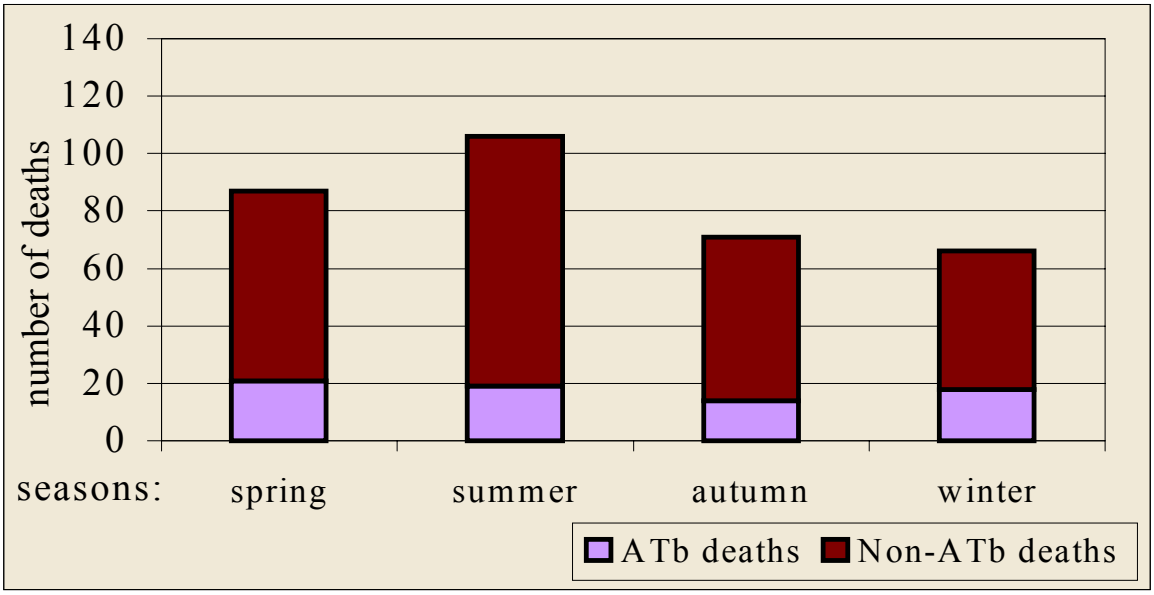
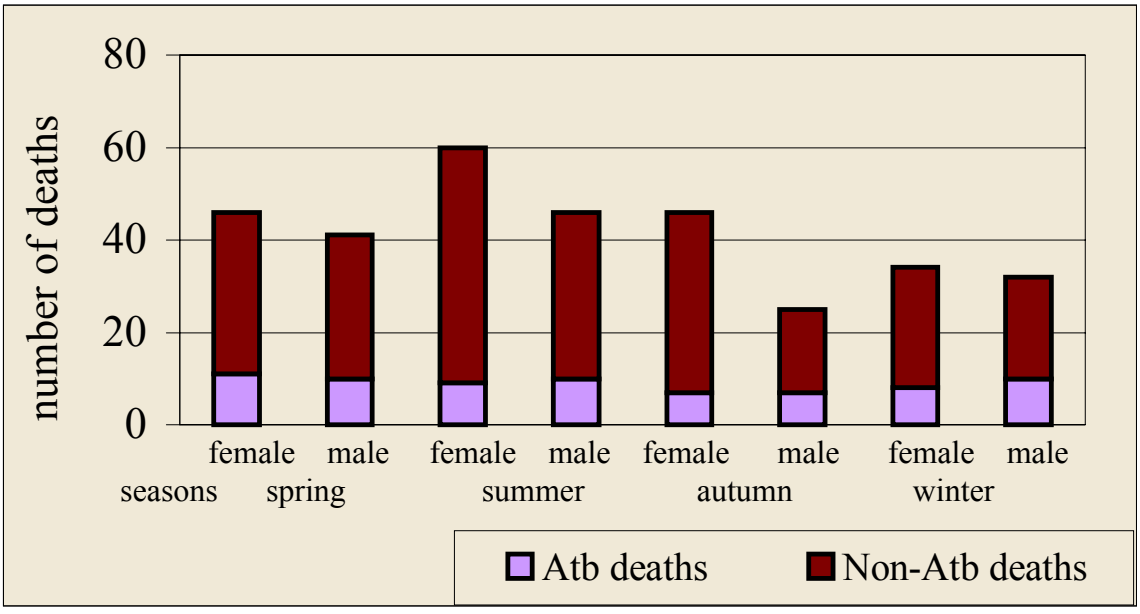


Figure 18: Number of birds dying with post mortem evidence of avian tuberculosis grouped by sex and season, as a proportion of the total number of birds in each group (n=330).



E. Discussion

1. Evaluation of the enzyme-linked immunosorbent assay (ELISA)

1.1. Antigens used in the ELISA

The use of a broad-spectrum polyclonal assay is advisable when screening a whole collection of captive wildfowl such as at the WWT centre at Llanelli. Highly specific monoclonal assays would increase specificity, but may restrict sensitivity. In this study five antigens from mycobacterial species were used, all of which are considered to correlate with the pathogenicity of these mycobacteria for birds. A bird is classified as positive for avian tuberculosis when at least one of the absorbency values is above the cut-off point of 50% for one of the five antigens. The simple average of the absorbency values of all five antigens was considered inappropriate, as it would consist of five independent tests. In another study (PAINTER, 1995), Kendall's Coefficient of Concordance is used to rank results of tests, which do not distinguish between antigens. As there is consistency in the results of all antigens in that study, only one or two antigens are considered in interpreting the results. However, the antigens used in the current study represent two serotypes of *Mycobacterium avium* (serotype 1 and 3), which were both previously isolated from birds in the WWT centres at Llanelli and Slimbridge (CROMIE et. al., 1993a and PAINTER, 1997). The use of a statistical test, such as the Kendall's Coefficient of Concordance, does not take into account the fact that individuals can react differently to single antigens. If, for example, there are two individuals, one with a low antibody titre for all five antigens and the second with a low antibody titre to four antigens but a high antibody titre to the fifth, those two individuals would be considered equal in the test due to a high consistency of results. In fact, the second individual should be considered positive for *Mycobacterium avium* infection using the assay described within this thesis, as there is a high antibody titre to one antigen, possibly representing infection with *Mycobacterium avium*. The aim of this screening programme is to identify birds infected by *Mycobacterium avium* without specifying the causative specific serotype.

1.2. Sensitivity and specificity of the ELISA

Sensitivity represents the fraction of subjects with disease who have a positive test result, while specificity represents the fraction of subjects without disease who have a negative test result. If the cut-off point is changed to a higher upper limiting value, e.g. from 20-50% to 20-60% or 70% the sensitivity of the ELISA would be improved, but there would be a loss of specificity. Likewise, if the cut-off point is lowered from 20-50% to e.g. 10-50%, the specificity would be increased at the expense of the sensitivity. The ideal specificity of any assay depends on the reasons for testing. Screening requirements may be best served by broad-spectrum polyclonal assays, whereas highly specific monoclonal assays may be preferable for confirmation of disease in an individual, prognosis or for monitoring drug therapy. In screening tests for collections, diagnostic accuracy is considered acceptable if it attains a sensitivity and specificity of approximately 70% (CROMIE, personal communication; SPANGLER et al., 1992). It is on this basis that the cut-off point in this study was determined.

The evaluation of sensitivity and specificity was based on results of the first ELISA and available post mortem data from birds tested that died during the period of this study. As shown in **Tables 16 and 17** and **Figure 8** (**Results, 3.2. Validation of sensitivity and specificity of the ELISA**), the sensitivity of the ELISA in this study was 76.9%, and the specificity reaches 55.6%, i.e. 44.4% of the results are false positive, and 23.1% are false negative. The difference between those groups is demonstrated statistically. Chi-square tests comparing the number of false positive and false negative results, according to tribe, showed no significant difference. The number of birds in each tribe is very small, and analysis of false positive and false negative results must be considered in this light. **Table 18** (**Results, 3.2. Validation of sensitivity and specificity of the ELISA**) presents the number of birds tested positive or negative for avian tuberculosis by ELISA. There appeared to be a high number of false positive in the *Dendrcygnini* (tribe 2) (50%). These findings were in contrast to other studies in which *Dendrocygnini* were found to be very susceptible to avian tuberculosis, but generally showed very low absorbency values, with a high number of false negative results (CROMIE, 1991; CROMIE et al, 2000). The *Anserini* (tribe 3) demonstrated a very high number of false positive (71.4%) and false negative results (50%). This was

found contradictory to earlier work,in which the *Anserini* gave generally very reliable results (CROMIE et al., 1993a). The *Somateriini* show a high number of false positive results (50%) and the *Oxyurini* a high number of false negative results (50%). Thus, ELISA results in those tribes need to be interpreted with caution.

There are several explanations for the occurrence of false positive results, some of which are discussed below. Non-specific binding of antibodies, indicative of infection other than mycobacteria which did not show pathological-anatomical lesions on gross post mortem, may yield false positive results. However, one of the four mycobacterial antigen groups (group i) is common to all species of mycobacteria (GRANGE et al., 1980) and so, in using a complex (polyclonal) ELISA, antibodies to other mycobacteria may cross react with *Mycobacterium avium* antigens in the ELISA. The use of specific antigens isolated from tuberculous birds from the WWT centre at Llanelli, is an attempt to overcome this problem. However, the use of crude sonicated antigens as in the current study reduces specificity. The WWT centre at Llanelli is a relatively young site compared to the WWT centre at Slimbridge, for example, where wildfowl has been kept for over 50 years and avian tuberculosis has been endemic for 30 years, and so, it can be assumed that the birds in the WWT centre at Llanelli have less contact with mycobacterial antigens. Decreased exposure to antigenic stimulation may lead to an overall slower development of immunological tolerance, and some individuals may lag in a sensitisation phase, with a high level of universal mycobacterial antibodies. Birds in a more highly contaminated environment, which experience more antigenic stimulation, can be expected to emerge from this sensitisation phase relatively early in life (BROWN, unpublished data). There were no antigens from saprophytic environmental mycobacteria included in the ELISA of this study due to poor antigen-antibody reactions in the preliminary experiments. Nevertheless, it cannot be excluded that sensitisation to those saprophytic environmental mycobacteria may be responsible for false positive results. Some authors believe that positive results caused by non-specific binding of antibodies indicate birds which are in early stages of infection with avian tuberculosis (FORBES et al., 1993).

As discussed below in more detail, the humoral response occurs relatively late in mycobacterial infections and increases with time, while the cell-mediated response decreases (HINES, 1995). Late in the course of tuberculous infection, some birds may reach a state of anergy. This brings about a reduced humoral response, and low antibody titres in the ELISA

(ASH and CROMIE, 1998; CROMIE, 2000). In 1967, GREY described the considerable variation in antibody content of serum, due to the immunisation status and/or the species of duck involved. Furthermore, a rabbit anti-duck antibody conjugated to HRP is used in the ELISA to test birds from various tribes. Differences in the structure of the primary and secondary antibodies may account for false negative results caused by poor or unreliable binding.

The antigens used in the ELISA influence specificity and sensitivity. Secreted antigens are produced by actively proliferating mycobacteria and are, therefore, potentially indicative for an active infection. Secreted antigens may also be more species specific than sonicated antigens which are produced by a breakdown of whole bacteria (PAINTER, 1995). Antigens containing mainly cell wall proteins have been shown to yield more specific results than, for example, sonicated antigens containing cytoplasmic antigens, which have a low specificity (HAAGSMA and EGER, 1990). CROMIE et al. demonstrated in 1993(c) that the use of sonicated *Mycobacterium avium* antigens yielded the best correlation between ELISA results and the presence of disease, while response to the secreted antigen was less conclusive. In the current study five sonicated antigens of *Mycobacterium avium* serotype 1 and 3 were used (see **Methods and Materials, 4.3. Antigens used in the ELISA**). *Mycobacterium avium* serotype 3 was found at the WWT centre at Llanelli Centre. Further research is required to improve the production of more specific and appropriate secreted and sonicated antigens, but it is still advisable to use antigens from those strains of bacteria isolated from the collection in question.

For statistical analysis and determination of sensitivity and specificity, birds with negative and borderline ELISA results were partly grouped. This is necessary because of the small number of birds in each group. This procedure is justified as from a control and management point of view, only birds with positive ELISA results are considered for culling. Some difficulty lies in the interpretation of borderline results. Borderline status may represent a bird in the early stages of infection. Such a bird may or may not develop progressive disease. Those that do not become diseased may retain ‘persistent’ bacilli, i.e. a low-level latent infection, which may be reactivated, particularly if the bird experiences physiological stress. This polyclonal B-cell activation is a recognised phenomenon in cases of *Mycobacterium tuberculosis* infection in man and in other diseases in which there is a marked increase in

auto-antibody production (FORBES et al., 1993). 'Borderline data' were obtained from birds that died in the period of the study. As seen in **Table 16** (**Results, 3.2. Validation of sensitivity and specificity of the ELISA**), the data appeared to suggest that 40.7% of the birds with borderline results were non-tuberculous on gross post mortem. Polyclonal B-cell activation, however, is not unique to infection with mycobacteria, and so, some or all of these birds likely contracted a non-mycobacterial infection. Although avian tuberculosis could not be diagnosed in those 'borderline' birds on gross post mortem, removal from the collection of birds showing immunological activity on the ELISA may have some merit on the basis of animal welfare and management of the collection.

1.3. Gross post mortem findings referred to histopathology and PCR results as basis of the determination of the cut-off point

The cut-off point was determined by evaluating the ELISA results in light of the results of sera from birds known to be positive or negative for avian tuberculosis. The positive or negative tuberculous status of these birds was determined by gross post mortem examination.
However, gross post mortem examination is not a gold standard diagnostic test for avian tuberculosis. A previous study (CROMIE et. al., 1993c) compared diagnostic techniques such as haematology, ELISA and agglutination with the results of findings at necropsy. A Ziehl-Neelsen stained smear from the spleen of one bird revealed a small number of acid-fast organisms although no mycobacteria were isolated on culture. In the case of another bird, which had negative results in all other diagnostic tests performed, and had no gross pathological changes at necropsy, *Mycobacterium avium* was isolated. It is recommended that a complete post mortem examination be performed, including microscopical examination with Ziehl-Neelsen staining, and ideally culture of organisms to identify the strain. In a study of avian tuberculosis in the WWT centre at Slimbridge, *Mycobacterium avium* was isolated from nearly 50% (10 of 21 birds) of birds, which based on gross post mortem were considered negative for avian tuberculosis at necropsy (SCHÄFER et al., 1973).
These findings suggest that birds infected with *Mycobacterium avium* do not necessarily develop grossly visible signs of disease. It remains to be determined whether birds which have not yet developed disease that can be confirmed on gross post mortem do excrete

Mycobacterium avium, thereby spreading it in a collection. Post mortem examinations at the WWT centres have been carried out by the same investigator (Martin Brown, animal health officer at the WWT centre at Slimbridge) for over twenty years. Therefore, a high degree of consistency in the post mortem technique is to be expected. For 15 years after the first diagnosis of avian tuberculosis at the WWT centres, histopathological examination, including Ziehl-Neelsen staining, and identification of the organism by culture and isolation were conducted. There was a high degree of correlation between laboratory results and those of gross post mortems alone. For this, and financial reasons, it was decided to renounce those additional examinations in routine procedures. Those examinations are still carried out if avian tuberculosis may be suspected due to indefinite post mortem findings (CROMIE and BROWN, personal communication).

During this study, tissue samples from 19 birds were obtained in the post mortem examination to confirm the diagnosis by histopathological examination, including Ziehl-Neelsen staining. Tissue samples were taken from liver and spleen as those are the organs which are most often involved in tuberculous processes (liver in 95% and spleen in 90%) (SCHULZ, 1992). As shown in **Table 12 to 15** (**Results, 3.1. Post mortem and histopathological examination**), there were no false negative results and 27.8% false positive results (five out of 18 birds). Chi-square test comparing the number of false positive and false negative results confirmed no statistical difference. As nearly all of the stainings showed similar (negative) results and there were 27.8% of false positive results when comparing post mortem to histopathological examination, a problem in processing of the section and/or in staining cannot be excluded. Acid-fast staining of granulomatous tissues caused by *Mycobacterium avium* typically reveals large numbers of acid-fast bacilli in contrast to other *Mycobacterium* spp. such as *Mycobacterium bovis* and *Mycobacterium tuberculosis*, in which organisms are rare within tubercles (VAN DER HEYDEN, 1997a). In the current study acid-fast clearly rod-shaped mycobacteria, were found in one out of 19 tissue samples (White-faced Whistling Duck, female, seven years old, ring number LC0316) when stained by Ziehl-Neelsen staining (see **Table 12 and 14, Results, 3.1. Post mortem and histopathological examination**). Ziehl-Neelsen staining is considered as very accurate in detecting mycobacteria, nevertheless, several factors can influence the results. Acid-fast bacteria do not take up the dye accurately when the sections are too thick or when they have

not been heated enough during the initial process of staining (CROMIE, personal communication). Therefore, in lack of the presence of obvious red acid-fast rod-shaped bacilli, tissue samples are diagnosed as negative for avian tuberculosis, leading to questionable results. It is also possible that in the progressed stage of mycobacterial infections the acid-fast bacteria are demarcated and melted in the centre of the granulomas. This phenomenon may be caused by the relatively short primary stage of the tuberculous cycle due to the lack of well-developed lymph nodes in birds. Particularly in older birds, as in those cases (age range from seven to eleven years), with advanced disease granuloma formation is then the only finding on histopathological examination. In those cases Ziehl-Neelsen staining may fail to detect mycobacteria. Some reports confirm that birds with more advanced disease had the more typical granulomas that contained often extensive amounts of caseous necrosis. Fewer intact mycobacterial organisms were evident in acid-fast stains of those caseous 'older' lesions as in the extensively necrotic 'earlier' granulomas (MONTALI et al., 1976; THOEN et al., 1977a). *Mycobacterium avium* tends to be partly pleomorphic with a very small, almost coccoid-shaped appearance on Ziehl-Neelsen staining (ARANAZ et.al., 1997).

On the other hand it is well documented that a diagnosis based only on gross post mortem is not without doubt. Granuloma formation in liver, spleen and multiple other organs similar to those caused by mycobacteria can occur due to an infection with *Escherichia coli*, *Salmonella typhimurium*, or *Yersinia pseudotuberculosis* (LUMEIJ, 1994). Granulomas caused by *Escherichia coli* can easily be mistaken for mycobacterial granulomas on histopathological examination employing haematoxylin-eosin staining (PALLASKE, 1969). Further differential diagnoses with granuloma formation are typhlohepatitis and leucosis (KÖHLER, 1991). Necrotic lesions in the liver are found on necropsy in cases of atoxoplasmosis, aflatoxicosis, haemocromatosis and Chlamydia infection (LUMEIJ, 1994).

An accurate diagnosis would only be possible by culturing suspicious organ specimens followed by DNA sensitive diagnostic techniques such as PCR, in which remaining fragments of mycobacterial DNA in the granulomas are detectable. In a 1997 study PCR was performed on samples of routiney-fixed, paraffin-embedded tissues from 29 humans which showed symptoms suspicious for tuberculosis. Histopathological features such as necrotising granulomas were found in the specimens, but Ziehl-Neelsen staining did not show any

mycobacteria in those samples. PCR enabled the detection of mycobacterial DNA in 13% of those specimens (GOLDMANN et al., 1998).
In the current study PCR did not confirm the presence of any mycobacterial DNA in any of the 13 of the tissue samples which were also examined by histopathology, therefore, confirming histopathological findings rather than gross post mortem findings. Performing PCR on specimens after having been processed for routine histopathology can not be considered ideal. PCR is more reliable for frozen tissue samples (RICHTER, personal communication) due to a slight degradation which is commonly found in DNA prepared from paraffin-embedded tissues (RICHTER et al., 1995). Possible reasons for fragmentation of DNA during PCR are the rigid cell wall of mycobacteria, which cause difficulties to access the DNA and the process of formalin-fixation, paraffin-embedding and extraction itself (GOLDMANN et al., 1998).

1.3.1. Determination of the cut-off point

Specification of an absorbency value cut-off point allows assessment of positive and negative results. In a direct sandwich ELISA, as performed in this study, samples which have an absorbency value above the cut-off point are positive and those below the cut-off point are negative. The results were expressed as a percentage of the difference between the positive and negative controls, after subtraction of background responses (responses from those wells containing coating buffer, but no antigen). The results were categorised as follows:

Avian tuberculosis negative:	values < 20% of the adjusted positive control
Borderline:	values of 20-50% of the adjusted positive control
Avian tuberculosis positive:	values > 50% of the adjusted positive control

To evaluate the amount of primary binding from conjugated anti-antibody to mycobacterial antigens and the plastic mircrotitre plates, preliminary experiments were necessary. Therefore, an ELISA was run with four microtitre plates to which no sera were added apart from the positive and negative control. In the experiment a non-specific binding of 3-5% was shown (see **Results, 1. Preliminary investigations**). This degree of non-specific binding can

be expected in most ELISAs and so the cut-off point needed to be raised by 5%. Although the anti-antibodies were purified, other components may still be present as the purified anti-duck antibodies are raised in a rabbit, with complete Freund's adjuvant. Complete Freund's adjuvant uses contains mycobacteria as an immunogen. Those could react with the mycobacterial antigens and so influence the absorbency values and falsify the ELISA results.

1.4. Conjugate used in the ELISA

In this study, HRP was conjugated to an anti-duck antibody. This conjugate was commercially available in this form, facilitating its use in screening programmes intended for large collections. Development of specific conjugated antibody for each species in question is prohibitively time consuming and expensive. The disadvantage is that many species of wildfowl ranging from various ducks and geese to swans are tested by the same anti-duck antibody conjugate. This is a known problem when using an ELISA for screening collections with multiple species. The advantages and disadvantages, as found in the literature, are discussed below.

In a study using anti-duck antibody, the ELISA was considered unsuitable for other avian genera. Wildfowl are a diverse and -from the evolutionary point of view- primitive group of birds, but there are still a number of anomalies in the ELISA reactions which may be ascribed to low binding to the anti-duck antibody (CROMIE et al., 2000). Another ELISA study used goat anti-chicken immunoglobulin G horseradish peroxidase conjugate when testing chicken serum, and a goat anti-turkey immunoglobulin G horseradish peroxidase conjugate when testing quail serum. Although sensitivity and specificity in this study were acceptable, the extent of cross-reactivity among immunoglobulins G of diverse avian species are thought to determine the usefulness of ELISAs for diagnosis of *Mycobacterium avium* infection in birds (CLARK et al., 1995). In a 1998 study, ASH and CROMIE found a notable variation in test reliability between tribes. There were discrepancies between ELISA and post mortem results in the *Dendroygnini* and *Cairinini*. They suggest that results could be improved by the use of more specific secondary antibodies or of staphylococcal Protein A. Protein A, produced by certain strains of *Staphylococcus aureus*, binds to the Fc portion of immunoglobulins, chiefly

immunoglobulin G, of several mammalian species. Studies were conducted to explore the use of protein A conjugated with horseradish peroxidase, in ELISAs involving sera from different species of animals. The conclusions support the use of enzyme-labelled protein A as a general-purpose reagent capable of detecting antibodies in tuberculous exotic animals (THOEN et al., 1980). Since zoo animal anti-species immunoglobulins G were not available, protein A and protein G were used in a 1990 study. A screening programme for tuberculosis using this modified ELISA was considered successful when applied to several zoo and wild animal species (HAAGSMA and EGER, 1990).

As presented below, there are many studies, which support the transferability of tests detecting antibodies from one avian species to another. An indirect antigen-capture ELISA was used for the diagnosis of type C botulism in wild birds. The presence of antibody was confirmed by membrane immunoassay with enzyme-labelled goat anti-chicken IgG. The test was found to be sensitive and specific in 236 individuals from 50 avian species, representing nine orders (ROCKE et al., 1998). In another study TORO et al. (1996) demonstrated the successful binding of human C1q to antigen-antibody complexes of two avian species, using a sandwich immunoflourescence complement fixation test (virus in cells + decomplemented avian antiserum + human-C1q + goat anti-human-C1q + fluorescein isothiocyanate rabbit anti-goat-IgG). Since the avian species tested belonged to different and phylogenetically distant avian orders, it is likely that antibodies of other avian species will also bind to human C1q. Consequently, the sandwich immunoflourescence complement fixation test is considered to be a promising method for serological surveys in wild birds (TORO et al., 1996).

However, in a further study, an indirect ELISA using rabbit anti-duck antibodies conjugated to horseradish peroxidase, rabbit anti-duck and rabbit anti-chicken monoclonal antibodies were tested and compared to an ELISA performed with protein A, also conjugated to peroxidase. It was shown that protein A is unlikely to be a satisfactory conjugate in the development of an avian tuberculosis test due to its weak binding to immunoglobulins from Galliformes and Guiformes. ELISAs using anti-sera were approximately 75% accurate, and it was shown in this study on comparative binding capacities that this anti-duck serum may be used for any bird within the order Anseriformes, and possibly in other closely related orders. Although there was a poor binding reported within the *Dendrocygnini* (AIKEN, 1999).

To interpret negative, borderline and positive results it is important to consider the susceptibility to avian tuberculosis of the bird species. Data from earlier WWT studies are available to assess the susceptibility of individuals of different tribes. The use of non-specific anti-antibodies is not ideal, and may produce false positive and false negative results. The reliability of the ELISA for birds of the different tribes in this study is discussed in conjunction with the validation of specificity and sensitivity in **section 1.2. (Sensitivity and specificity of the ELISA)** of this chapter.

1.5. Antibody related reactions in the ELISA

In this study, samples from multiple birds were tested either twice or three times by the ELISA. Although the technique has been shown to be reliable, sensitive and specific (FORBES et. al. 1993), the repeated testing revealed inconsistency in the ELISA results in this study (see **Results, 2. ELISA results**). It was statistically proven that the results of the second ELISA had higher absorbency values than those of the first ELISA, as is demonstrated in **Figures 5 and 6** (**Results, 2. ELISA results**) (and in more detail in **Tables I and II in Appendix 1**). When the ELISA results were examined in light of gross post mortem data, each ELISA series had a different sensitivity and specificity, as shown in **Tables 11a to 11d** (**Results, 3. Post mortem examination and comparison of post mortem findings with the ELISA results**). The first ELISA showed a sensitivity of 62.5% and a specificity of 93.3%. Sensitivity of the second ELISA was 37.5% and its specificity was 44.4%. The combination of the first and the second ELISA demonstrated a sensitivity of 61.5% and a specificity of 68.4%. The third ELISA had a sensitivity of 33.3% and a specificity of 84.5%. For the evaluation of the ELISA in general, the results of the first ELISA were combined with the results of the 19 birds, which were culled for gross post mortem and histopathological examination. A sensitivity of 76.9% and a specificity of 55.6% were obtained (see **Table 17, Results, 3.2 Validation of sensitivity and specificity of the ELISA**).

A change in the reaction conditions such as alteration of any of the reagents or a change in environmental conditions is unlikely to account for this difference as all the sera samples

were tested under the same environmental conditions, with the same reagents and the same antigens and positive and negative controls. A number of serum samples from the first and second catches were tested in the same assay, and in some cases on the same microtitre plate. All the antigens used were produced especially for this study, and so were not subjected to multiple freeze-thaw cycles, which can initiate precipitate development. The only difference in the handling of the first and second serum samples is that samples from the first catch were frozen at -20°C for eleven months prior to processing, while the samples from the second catch were frozen for four months. Previous work has shown, however, that freezing time differences of this length did not influence the response of the antibodies to the antigens (CROMIE, personal communication).

Differences may possibly be explained by seasonal variations in antibody levels. Summer and autumn represent a time of high stress and energy expenditure due to the demands of breeding and moulting. This manifests as a loss of body condition and a possible general depression of the avian immune system. Birds with a compromised immune system are susceptible to infections, and so high titres of antibodies may be expected in the blood. Non-specific antibodies to a variety of antigens, or antibodies to common mycobacterial antigens may be present.

Marshes, ponds, surface waters estuaries and soil, particularly acidic soil that is marshy or subject to periodic flooding, are habitats of mycobacteria (GRANGE, et. al., 1990). This is also the nature of the physical environment of the WWT centre at Llanelli (see **Results, 4. Physical environment and husbandry details**). In summer the water level is often low, and it is possible that there are high levels on environmental mycobacteria in the grounds, which may prompt an antibody response in the birds. The presence of such a response may lead to cross-reactions and false positive ELISA results. Mycobacterial antigens have been classified into four groups, of which one (group i) is common to all species of mycobacteria (STANFORD and GRANGE, 1974). Antibodies to other mycobacteria may, therefore, cross react with *Mycobacterium avium* antigens used in the ELISA.

Further studies are necessary to investigate the changes in the antibody levels in birds with chronic disease such as avian tuberculosis and research involving serial testing throughout in the year for at least two consecutive years is essential to elucidate possible seasonal changes

in antibody levels. Some work in this area was initiated by CROMIE in 1991, in connection with vaccination trials.

The ELISA measures antibodies constituted by the humoral response of the individual. The quantity of antibodies is expressed as an absorbency value. In virtually all mycobacterial infections, cell-mediated immunity is of paramount significanse initially, with a relatively late humoral response. Typically, as the disease progresses, the humoral response increases as cell-mediated immunity decreases (HINES, 1995). Late in the humoral response in wildfowl 5.7S IgG or IgY(ΔFc) predominate. It is known that duck serum immunoglobulins are considered relatively inefficient at secondary function, such as complement fixation, agglutination, precipitation and tissue sensitisation (HIGGINS and WARR, 1993; ZIMMERMANN et al., 1971). This may explain the weak protective nature of these serum immunoglobulins. Similar research concerning swan or geese antibodies has not yet been undertaken. During the late tuberculous infection a state of anergy in the bird may also yield false negative results due to the reduced humoral immune response to the mycobacterial antigens (ASH and CROMIE, 1998; CROMIE et al., 2000). Studies have confirmed that the relative quantities of the immunoglobulins in serum vary considerably, depending on immunisation status, as well as on the species of duck (GREY, 1967). This may also explain the decrease in antibody levels seen in same birds in the repeated tests. Further reasons for false negative results are discussed in **section 1.2.** (**Sensitivity and specificity of the ELISA**) of this chapter.

Vaccination has been used in several centres of the WWT, including the centre at Llanelli, in an attempt to control avian tuberculosis. There is no significant rise in antibody levels in the birds vaccinated compared to the control group (CROMIE, 1991). A controlled trial of the vaccination carried out in the WWT centre at Slimbridge assessed the degree of protection afforded by the vaccine and the vaccine gave protection to only one of the taxonomic groups of birds in trials (BROWN, 1995, unpublished data). The efficiency of the vaccine varies depending on species or tribe and age of the birds vaccinated. As the birds are vaccinated at a very young age, there is probably little effect on antibody levels. But further research is needed. Further research is being conducted currently to determine the optimal dose rates and timing of vaccination (CROMIE et al., 2000).

1.6. Evaluation of the ELISA as diagnostic tool in a screening programme

The analysis of the ELISA results to evaluate sensitivity and specificity was based on gross post mortem examination. Also from those 19 birds, which were included in histopathological examination, only the findings on necropsy were taken into account, to remain consistent with the other findings. As pointed out above, there was only little agreement between gross post mortem and histopathological findings with regard to avian tuberculosis. Therefore, the evaluation of the ELISA based only on post mortem findings as gold standard has to be considered very critically. Further research, for example identifying species-specific mycobacterial DNA fragments by molecular biological techniques such as PCR, preferably on frozen specimens, is necessary to obtain a final diagnosis. Analysing the ELISA results by referring to such highly precise diagnostic techniques may reveal a somewhat different sensitivity and specificity than the ones obtained in this study.

The chronic nature of avian tuberculosis, with its protracted and complex immunological responses to the mycobacterial infection, makes reliable *ante-mortem* diagnosis notoriously difficult. The results of screening by ELISA showed a poor correlation with the post mortem data. With a sensitivity of 76.9% and a specificity of 55.6% the test cannot be considered acceptable. Nevertheless, culling 55.6**%** (100% minus percent of false positive birds) of the birds which are tested positive for avian tuberculosis by the ELISA, would significantly reduce the amount of environmental *Mycobacterium avium* contamination. This would ultimately reduce the risk of infection to birds of the collection and wild birds.

Further research might help to identify other factors indicative of infection with *Mycobacterium avium*, which in conjunction with the ELISA would improve diagnosis. One possibility is to correlate weight loss with the ELISA results. Body condition in this study is assessed by evaluating the development of the pectoralis muscles, the subcutaneous fat and the intra-abdominal fat stores. Body condition score is expressed on a scale from 1 to 4, with 1 being the thinnest and 4 the fattest. In animal ecology research it is common to use the residuals from an ordinary least squares linear regression of body mass against a linear measure of size as an index of body condition. Indices computed merely from measures of body mass and size do not correlate well with true physiological condition or measures of fat

and other energy stores (GREEN, 2000). Weight is not a suitable measure of body size because it can vary within physiological limits, therefore only skeletal variables should be used to determine size. The combination of several external measurements, however, may explain only 40-60% of the total variance in overall body size (FREEMAN and JACKSON, 1990). It is implicit that a measure of body size should be comparable across all species being examined, however avian species show considerable range in size and shape, an univariate measure of body size should not be used as a body size variable in interspecies comparisons (BARTON and HOUSTON, 1994).

In this study the body condition of the birds did not indicate avian tuberculosis status. There was no correlation between a poor body condition and evidence of avian tuberculosis on post mortem examination. Similar findings were found in a 1986 study, in which 68% of chickens infected by avian tuberculosis, had a good to very good body condition (GÖTZ et. al., 1986). A survey comparing different techniques for diagnosis of avian tuberculosis on post mortem examination involved 14 birds showing gross signs of avian tuberculosis and seven birds which had no gross pathological changes at necropsy, found all but one tuberculous bird in good clinical condition (CROMIE et. al., 1993c). There are further reports about pets birds being able to maintain good body condition until avian tuberculosis becomes disseminated (VAN DER HEYDEN, 1997a).

Several studies have examined changes in the blood profile that occur with avian tuberculosis. Clinical biochemistry is often of little diagnostic help. Tissue enzymes and hepatic function (bile acids) values may be abnormal, particularly in birds with marked hepatomegaly. The levels, however, are rarely as high as those seen with the same degree of hepatomegaly in the course of to viral, chlamydial and other bacterial diseases that typically cause more hepatic necrosis than do mycobacterial infections (MONTALI et al., 1976; VAN DER HEYDEN, 1986; FORBES et al., 1993). Diagnostic tests usually reveal extremely high white blood cell counts, characterised by a monocytosis and a heterophilia. Hyperfibrinogenaemia and microcytic, hypochromic anaemia are often present (MONTALI et al., 1976; HAWKEY et al., 1990; FORBES et al., 1993). BUSH et al. (1978) reported that in quail a case was considered to be positive when the white blood cell count reached 18 x 10^9/l.

Although leucocytosis, heterophilia, hyperfibrinogenaemia and a hypochromic microcytic anaemia are non-specific reactions to chronic infectious and inflammatory conditions in birds, haematology could be used in conjunction with the ELISA in a screening programme. Basic haematologic tests consisting of haematocrit, fibrinogen, white blood cell count and possibly differential blood cell count would be sufficient. The blood sample for haematology could be obtained at the time of sampling for the ELISA, and the tests themselves are not technically demanding. Further research may show the combination of haematology and ELISA to have improved sensitivity and specificity over either test alone, making the combination a useful screening tool in the control of avian tuberculosis in collections.

2. Discussion of the post mortem results from 1989 to 1999

This analysis was based on data from post mortem examinations of birds that were found dead within the grounds. The advantages and disadvantages of gross post mortem examinations are discussed in **section 1.3.** (**Gross post mortem findings referred to histopathology and PCR results as basis of the determination of the cut-off point**) of this chapter. A certain number of bodies will not have been found, due to the dense cover in many pens, and the tendency of diseased birds to hide. It is to be expected that the greater proportion of undiscovered bodies will be those of the smaller, more numerous birds.

2.1. Annual incidence of avian tuberculosis, from 1989 to 1999

As seen in **Figures 10 and 11** (**Results, 5.1. Incidence of avian tuberculosis over a ten year study period**) (and in detail in **Table V in Appendix 1**), there was a notable increase in the overall number of deaths due to avian tuberculosis in the years 1994 to 1999 with highest incidence of over 40% in the last two years. Analysis of the annual incidence in each tribe revealed the same marked increase in 1998 and 1999.

Several factors might have influenced these figures. The WWT centre at Llanelli was founded in 1989, with a population of juvenile birds and no adult birds were introduced to the collection since it was founded with the exception of the flamingos. Over the last ten years many of the birds have reached the end of their normal life span. An immune system compromised by age, increases susceptibility to infectious disease. In addition to age, the immune system is influenced by other stressors. A high stocking density increases the likelihood of exposure to contaminated material and increases the level of social stress experienced by the birds. A study in 1989 showed that social stress due to high stocking density affected both the extent and nature of avian tuberculosis lesions in chickens (GROSS et al., 1989). Since a study of stocking density in the WWT centre at Llanelli in 1995 (PAINTER) there has been a distinct decrease from approximately 1100 to approximately 800 birds on the entire grounds at the time of this current study. The overall number of birds, particularly the number of geese, has been reduced by transferring birds to other centres and

by a restricted breeding programme. Visitor numbers have increased in recent years, and although there is cover in the pens, a high number of visitors represents a certain amount of disturbance and stress to the birds (EDWARDS, personal communication).

Avian tuberculosis is a disease with a long incubation period, seen mainly in birds over two years of age or older. Some species, like the White-winged Wood Duck, do not breed until they are two or three years old, and so experience a period of stress which may coincid with development of disease (CROMIE et al., 1992; CROMIE et al. 1993a). It may be that in 1998 and 1999 the extent of the problem became clear as the population in the collection aged and began to manifest clinical signs and die of avian tuberculosis after a long incubation. Infected birds excrete in their faeces mycobacteria long before they show clinical signs, allowing the degree of contamination of the environment to increase (MONTALI et al. 1976). Further research could investigate the effects of age structure of the collection on the incidence of avian tuberculosis.

If the infection rate has remained constant over the period, then the increased mortality from the disease must be due to an increase in susceptibility to the disease within the population. This can occur in small populations, with low levels of heterozygosity and an associated reduction of genetic immunity. However, there is no evidence to suggest a significant change in levels of heterozygosity in these birds over this study period. Again assuming that the infection rate remains constant, increased contamination of the environment will lead to increased mortality from disease. The greater the environmental contamination, the greater is the risk of infection. That is, with time more birds were exposed to the organism. The pattern in numbers of deaths due to avian tuberculosis from year to year points to the eventual attainment of a plateau in the future.

As demonstrated in **Table 19** (**Results, 5.1. Incidence of avian tuberculosis over a ten year study**), the overall incidence of avian tuberculosis was doubled from the period from 1994 to 1997 (21.3%) to the period from 1998 to 1999 (42.4%). It is worth noting that in 1998 the mud on the bottom of a number of ponds was removed. During this process, mud and soil were disturbed and spread onto the shore and on the adjacent vegetation. There was no complete water exchange but the ponds were filled up to level with water from the river,

which is stored in a common tank (EDWARDS and WALTERS, personal communication). The advantages of cleaning the ponds are obvious, but at the same time mycobacteria which are frequently isolated from mud and soil (GRUFT et al., 1979), may be brought to the surface.

2.2. Incidence of avian tuberculosis according to sex and season

As demonstrated in **Figure 15** (**Results, 5.4 Analysis of the data according to sex and season**) (and in detail in **Table VIII in Appendix 1**) and in **Table 21**, this study found no sex predilection for avian tuberculosis. Other surveys, also involving collections, report similar findings (MONTALI et al., 1976; CROMIE et al, 1991; PAINTER, 1995). A study, which involved only White-winged Wood Ducks, also found no difference between the females and males affected by avian tuberculosis (CROMIE et al., 1992).

The investigations presented herein found no statistically demonstrable differences in the number of deaths due to avian tuberculosis depending on the different seasons, or in the numbers of males and females dying in each season (see **Figures 13 and 14, Results, 5.4 Analysis of the data according to sex and season**, and in more detail **Tables IX and X in Appendix 1**). These findings do not agree with those of other surveys. In 1991 CROMIE et al. found the greatest percentage of avian tuberculosis deaths in winter and summer. In summer, a significantly higher mortality among females was observed. In summer females lose body condition due to the stress and energy expenditure of breeding and the summer moult. In addition to egg laying, the incubation and brooding are often carried out entirely by the females. During incubation, females leave the nest less often to feed and drink. Long and hot summers exacerbate the situation, increasing the likelihood of dehydration (CROMIE et al., 1991). The highest percentage of male mortality was in the winter. The increased winter mortality due to avian tuberculosis may be brought on by increased physiological stress during cold spells; cold weather has been shown to be particularly stressful to those birds which are native to hot climates (CROMIE et al., 1991; CROMIE et al., 1992). Cold weather has a greater effect on smaller and medium sized birds (BEER, 1964, cited by CROMIE, 1991). Some studies also show a seasonal variations with low body weights and body

conditions in summer and winter (OWEN, 1980; OWEN and COOK, 1977, both cited by CROMIE, 1991). Seasonality of bacterial epornitic diseases is common in captive and wild populations. Given the long incubation period of avian tuberculosis, however, death from this disease can occur throughout the year (CROMIE et al., 1991). Observation of the birds in winter reveals that birds native to warmer climates, for example the whistling ducks, search for cover in hedges or straw provided (EDWARDS, personal communication). A possible explanation for male mortality is marked territorial behaviour in spring (SOOTHILL and WHITEHEAD, 1978) which might be intensified by small pens, a small number of suitable breeding sites and a possibly high number of male birds and which leads to increased stress among male birds due to competition.

2.3. Incidence of avian tuberculosis according to taxonomic tribes

As shown in **Figure 13** (**Results, 5.3 Analysis of the data according to different tribes**) (and in detail in **Table VI in Appendix 1**), the current study found a significantly higher incidence of avian tuberculosis in the *Aythini*, the *Cairinini* and the *Mergini* than in other tribes. Several studies support this finding, but also find the *Anatini* and the *Dendrocygnini* particularly susceptible to avian tuberculosis, and the *Anserini* less so. The *Anhimidae*, *Oxyurini* and *Phoenicopteridae* show a relatively low susceptibility (CROMIE et al., 1993a; PAINTER, 1995; BROWN, personal communication). Those findings were confirmed by the current study.

In this study avian tuberculosis was not diagnosed in the *Somateriini* or flamingos, as shown in **Figure 13** (**Results, 5.3 Analysis of the data according to different tribes**) (and in detail in **Table VI in Appendix 1**). The very low incidence of disease among in the flamingos may be related to their food and feeding habits. The high quality, enriched food may also help the birds in resisting the disease and in reducing susceptibility (CROMIE, 1991).

The *Mergini* and *Somateriini* share similar life-styles and environments in the wild and in captivity. Given the notable difference in incidence of disease the latter appear to have a high level of genetic immunity to avian tuberculosis, whilst the mergansers, scoters and

goldeneyes (*Mergini*) are exceptionally susceptible to this disease (CROMIE et al., 1991). As shown in **Table 21** (**Results, 5.4 Analysis of the data according to tribes**), this study found that Hooded Merganser and Smew were also highly susceptible with incidences of avian tuberculosis of over 78% or 66%, respectively. The high incidence in the *Mergini* may indicate genetic susceptibility. As these birds evolved for partly sea life, they would rarely come into contact with *Mycobacterium avium*, and so there would not have been an evolutionary pressure towards mycobacterial immunity. It has been shown that strains of MAI complex are more abundant in waters of low salinity than in marine waters (WENDT et al., 1980). Many of these birds live and breed in very cold climates, thus encountering fewer pathogenic organisms, including mycobacteria. Birds like the *Oxyurini* have a substantial layer of subcutaneous fat, as an adaptation for long periods on the water. This may decrease the stress they experience during cold weather, thereby increasing resistance to disease (CROMIE, 1991).

The current study showed a statistically significant difference in the incidence of avian tuberculosis between the perching ducks (*Cairinini*) (27.0%) and most remaining tribes (see **Figure 13**, **Results, 5.3. Analysis of the data according to tribes**) (and in detail **Table VI in Appendix 1**). Other studies have also found a high incidence of avian tuberculosis in adult *Cairinini* in the WWT centre at Slimbridge (CROMIE et al., 1991). If the high incidence of the disease in this group is a reflection of increased genetic susceptibility, it may be due to the fact that as perching ducks they would normally have less contact with the ground, and therefore with mycobacteria, than would other wildfowl groups. Mycobacterial immunity may have exerted less selective influence in the evolution of arboral species than in other species (CROMIE et al., 1992). Additionally, in captivity the birds are pinioned and therefore brought into for more frequent contact with the ground, where they encounter environmental mycobacteria and both their own and other birds' potentially infected droppings (CROMIE et al., 1991). The relatively low incidence that was observed in this current study in the WWT centre at Llanelli compared to the survey on the WWT centre at Slimbridge might be explained by the fact that species belonging to the order *Cairinini* are living in different pens of the WWT centre at Llanelli that are less muddy in winter and after heavy rain compared to the pens in the WWT centre at Slimbridge, which indicates a better drainage and thus, a potentially smaller accumulation of mycobacteria in the grounds. As seen in **Table 21**

(**Results, 5.3. Analysis of the data according to tribes**), among the *Cairinini*, the White-winged Wood Duck showed a particularly high susceptibility to avian tuberculosis in the current study (58.3%). Similar findings were seen in studies from 1991 (CROMIE) and 1992 (CROMIE et al.). Mycobacteria thrive in the absence of the sterilising effects of ultra-violet radiation (CROMIE et al., 1992). White-winged Wood Ducks require a shaded pen to achieve breeding success. Other species will also actively seek the shelter of vegetation, and so can be expected to encounter higher concentrations of mycobacteria.

Other than environmental factors, several endogenous factors, such as phylogenetic susceptibility or loss of heterozygosity may explain the variable susceptibility seen to *Mycobacterium avium*. It is known that resistance of mice to early growth of various mycobacteria including *Mycobacterium avium*, is controlled by a single dominant autosomal gene, *Bcg* (BERMUDEZ, 1994). As inbred strains of mice exhibit greater mortality when infected with *Mycobacterium tuberculosis* than do hybrid strains, CROMIE et al. (2000) suspect that the high degree of inbreeding in the population of White-winged Wood Ducks influences their susceptibility to avian tuberculosis.

Although the results are not statistically significant, this study found a relatively high incidence of avian tuberculosis (25%) in the *Tadorinini*, as seen in **Figure 13** (**Results, 5.3. Analysis of the data according to tribes**) (and in detail in **Table VI in Appendix 1**). CROMIE also reported this disease as the primary single cause of death within this tribe (1991).

There appears to be a correlation between the incidence of avian tuberculosis and feeding habits. Birds which feed by diving and dabbling seem more at risk than birds which feed mainly by grazing (CROMIE et al., 1991). The results of this current study showed a significantly higher incidence in the *Aythini* than in the *Anatini*, the *Dendrocygnini* or the *Anserini* (see **Figure 13**, **Results, 5.3. Analysis of the data according to tribes**) (and in detail in **Table VI in Appendix 1**). CROMIE's findings in a previous study were similar (CROMIE, 1991). She noted, for example, the tendency of the *Anserini* to spend less time on potentially contaminated water. Furthermore, it is notable that some of the sea and perching ducks (*Mergini* and *Cairinini*) are fed a special diet consisting of floating pellets. Particularly

species such as Eiders, Bufflehead, Smew, Hooded Merganser and Goosander tend to feed from those pellets, which are in direct contact to the potentially contaminated water or mud and not on the more dry edges of the ponds (EDWARDS, director of the WWT centre at Llanelli, personal communication). Those species are the ones that shown a high incidence of avian tuberculosis (see **Table 21**, **Results, 5.3. Analysis of the data according to tribes**).

The *Anserini* are divided into geese and swans. Results of a study comparing the incidence of avian tuberculosis in wild Barnacle and Canada Geese and in wild Whooper Swans show a minimal impact on the population of the geese, but a comparatively high incidence among the swans. Unlike geese, which feed almost exclusively by grazing on land, swans spend a proportion of their time feeding in water, where exposure to mycobacteria can be expected to be greater (CROMIE et al., 1991; CROMIE et al., 1993b). Statistical analysis of the incidence of avian tuberculosis within species in the order *Anserini* showed no significant differences in the current study (see **Figure 13**, **Results, 5.3. Analysis of the data according to tribes**) (and in detail in **Table VI in Appendix 1**). Nevertheless, there seemed to be a higher incidence within the geese than the swans. This inconsistent finding to the study mentioned above may be explained by the fact that the current study analysed data from birds in captivity. As discussed below, captivity influences stress susceptibility, behaviour and most importantly there is an accumulation of contagious organisms due to the permanent use of the same sites by the birds. In the current study there was no reported incidence of avian tuberculosis in southern swan species (see **Figure 13**, **Results, 5.3. Analysis of the data according to tribes**) (and in detail in **Table VI in Appendix 1**). This is confirmed by a 1998 study, in which the northern swan species all showed a higher incidence of avian tuberculosis than the Black, Black-necked and Coscoroba Swans. The apparently greater susceptibility of Northern Hemisphere species may in fact be a reflection of the higher mortality among the Southern Hemisphere species at an earlier age and from causes other than avian tuberculosis. Black-necked Swans have a shorter live expectancy, relative to Bewick's and Trumpeter Swans, and so they may be less likely to die from avian tuberculosis, which as a chronic disease is more commonly found in older birds. This is supported by the fact that adult females swans that died of avian tuberculosis were significantly older than those that died of other causes (BROWN et al., 1992).

The susceptibility of a tribe to avian tuberculosis depends at least in part, on factors, which affect the immune system or behaviours, which affect exposure to mycobacteria. Some of the factors influencing susceptibility to avian tuberculosis are discussed below. Further work, looking at species susceptibility, would possibly clarify the differences in incidence of avian tuberculosis in the given tribes in the WWT centre at Llanelli.

Stress plays a role in susceptibility to *Mycobacterium avium* and in disease progression. In a 1989 study, chickens were infected with *Mycobacterium avium* and then subjected to variable levels of stress by housing them in cages with higher or lower stocking densities. It was found that the first five days after inoculation were important in determining the number of lesions and the percentage of lesions that develop necrotic centres. Both figures increased with the level of stress, suggesting that initial defence factors are suppressed by stress. The study suggests that effective defence strategies against *Mycobacterium avium* varied with the social environment of the chickens. Interaction between genetic traits and their environment may also influence the response to stress, and therefore, the response of the immune system (GROSS et al., 1989). Different species perceive and respond to stress in different ways. Among the sources of stress in captivity, birds are being kept in unnaturally dense populations, involving relatively frequent handling. The presence of visitors in close proximity to nesting sites, during the breeding season may exacerbate stress at this time of year. Additionally there may be stress of mixing with species with which they would not naturally mix (CROMIE et al., 1992). The birds in the collection are pinioned and so spend all of their time at ground level, in contact with their own and other birds' droppings, increasing the risk of infection. Pinioning in itself is a source of stress. Birds, which would naturally fly are unable to escape real or perceived danger; those species, which would normally migrate, are unable to do so. The physiologic effects of being unable to perform the basic, natural behaviour of flight cannot be quantified, but are considered as a source of stress. Birds, such as the *Oxyurini* (Stiff-tailed ducks), with their particularly short wings, fly less than many other species. The relatively low incidence of avian tuberculosis in this group of birds may reflect in part that they are less stressed by being unable to fly, than are some other species (CROMIE et al., 1991). Other sources of stress may be identified when the ecology and social structure, such as the degree of social interference or whether males are

usually found with the females, in captivity are compared to those in the wild (CROMIE, 1991).

Patterns in the incidence of avian tuberculosis in captive collections are evident in the climatic origins of the species studied. The lowest incidence is seen in birds from temperate climates (mean temperatures of 10°C-20°C). Higher incidences are seen in birds from cold (below 10°C) and hot (21°C and over) climates. These last two groups experience in their captive situation temperatures most different from their climates of origin. A lower incidence of disease is seen in birds which are adapted to the temperatures found in Great Britain (CROMIE et al., 1991). An analysis of the incidence of avian tuberculosis according to climatic origins of the species studied has not been carried out in the current study due to small sample size, but could possibly offer further information.

Susceptibility to avian tuberculosis is influenced by the level of genetic variation within a population. GROSS et al. (1989) find an increased susceptibility in inbred chickens. The effects of a genetic bottle neck on immunity to disease are well documented in such cases as of the Black-footed Ferrets (*Mustela nigripes*) and canine distemper, and the African Cheetahs (*Acinonyx jubatus*) and feline infectious peritonitis (O'BRIEN and EVERMAN, 1989).

Genetic disposition also influences susceptibility to avian tuberculosis. Birds infrequently challenged with *Mycobacterium avium* in their natural habitat often have greater exposure to mycobacteria in captivity. Although captive bred, birds in a collection will exhibit the genetic tendencies towards immunity or susceptibility to infection, possessed by their wild counterparts. The birds in the collection are several generations removed from the wild, and while this is insufficient to allow for development of genetic resistance to mycobacteria, it may be that due to the smaller flock sizes and restrictions of captivity some degree of inbreeding and loss of genetic diversity occurs. Species from temperate zones and freshwater habitats would be expected to have been selected for resistance to *Mycobacterium avium* infections (CROMIE, 1991). This was also reflected in the incidence of avian tuberculosis among such species in the WWT centre at Llanelli during this study, as demonstrated in **Table 21** and **Figure 13** (**Results, 5.3. Analysis of the data according to tribes**) (and in detail in **Table VI in Appendix 1**). Some of the marine wildfowl (*Mergini*, including Hooded Merganser and Smew) exhibit the increased incidence of disease that would be

expected of them. Contrarily some species of the arctic regions such as the *Somateriini* did not show an increased incidence of avian tuberculosis in the current study.

There is more research needed to clarify the effect of diet on the incidence of avian tuberculosis, but it seems as if the method of feeding may have some influence. There is a significant difference in incidence of disease between grazers and divers, and grazers and dabblers (CROMIE et al, 1992). Much of the food of grazing birds is exposed to the sterilising effects of ultra-violet radiation. These birds may also selectively avoid food, which is potentially contaminated, for example, by faeces. Mycobacteria live in damp environments and so those wildfowl that dabble or dive for food in the water are at greater risk of becoming infected than are those that graze on dry land. Faecal material accumulates in the shallow waters in which these birds spend a great deal of time feeding. It has been shown experimentally that MAI complex is readily aerosolised (WENDT et al., 1980; GRUFT et al., 1979). Dabbling may facilitate aerosolisation, but there is no evidence that this is decisive in transmitting the disease (CROMIE et al., 1991).

All ponds in the WWT centre at Llanelli have anti-erosion concrete edges. In time these are covered by mud and soil and birds feeding from the ground will contact potentially contaminated material. The low incidence of disease among Flamingos may be ascribed in art to their relatively low genetic susceptibility, but also to the practice to feeding them a prepared diet, fed from troughs which are cleaned daily. These feeding practices would be expected to minimise the risk of infection and spread of the disease. Further details about special feeding facilities and underwater pellets are given in **section 3.2.** (**Sanitation**) of this chapter.

Due to different life expectancies between tribes data regarding the age of the birds can only be analysed within a given tribe. **Figure 14** (**Results, 5.3. Analysis of the data according to tribes**) (and in detail in **Table VII in Appendix 1**), demonstrate that the average age of birds dying from avian tuberculosis is higher than the average age of the birds that died due other causes of death. This finding confirms the common statement that avian tuberculosis is a disease that affects mainly older birds (MONTALI et al., 1976; CROMIE et al., 1991c). **Figure 15** (**Results, 5.3. Analysis of the data according to tribes**) represents to probability that birds of a given tribe die from avian tuberculosis at a given age.

2.4. Incidence of avian tuberculosis according to pen with regard to environment and husbandry practices

An analysis by pen of the incidence of avian tuberculosis in the WWT centre at Llanelli (see **Results, Analysis of data by pens**) showed no area harbouring wildfowl that is free from disease, except from the outdoor Duckery and the European side-pen.
This study showed statistically significant differences between individual pens. As seen in **Figure 12** (**Results, 5.2. Analysis of data by pens**) (and in detail in **Table V in Appendix 1**), the incidence of disease in the North American pen, the Smew pen and the Asian pen (in this order) proved to be higher than in the others. Members of nearly all tribes are found in each pen, and if the distribution of susceptible tribes were uneven, this would bias the results. Comparison of the tribes in a given pen did not yield statistically significant differences in the incidence of avian tuberculosis. Nevertheless, it was notable that although there is a relatively even distribution of most tribes in all pens, there were differences in the number of birds of a given tribe having died from avian tuberculosis in the different pens. There was a statistically not significant correlation between a high number of deaths due to avian tuberculosis in the highly susceptible tribes and the pens with the highest incidence of avian tuberculosis. Within the *Aythini* a high number of deaths due to avian tuberculosis was found in the North American pen and the Smew pen; within the *Cairinini* the highest number was found in the Smew pen and the Asian pen; and within the *Mergini* the highest number of birds with evidence of avian tuberculosis on necropsy was found in the North American pen. The small numbers of birds in some pens (Stifftail pen and side pens) may affect the results. As there were differences in the number of birds from the same tribe dying in different pens (although statistically not proven), one could suggest that highly contaminated pens influence the incidence of avian tuberculosis within the tribes that are housed in those pens. Further research at the WWT centre at Llanelli is necessary to clarify this situation.

As shown in **Figure 12** (**Results, 5.2. Analysis of data by pens**) (and in detail in **Table V in Appendix 1**), the incidence of disease was highest by far in the North American pen. There is a continuous water flow from one pen to the other and the North American pen is situated close to the end of this chain. Therefore, an accumulation of mycobacteria in this pen is to be expected. This pen also has poor drainage and is muddy after rain, again conditions

conducive to survival of mycobacteria (GRUFT et al., 1979). The high degree of birds mixing between this pen and the Asian pen may contribute to distribution of the disease outside the pen.

As demonstrated in **Figure 12** (**Results, 5.2. Analysis of data by pens**) (and in detail in **Table V in Appendix 1**), the Asian pen showed the third highest incidence of avian tuberculosis. This pen is also found to the end of the water supply, immediately upstream to the North American pen. Birds from this pen mix not only with birds from the North American pen but also with those from the Island pen, which shows also a relatively high incidence of avian tuberculosis.
As described in **section D.4.** (**Results, 4. Physical environment and husbandry details**), the ratio of water to land surface is relatively high in the Smew pen, which showed the second highest incidence of avian tuberculosis (see **Figure 12** (**Results, 5.2. Analysis of data by pens**) (and in detail **Table V in Appendix 1**), and the water has flowed through only the Top pond. Wild ducks, mainly Gadwall and Tufted Ducks frequent this pen, which may contribute to the spread of the disease.

In summary, features such as vegetation and amount of open space, as described in **section D.4.** (**Results, 4. Physical environment and husbandry details**), do not seem to influence the incidence of avian tuberculosis significantly. A low incidence in the European pen and the South American pen, which have only a little open space but a great deal of dense vegetation, appears to diminish the importance of the sterilising effect of ultra-violet radiation. Factors such as water surface to land ratios, drainage after raining, water supply (see **Figure 9**, **Results, 4. Physical environment and husbandry details**) and the degree of mixing with wild birds and birds from other pens appear to affect incidence of disease, although this is not proven statistically. The Top pond for example, is the first pen through which water travels and has a very low incidence of avian tuberculosis. Mycobacteria grow best in microaerobic conditions, equally well, in water with salt (up to 2%) or without; MAI complex organisms can be found in large numbers in brackish swamps and estuaries (FALKINGHAM III, 1996). These are the conditions in the WWT centre at Llanelli, where the river Loughor is tidal and pens can be extremely muddy. Susceptible species are found in all the pens. But Smews and White-winged Wood Ducks, which are particularly susceptible

to avian tuberculosis (CROMIE et al., 1992) were found in the Smew pen. Hooded Merganser, which also showed a high incidence of avian tuberculosis, was found in the North American pen. The Flamingo pen had a relatively high incidence of avian tuberculosis, although the flamingos themselves are relatively resistant to the disease (CROMIE, 1991). In analysing the mortality data from this pen, most of the deaths from avian tuberculosis are seen to be among the *Anatini* and the *Cairinini* housed in this pen. The Llanelli collection is divided primarily by continent of origin. Grouping them by tribe, and isolating highly susceptible species like the White-winged Wood Ducks should facilitate the control of diseases like avian tuberculosis. Control of disease also depends on education of the visiting public, and maintenance of fencing and stiles would minimise movement of birds between pens.

All the pens receive water from the same source (see **Figure 9**, **Results, 4. Physical environment and husbandry details**). From a small open tank there is a constant water flow from one pen to the next, allowing the spread of contaminated material. Disease control and hygiene would be improved if there were a direct supply of water to each pen from the main source, and no drainage between pens. The ditch carrying water from the river to the tank is lined by reed beds, harbouring populations of wild birds, which may contaminate the water before it reaches the centre. It had been speculated that reed beds might serve as a filter system for *Mycobacterium avium* (BILLINGTON, 2000). However, other work calls this into question and seems to indicate that reed beds provide perfect conditions for mycobacteria to survive (KIRSCHNER et al., 1999). PAINTER (1995) suggested that environmental sampling, particularly from the watercourse, for bacteriology and serotype isolation, would reveal the degree of contamination by *Mycobacterium avium*. Identification of environmental bacteria, which might be responsible for false positive ELISA results, could also lead to refinement of the ELISA. Highly sensitive detection methods allow the identification of small numbers of disease-causing organisms in large sample volumes, containing high numbers of non-tuberculous mycobacteria. Since it has been shown that a single infectious organism in one litre of water can be of no significance to an animal of a susceptible species, the relevance of such sensitive testing to assessing the risk of infection in an environment is unknown (FALKINGHAM III, 1996).

Along the river Loughor there are steel works that drain their waste water into the river which then supplies the WWT centre at Llanelli with water (EDWARDS, personal communication). The degree of dilution is high, but it remains to be determined whether this pollution plays a role in susceptibility to disease. Low grade zinc toxicity in birds impairs the immune system (DUMOCEAUX and HARRISON, 1994). Non-tuberculous mycobacteria are relatively resistant to heavy metals and oxyanions. Metal metabolism and metal requirements directly influence MAI complex populations, as numbers of these bacteria in natural waters directly correlate with zinc levels. It has been suggested that the persistence of MAI complex organisms in hospital water systems and drinking-water distribution systems is a result of the use of galvanised (i.e. zinc-coated) pipes (FALKINGHAM III, 1996).

Until 1986 the site occupied by the WWT centre at Llanelli was used as farmland, from 1986 to 1989 it was under construction. The farm had held cattle, chickens and some pigs. Pigs and chickens were housed inside the buildings that are now used for storage (WALTERS, personal communication). Infection with *Mycobacterium avium* in pigs and chicken is well documented (THOEN et al., 1978; THOEN et al. 1979). Serotypes 1, 2 and 3 have been isolated from chickens (THOEN et al., 1978; SCHÄFER et al., 1973), but also from swine and cattle. (GÖTZ et al., 1986; SONGER, 1980; THOEN, 1994). Dung and liquid manure were spread on a field, which is now the European pen, near the sluice. There is no record of mycobacterial infection on this farm, but the possibility of contaminated faeces draining into the ground and into the ditch remains. Mycobacteria could spread widely in this way. Surveys show that mycobacteria are particularly abundant where the soil is contaminated with the faeces of infected animals, as in piggeries, and organisms may be washed from such sources into surface waters (GRANGE et al., 1990). It needs to be considered that the WWT centre may have been built on previously contaminated ground, exposing captive, infection-susceptible birds. This may also explain the isolation of *Mycobacterium avium* serotype 3 from birds in the WWT centre at Llanelli (PAINTER, 1997).

3. Recommendations for management and control of avian tuberculosis

3.1. Disinfection

Sanitation and disinfection are fundamental aspects of disease prevention. Phenolic disinfectants, such as Environ and TBQ (Vestal Laboratories, St. Louis, Missouri) have been the disinfectants of choice because of their tuberculocidal properties and efficacy under diverse environmental conditions (DERRICKSON and PICKETT, 1991; BEEHLER, 1990). Other disinfectants with known tuberculocidal properties are cresols such as Calgonit sterizid P24 (Kleencare Hygiene GmbH, Ladenburg, Neckar,), Dessau DES SPEZIAL N (Impfstoffwerk Dessau-Tornau GmbH, Rodleben), Endosan Forte S Neu (Schaumann, Pinneberg) or organic acids such as Dessau DES Forte (Impfstoffwerk Dessau-Tornau GmbH, Rodleben), Organosept Neu (Schaumann, Pinneberg) By using any kind of desinfectant for ground desinfection outside it is crucial to consider its polluting effect. No noxious influence of those substances on ground or ground water can be accepted, particularly with drinking water reservoirs or agriculture nearby. None of those disinfectants mentioned above is recommended to be used in outdoor exhibits as there are no sufficient information about their effect on the environment (personal communication, Professor BÖHM, University Hohenheim, Germany). Large exhibits with Solite-mix substrates (40% Solite, a light volcanic gravel (Solite Corporation, Richmond , VA, USA), 20% peat moss and 40% pine bark chips) are routinely disinfected twice a year, although it is recommended that areas of faecal build up have to be treated more often. After birds are removed, the entire exhibit is soaked with disinfectant, delivered by a hose with a spray attachment. The exhibit is then rinsed with tap water before the birds are returned. Plants generally benefit from treatment, as many plant pathogens are eliminated by the phenolic disinfectants (DERRICKSON and PICKETT, 1991). Some authors recommend spraying all surfaces with chlorinated foam cleaner prior to rinsing with water. This includes the interior of the air handling system. The soil should be sprayed thoroughly with three applications of 1 Stroke Environ using a high-pressure spray that penetrates the soil to a depth of approximately 25 cm. The 1 Stroke Environ should not be rinsed. The exhibits can also be cleaned and decontaminated by using a 5% cresylic compound or a derivative of phenol such as sodium orthophenyl phenat. If possible, the enclosures should be disinfected and rinsed three times,

every seven days, before introducing new birds. Hypochlorites, halides and benzalkonium chloride, which are commonly used disinfectants, are not suitable for killing tuberculous bacilli (THOEN, 1993). Mycobacteria are also relatively resistant to chlorine, chloramines, carbon dioxide and ozone (MIYAMOTO et al., 2000; TAYLOR et al., 2000). Following disinfection, a site must be left closed for one year (BEEHLER, 1990). MIYAMOTO et al. (2000) showed that strains of *Mycobacterium avium* are killed on exposure to water at 70°C for three minutes. This offers ideal means of cleaning equipment, and indoor and outdoor facilities prior to disinfection. These authors also cite the sterilising effect of ultra-violet light.

3.2. Sanitation

The first step in disease control is to move the birds away from severely contaminated pens into new facilities. Substrates (soil, sand, mulch, plants, perching, etc.) in indoor exhibits are soaked several times with a phenolic disinfectant, and then removed and buried under a minimum of 30 cm of soil, away from the collection. All trees and other plants should be cut down or uprooted, sprayed thoroughly with a tuberculocidal detergent, and removed from the site. After air drying for several days, the plants must be buried. Incineration is also possible (BEEHLER, 1990).

Indoor enclosures are commonly furnished with a shallow, soil-mix substrate that is layered over sand and pea gravel to promote drainage. Use of this substrate has several distinct disadvantages. Substrate removal and replacement is difficult, time consuming, and labour intensive. Progressive compaction of such a substrate increasingly interferes with drainage, plant growth, and sanitation efforts. Soil in indoor exhibits can be replaced with a mixture of 40% pine Solite (a light volcanic gravel; Solite Corporation, Richmond, VA), 20% peat moss, 40% pine bark chips, and trace elements for plant growth, layered over a filter blanket and a Solite or gravel base (DERRICKSON and PICKETT, 1991). MONTALI et al. (1976) also recommend a shallow layer of coarse gravel which can be periodically discarded and which should minimise the accumulation of organisms shed by asymptomatic infected birds. Floors in off-exhibit and in indoor breeding enclosures are maintained as bare concrete, or are covered with a layer of laboratory grade wood shavings (Northern Products Corp.,

Warrensburg, NY). When wildfowl are kept on bare concrete attention must be paid to good foot hygiene and the potential risk of pododermatitis. The use of rubberised wire mesh as flouring can help to overcome this problem. Outdoor exhibits would ideally be bulldozed, rebuilt and receive new sod before birds are returned (DERRICKSON and PICKETT, 1991).

If radical sanitation measures are not feasible, sub-optimal environmental conditions for mycobacteria can be created. Mycobacteria are more abundant in conditions of warm temperatures, low pH (pH of 5-5.5), low dissolved oxygen, high soluble zinc, high humic acid and high fulvic acid concentrations (GRANGE et al., 1990; FALKINHAM III, 1996; KIRSCHNER et al., 1992). To avoid creating these conditions, it is necessary to reduce organic matter within the water system and to decrease acidity by the addition of lime to the soil and watercourse. Water oxygen content can be increased by actively oyxgenating the water. Cleaning with hot water helps to reduce the number of viable mycobacteria in the environment, and ultra-violet radiation is an effective tuberculocidal disinfectant. The use of ultra-violet radiation for exhibits is obviously not feasible, but it may be helpful for the disinfecion of equipment, particularly in the quarantine and hospital pens, or brooders and incubators.

The stringent cleaning protocols detailed above would be prohibitively difficult to apply in the context of outdoor enclosures, such as are found at the WWT centre at Llanelli. A consequence of captivity is that birds are kept in close contact with faecal material. Frequent removal of faeces, grass cuttings and other organic refuse or prevention of exposure to such materials is the single most important factor in preventing transmission of mycobacteriosis.
Practical recommendations of sanitation with improved hygiene methods are outlined below.

1. Each pen needs to undergo sanitation according to a strict rotation. This programme should include mudding out the pond and the pen when mud and silt accumulation is compromising water flow. At a minimum, in those pens showing the highest incidence of avian tuberculosis, soil or substrate should be removed to a depth of at least 15 cm and replaced by fresh soil every year. Suitable areas within the pens can be covered with coarse gravel, wood shavings or a mixture of 40% Solite - a light volcanic gravel, 20% peat moss, 40% pine bark chips, with trace elements for plant growth, to facilitate the

regular replacement of substrate. It would also help to improve drainage to minimise mud after heavy rains.

2. Nesting materials and nesting boxes should be cleaned out annually.
3. Pruning or cutting down excessive vegetation will allow sunlight and the sterilising effects of ultra-violet light to reach areas which might harbour contaminated material. Since the Centre is open to the public, aesthetic considerations will influence decisions regarding the vegetation in enclosures. Adequate cover for shy birds and suitable nesting sites must also be supplied for each species.
4. Plant material, soil or mud removed from one pen should not to be used in another pen. It needs to be burnt and buried to prevent the spread of potentially contaminated material.
5. Pest control is necessary to prevent the spread of potential avian pathogens and parasites. Barred traps and poisons will help to control rodents and pesticides. Feeding only during the day will discourage rodents. Food can be presented in such a way as to make access difficult. Many wildfowl either dive or dabble when foraging for food in the wild, and some species have developed courtship or pair-maintaining displays that mimic these methods of feeding. To promote reproduction, it may be important to allow captive wildfowl to express natural foraging behaviour. With the conventional method of surface feeding, whether from the ground or from pans or hoppers at the water's surface, this behaviour may not be adequately reinforced. The underwater method of feeding makes access to food difficult for such birds as starling, pigeons, and sparrows, as well as unwanted mammals. If food is not readily available, unwanted mammals and birds will be less likely to frequent the enclosure. An overflow pipe located near the feeder, or a type of automatic feeder which disperses food throughout the day encourage wildfowl to forage continuously, keeping excess pellets at a minimum. For wildfowl, a complete pelleted diet has been developed to permit underwater feeding. Nutri-Binder needs to be included in the diet at a minimum of 3% to maintain pellet integrity, for about 110 minutes (CRISSEY et al., 1989). Provision of food in troughs, along with sufficient grit, will reduce the time birds spend feeding from the ground, which may be contaminated. This method is not practical when large numbers of birds are being fed, and may lead to increased competition for food. Feeding from a trough allows birds to consume a great deal of food in relatively little time, and may inhibit the behaviours associated with foraging for food naturally (CROMIE, 1991).

3.3. Husbandry and modification of enclosures

1. In addition to improved screening and sanitation protocols, reduction of the size and diversity of the collection is crucial to disease control. Management of a disease such as avian tuberculosis within a collection, requires progressive compartmentalisation of operations and procedures. Each compartment should be operated independently, and no cleaning tools should be transferred between facilities. This is necessary to minimise the spread of *Mycobacterium avium*. Ideally, traffic flow should be co-ordinated such that visitors do not travel through one exhibit to reach another. Foot-baths should be situated between exhibits, and the disinfectant should be changed weekly. However, this is probably impractical, as zoo layout depend on a flow of visitors from one exhibit to the next with no doubling back. High standards of hygiene have to be maintained among staff. Wardens' footwear, crates, feed barrows, and sacks are potentially serious sources of contaminated material, education of staff in the principles of hygiene and disease control is crucial. Water margins where dabbling species are fed and food troughs should be cleaned daily. Even cleaned boxes or troughs should not be exchanged between pens. The movement of keepers between the different aviaries and pens should to be minimised

2. A parallel watercourse will avoid the build up of mycobacteria within the chain. Each pen should have its own water supply from a clean water source and should discharge the water into a channel not bound for any other pen. Separate drainage systems allow pens to be emptied and disinfected regularly. Stagnant ponds should be drained and dredged frequently. Water pipes must be well maintained to keep up a strong through-flow of water.

3. More research is needed into the use of biofilters such as reedbeds between pens. Although their effect on mycobacterial levels remains unknown (BILLINGTON, 2000) they are effective at reducing organic content of water and may, therefore, be of benefit in controlling survival of MAI complex.

4. Currently the water flows first through a pond by the visitor centre. Wild wildfowl and other species inhabit this pond. No procedures to control disease have been undertaken in

this area and the dense vegetation and muddy shore of this pond are conducive to the survival of mycobacteria. The present exhibit is particularly attractively designed and represents a valuable entertainment to the public. To maintain the appearance of the site in light of a disease control programme, a contained, designated water system is necessary which is separated from the water system in the remaining grounds.

5. Species that are particularly susceptible to avian tuberculosis such as the White-winged Wood Duck, Smew or the Hooded Merganser should be housed separately, with an independent water supply. Netting over aviaries would prevent contact with wild birds. This would limit the spread of contaminated material and so, of disease, around the site. Covering and heating these pens would create more suitable conditions for tropical species such as the White-winged Wood Duck. A more natural climate may effectively reduce stress for the birds. Furthermore, those birds would not need to be pinioned in covered pens. In that way there would be less risk of infection as the birds would spend more time away from the contaminated ground and they would experience less stress, as mentioned in **section 2.3.** (**Incidence of avian tuberculosis according to tribes**) of this chapter.

6. Certain highly susceptible species can be used as avian tuberculosis sentinels in larger, mixed-species exhibits. Birds, such as quail, can be included in most exhibits and can be euthanized and examined for evidence of avian tuberculosis on a regular basis. Complete necropsies should be performed on all deaths from the collection (BEEHLER, 1990). When introducing those birds there is a risk of bringing diseases other than avian tuberculosis into the collection. The health status of those birds needs to be elucidated thoroughly

There is the potential for disease transmission between wild and captive populations of wildfowl in the same environment. A survey of wild birds either captured or found dead on the grounds of collections with known cases of avian tuberculosis, found none positive for avian tuberculosis (MONTALI, et al., 1976). A number of bodies will remain undiscovered, be predated upon, or will decompose before post-mortems can be performed. It is important to examine birds, which appear to have died from other causes such as trauma or predation.

Birds weakened by avian tuberculosis (as with other underlying illnesses) are often excluded from the flock, or may be treated aggressively by their flock mates. These birds are also more often the victims of predators (MONTALI et al., 1983).

Some authors stress the importance of contact between wild birds and birds in the collection as mean of spreading disease. There are many citations in the literature of free-living birds infected with mycobacteria. Wild birds or mammals can function either as sources for infection of birds in captivity. Migratory birds can spread disease over long distances. Avian tuberculosis has been diagnosed in hawks and owls (PIECHOCKI et al., 1981), in pigeons (NESIC et al., 1992), in Rooks (KOVACIC et al., 1985; HEJLICEK and TREML, 1994a), in Collared Doves and Turtle Doves (HEJLICEK and TREML, 1993a, VOLNER, 1978), in domestic pigeons (HEJLICEK and TREML, 1994b), in House Sparrows and Tree Sparrows (HEJLICEK and TREML, 1993b; KOVACIC and TUNKL, 1978), in pheasants and partridges (HEJLICEK and TREML, 1993c), in Black-headed Gull (HEJLICEK and TREML, 1994c) and in Sandhill Cranes (THOEN et al., 1977b). BROWN et al. (1992) describe the incidence of avian tuberculosis in wild swans which were found dead in the grounds of the WWT centre at Slimbridge between 1951 and 1989. Of 264 adults examined, 6% showed signs of avian tuberculosis, while 1% of 94 juveniles were determined to be affected. Since captive wildfowl have been present at the WWT Centre at Slimbridge since the 1940's, *Mycobacterium avium* may be more prevalent in the pond mud and soil than in the mud and soil of younger collections or natural habitats. In a previous study (CROMIE et al., 1993b) compared the incidence of avian tuberculosis in wild birds having had contact to captive wildfowl to those, which had no contact. They found a greater incidence in the contact group (24.7%) than within the non-contact group (11.1%) of the Mallards tested. Findings among the Bewick's Swans were similar, with an incidence of 27.6% within the contact group compared to 9.5% in the non-contact group (CROMIE et al., 1993b).

7. The use of aviaries for susceptible species of high conservation value, such as the White-winged Wood Duck will prevent their interaction with wild birds. Wild birds, especially wildfowl, should be denied access to the collection, through placement of physical barriers, such as plastic or wire mesh screening around enclosures. Contact should be prevented with sparrows, blackbirds, and pigeons. The pens of the duckery are already covered with nets to protect the ducklings from predators.

MAI complex infections are a noted problem in zoo animals, possibly associated with the innate stress of captivity (GRANGE et al., 1990). A well-nourished and active bird may encase a tuberculous lesion in scar tissue, containing the organisms. Walled off infections may be controlled while the bird's immune system remains healthy. Under stressful conditions, or in the presence of a second pathological condition, mycobacterial infection may reactivate. Stressors include recent importation, and the associated change of diet, possible differences humidity and climatic conditions. Furthermore, the lack of sunlight, overcrowding and generally poor standards of hygiene usually associated with capture and transport may all predispose a bird to infection (CROMIE, 1991).

8. Introduction of new birds to the exhibit and to other birds has to be gradual. Compatibility with birds sharing the exhibit has to be considered when selecting species and individuals for display. Less aggressive birds should be introduced first to allow them to establish territories. Exhibits and holding facilities have to be designed to facilitate introduction (BEEHLER, 1990).
9. Overcrowding, stress such as pinioning and neglecting of social structures and incorrect nutrition must be avoided. Although the presence of visitors is also a unavoidable stressor, all efforts must be made to limit disturbance to the birds by visitors. Some provision must be made to provide warmth during cold weather. Pens should be stocked with young, preferably full-winged, birds in appropriate social groups and disturbance should be kept to a minimum (particularly with respect to the females during the summer) (CROMIE et al., 1992).
10. Being pinioned is a likely source of stress, particularly for strongly migratory species, or those that would naturally take to the air when in danger. When planning exhibits consideration must be given to such matters as the ecology of the birds, whether or not the birds are social, and whether males are usually found with the females and the approximate ratio of male to females (CROMIE, 1991).
11. In order to take species specific behaviour patterns, similar environmental and nutritional needs, and susceptibilities to disease into account, grouping birds by tribe instead of by continent of origin would be more appropriate. However, this may be of dubious educational value. Although birds origin from the same continent, they may inhabit vastly different ecological niches. Pens could be designed to meet the needs of specific tribes with respect to, for example, vegetation, cover and heat source in winter, trees for

perching ducks, appropriate large and deep ponds for divers. Conversion of pens needs to be planned carefully as redesign carries the risk of increased stress due to competition for the same niche in feeding, roosting or nesting. Compatibility details and the specific requirements of captive wildfowl can be found in the reports by FORBES and RICHARDSON (1996) and BROWN (1998).

3.4. Recommendations for management

When endangered species bred in captivity for conservation purposes are affected by avian tuberculosis, the problems of management gain significance. This situation has been encountered in the case of cranes held in a collection in London (HAWKEY et al., 1990). In that collection the risk of disease was perpetuated by the practice of using brooding hens that were suffering from covert avian tuberculosis, for hatching and rearing crane chicks. The risk of disease transmission was compounded by extensive mixed species paddock management of the juvenile and adult birds. An alternative to carefully screened brooding hens for hatching would be the use of incubator systems. In the WWT centre at Llanelli most birds are hatched in incubators and reared together in the outdoor duckery. If incubators are used to avoid transmission after hatching, there is the risk of imprinting the birds due to hand rearing. To prevent imprinting and to reduce the risk of disease, the birds are reared in small groups where they imprint on one another and contact with humans is kept to a minimum.

The unnaturally high stocking densities found in captive collections and the permanent presence of birds on the same ground allow the accumulation of high levels of contamination. Signs that a bird is infected with avian tuberculosis are only seen late in the course of the disease, usually shortly before death. Therefore, infected birds can spread the disease for a long period before they are noticed. In order to manage a collection and to permit disease control, but also problems such as cage mate aggression or cannibalism, a management plan must be tailored to each species, to include the following: enclosure(s); current and desired numbers; breeding priority and preferred rearing method; proposed disposition of surplus individuals; co-operative programmes; and specific exhibition, conservation, and research objectives (DERRICKSON and PICKETT, 1991).

1. An accurate record keeping system is of fundamental importance. A computer programme can be developed to take inventory of the collection and monitor the movement of individual birds between enclosures. An example of such a programme is the ‘Animal Record Keeping System (ARKS)‘ (ISIS, Apple Valley, Minnesota, USA) (DERRICKSON and PICKETT, 1991). It allows individual birds to be located, it records where birds have previously been housed, maps the movement of diseased birds within the collection and facilitates identification of potential problem areas. MEDARKS (ISIS, Apple Valley, Minnesota, USA) the veterinary software which interfaces with ARKS may also be useful.
2. Employees with chronic lung diseases or those taking immune-suppressive drugs should be informed about a potential health hazard (BEEHLER, 1990). Personnel working with birds known to be infected, should wear a well-fitted airtight mask (HOOP, 1997).
3. Although THOEN (1993) points out a certain risk of disease transmission by visitors to the birds in the collection, this is considered negligible. The risk of disease transmission to visitors, who have minimal direct contact with the birds, is similarly extremely low, given the lack of evidence for the zoonotic potential of MAI complex. Although as a result of walking through pens, visitors may be expected to contact organisms, enclosure, footpath design and placement of foot-baths will minimise spread of organisms.

An earlier study at the WWT centre at Llanelli showed that most avian tuberculosis positive birds were at least four years old (PAINTER, 1995). This is consistent with the higher incidence of avian tuberculosis in older birds (CROMIE et al., 1993a). Maintaining a relatively young collection by removing birds over a pre-determined age reduces the contamination of the environment with infectious organisms and therefore the risk of infection with avian tuberculosis within a collection (PAINTER, 1995). Age cohort mortality needs to be calculated at a tribal or better species level because of the range of life spans of the different species. In agreement with CROMIE et al. (1993a) and PAINTER (1995), this study finds the average age of birds dying from avian tuberculosis is approximately seven to eight years while, deaths from causes other than avian tuberculosis occur at approximately four to five years of age. **Figure 14** (**Results, 5.3. Analysis of the data according to different tribes**) (and in detail **Table VII in Appendix 1**) gives further details for each tribe.

4. As presented in **Figure 15** (**Results, 5.3. Analysis of the data according to different tribes**) it is possible to produce an estimate about the chances of dying from avian tuberculosis at a given age for birds of a given tribe. This can be used to fix the age at which birds of different tribes are to be culled from the exhibit. This practice may not be suited to some species, which are particularly valuable for the collection.

5. Vaccination

Development of a vaccine to protect avian tuberculosis negative birds from infection is being explored. Vaccination has been attempted using heat killed as well as live strains of different serotypes of *Mycobacterium avium* in chickens (ROSSI, 1974). There was evidence of incomplete immunity in one series of tests and the best results were obtained by the oral administration of live serotype 6 (ROSSI, 1974). Because *Mycobacterium avium* is potentially pathogenic for wild birds, only killed organisms or cell wall cytoplasmic fractions could be used for vaccination of birds on display in a collection (MONTALI et al., 1976).
A trial vaccine against avian tuberculosis exists, as developed by CROMIE (1991). Between 1991 and 1995 vaccination with killed *Mycobacterium vaccae* (strain R877R) has been performed in the WWT centre at Slimbridge. Analysis of data from all tribes revealed that the vaccine did not provide protection from the disease, whilst analysis of the data within the tribe *Cairinini* showed a statistically significant reduction (70%) in tuberculous deaths in the vaccinated group compared with the control group (PAINTER, 1995; CROMIE et al., 2000). Analysis of the numbers of deaths from avian tuberculosis in vaccinated and control groups shows no significant difference between the two, with the exception of the *Cairinini* which were afforded protection (CROMIE et al., 2000). However, this result must be treated with caution because of the marked variation in sample sizes between the tribes and the potential variation in the effect of vaccination on each tribe. The vaccine trial has been redesigned with a lower dose of vaccine and the effects of a booster dose of vaccine are being investigated (CROMIE, personal communication).

6. Clinical screening

Before birds are returned to renovated facilities they should be screened for avian tuberculosis. Since not even necropsy including histopathology can find a bird negative for avian tuberculosis without question, screening tests should be chosen in light of financial

constraints and the birds to be tested. The value of the bird, the susceptibility of the tribe to which it belongs, and the physical examination findings should, according to a pre-determined protocol, determine which tests are most appropriate. Screening programmes may include physical examination, haematologic, radiographic and laparoscopic examination, faecal acid-fast staining, clinical chemistry, intradermal tuberculin testing, lymphocyte transformation studies, and enzyme-labelled antibody and serum agglutination tests. In **chapter B.5.** (**Literature review, Diagnosis of avian tuberculosis**) the diagnostic reliability of those procedures is discussed in detail, particularly the questionable diagnosis by haematology and faecal acid-fast staining (see **Literature review, 5.3.3. Direct examination by staining an microscopy and 5.3.6. Haematological analysis and blood chemistry**). Because of the wide range of pathologic manifestations of tuberculosis in birds, the disease can be difficult to diagnose and may be missed at necropsy. For this reason, it is important to perform acid-fast stains on smears of any abnormal appearing organs at necropsy, and on all histologic inflammatory lesions (MONTALI et al., 1992). To minimise the number of birds being screened, individuals of taxa, which have previously shown a high incidence of avian tuberculosis can be euthanized and subjected to post mortem examination. Further considerations in the screening and culling process include age, history of movement within the collection and potential for exposure to avian tuberculosis, monetary value and ease of replacement. If one considers the control of avian tuberculosis strictly with a view to disease eradication, the nature of the disease dictates that any positive bird and its cage mates, as well as any contact birds be euthanized, and that the enclosure be disinfected and rebuilt. There is obvious reluctance to euthanase, in some cases, very large numbers of birds. The loss of so many birds, as well as the reconstruction of facilities could easily prove financially prohibitive (DERRICKSON and PICKETT, 1991; BEEHLER, 1990).

It is proposed that annual screening by ELISA and subsequent action will be of direct benefit in the control of avian tuberculosis. If a policy of euthanasia for ELISA positive birds is considered unacceptable in terms of animal welfare and conservation, a management system should be developed which allows isolation of potentially infected individuals of threatened species. Given the persistence of *Mycobacterium avium* in the environment, a strict initial regime of euthanasia is required. A sensitive microbial DNA detection assay, such as the polymerase chain reaction or the DNA probe being developed in the USA (MONTALI et al.,

1992) could potentially eliminate all birds harbouring *Mycobacterium avium*. This is the ideal form of prevention and control of the disease. Due to financial reasons such testing would be restricted to birds being sent to other facilities or those to be used in re-introduction programmes

7. Quarantine policies

The role of quarantine as the first line of defence against avian tuberculosis and other diseases must be emphasised. As far as possible new additions to a collection should be free from avian tuberculosis. Quarantine should last at least 30 days. All birds must receive physical and haematological examination preferably at the start and end of the quarantine, serial faecal checks, as well as laparoscopic and radiographic examination. In the case of a positive result, the bird has to be euthanized, and the source individual or institution has to be notified of the diagnosis (DERRICKSON and PICKETT, 1991; BEEHLER, 1990). Some authors recommend a quarantine of several months to a year (VAN DER HEYDEN, 1994), three to six months (HOOP, 1997) or a minimum of 60 days, but preferably 120 days (THOEN, 1993). HOOP (1997) suggests that animals having contact with avian tuberculosis positive birds should be removed from the contaminated area and quarantined for 2 years with, regular testing every 12 weeks. Birds that remain negative (not shedding the agent with the faeces) and are in good physical condition following the quarantine procedure can be considered free of the disease (RITCHIE, 1994). Wild birds infected with advanced avian tuberculosis are weak and are often found sick, emaciated or injured in the grounds of a collection. Such birds should not be hospitalised without a thorough examination, screening tests and preferably quarantine.

8. Quarantine facility

An isolated, roofed building is required for a quarantine facility, with its own water supply. Sick and newly introduced birds should not be housed in the quarantine facility. Hygiene practice should include foot-baths, the regular disinfectant of equipment which is specific to the quarantine area and weekly power hosing and cleaning of the enclosures with hot water and a tuberculocidal disinfectant. The floor of the enclosures should be concrete, from which the substrate should be removed weekly. The drainage system within the units should allow the water to drain to the front and flow into a channel to the sewage system without meeting

the rest of the watercourse. The site should be secure from the entry of pest species. If possible, each unit should have access to a separate outdoor pen. This area would have to be enclosed by a solid roof and side netting to prevent access of pest species or free flying birds or contact with their droppings. Outdoor units can be separated by using double layered side netting with a distance of 20 cm. The number of birds managed within this system, and their distribution within the facility should be determined in advance and should be adhered to strictly.

The widespread adoption by the poultry industry of management practices aimed at controlling contagious diseases has demonstrated amply that disease such as mycobacteriosis can be virtually eliminated when the environment of the bird can be controlled. These management practices are based on principles such as identification and elimination of infected birds and strict hygiene practices to minimise contact with faeces, soil and other potentially contaminated materials. These principles can be adapted for effective control of mycobacteriosis in pet birds, private and commercial aviaries, and zoological collections. With a strict programme of disinfection and culling of tuberculous birds, the incidence of avian tuberculosis in zoo aviaries decreased from 5.2% in 1976 to 2.7% in 1977, and to less than 1% for the years 1978 to 1981 (MONTALI et al., 1983). In another instance, after screening two different collections for avian tuberculosis by agglutination test, endoscopy and culture of samples, all birds positive for mycobacteriosis were culled. The tests were repeated every six months. After one year the incidence of avian tuberculosis was reduced from 12.8% to 0.8% in one collection, and from 13.6% to 2% in the other (GÖTZ et al., 1986). Studies at The WWT centre at Slimbridge show that removing tuberculous birds significantly reduces the number of birds found infected by *Mycobacterium avium*. A reduction in environmental contamination lessens the risk of infection both to the birds of the collection and to wild birds.

Reflecting all those recommendations it has to be borne in mind that health care is just one aspect of the WWT centre at Llanelli next to education for example. The efficiency of the centre is dependent on visitor numbers. Due to limited practicability and deterrence of visitors there is the need to balance disease control in exhibit settings. Areas of compromising

are the design of the enclosures, co-ordinating the traffic flow of visitors or the use of foot-baths situated between the different exhibits.

Avian tuberculosis in a collection of wildfowl can be reduced and maintained at acceptably low levels if strict screening, management and husbandry protocols are adopted. Such an aggressive approach carries high financial costs and ethical, medical and conservation choices. The consequences of inaction, however, are likely to be unacceptable. The eradication of avian tuberculosis from a collection is not currently a realistic aim because of the chronic nature of the disease, the existence of a carrier state and the tenacity of the organism. Control of the disease will, therefore, involve a programme of continous monitoring to allow the culling of birds at a relatively early stage of disease. Since regular screening of all stock may not be possible, the identification and screening of a proportion of the most susceptible birds in each pen, on an annual basis, may be an adequate compromise. As for instance, the incidence of avian tuberculosis in the WWT centre at Llanelli was 43% in 1999, a suitable proportion of susceptible birds in each pen to be tested would be 50%.

In 1872 CRISP defined avian tuberculosis as ‘contagious’ and described affected birds as displaying ‘symptoms of failing appetite, gradual wasting and diarrhoea with lesions in the liver, spleen, mesentery, and joints’. 129 years later, this remains an accurate description of a disease, which in spite of the aetiology being identified, and the pathogenesis understood, remains challenging to control.

F. Summary

With the involvement of collections of birds like those belonging to the Wildfowl and Wetlands Trust (WWT) in recovery and reintroduction programs for endangered and threatened species, disease has become a topic of considerable importance. Many diseases, such as avian tuberculosis, are difficult to detect, have long latency periods, and have no effective treatment. Because diseases with these characteristics can easily spread undetected, they represent a significant threat to both captive and wild populations in contact with infected birds. Within captive collections of birds avian tuberculosis is notoriously difficult to eradicate due to the tenacity of *Mycobacterium avium* which can persist in an environment for several years. It is the insidious nature and slow progress of the disease, coupled with the ability to survive outside the host, and therefore create an infectious reservoir, that makes avian tuberculosis such a problem.

Therefore it is important to consider different techniques of diagnosis and to develop a management plan to control the disease. This epizootiological study is undertaken in the WWT centre at Llanelli, South Wales, where the adult mortality of avian tuberculosis rose from 20% to 43% in the last six years (1994 to 1989).

First, an enzyme-linked immunosorbent assay (ELISA) was evaluated by testing blood samples from birds which were known to be positive or negative for avian tuberculosis. This ELISA was first developed by CROMIE (1991). The ELISA used in this study was now modified by using a commercially available rabbit anti-duck horseradish peroxidase conjugate and five antigens which were isolated from birds that died of avian tuberculosis in the WWT centres at Llanelli and Slimbridge. Samples which have an absorbency value above the cut-off point were considered positive and those below the cut-off point were considered negative. The results are categorises as follows:

Avian tuberculosis negative:	values < 20% of the adjusted positive control
Borderline:	values of 20-50% of the adjusted positive control
Avian tuberculosis positive:	values > 50% of the adjusted positive control

The ELISA was performed on blood taken from 418 birds of the collection including ducks, geese and swans. 219 birds have been repeatedly tested over the period of two years.

ELISA results from those birds tested in spring and autumn show significant differences with a tendency of a higher antibody titre for avian tuberculosis, in the blood samples obtained in autumn. Further studies with serial testing twice a year for at least two years are necessary to elucidate possible seasonal changes in antibody levels.

The analysis of the ELISA results to evaluate sensitivity and specificity is based on gross post mortem examination. To confirm diagnosis a histopathological examination was performed on liver and spleen biopsies of 19 birds. There was only little agreement between gross post mortem and histopathological examination with 27.8% (n=5) of questionable results and no false negative results. Therefore, the evaluation of the ELISA based only on post mortem findings as gold standard has to be considered very critically.

PCR performed on 13 of the specimens prepared for histopathology did not identify the presence of mycobacterial DNA in any of the samples, therefore, confirming histopathological findings rather than gross post mortem findings. Further research, for example, identifying species-specific mycobacterial DNA fragments by molecular biological techniques such as PCR, is necessary to obtain a final diagnosis. In performing PCR the greater degree of degradation in DNA prepared from paraffin-embedded tissue compared to frozen tissue should be considered. Analysing the ELISA results by referring to such highly precise diagnostic techniques may reveal a somewhat different sensitivity and specificity than the ones obtained in the current study.

51 of the birds tested died during the period of this study and a gross post mortem examination was performed. Only the ELISA carried out in spring proved reliable with a sensitivity of 76.9% and a specificity of 55.6%. Those figures are not ideal and the percentages of false negative (23.1%) and particularly false positive results (44.4%) are not acceptable in a screening program for larger collections. Screening of individual pet birds requires an even more specific test. Some possible tests are mentioned in the literature review.

Analysis of the ELISA and post mortem results according to different taxonomic tribes showed no significant differences. The ELISA results in the *Dendrocygnini*, *Anserini*, *Tadornini*, *Somateriini* and *Oxyurini* have to be interpreted very carefully due to a high percentage of false positive and false negative results.

There is need for further reappraisal and refinement of the ELISA to improve its sensitivity and specificity as a mean of ante-mortem diagnosis of avian tuberculosis. There must also be further considerations about the evaluation of the ELISA, as basing the data on gross post mortem examination may not be accurate enough, but more specific molecular biological techniques might be advisable.

Secondly, post mortem data from the last ten years were analysed to identify the distribution, prevalence and percentage mortality of avian tuberculosis in the WWT centre at Llanelli according to different sex, seasons, tribes, age and pens.

The statistically significant highest mortality of avian tuberculosis was found in the North American pen, the Asian pen and the Smew pen. As possible reasons environmental details, particularly the water course through the grounds, contact to other birds of the collection and to wild birds and the former use of the grounds as farmland are discussed.

The *Mergini*, *Cairinini* and *Aythynini* showed the highest mortality of avian tuberculosis including the very susceptible species Hooded Merganser, Smew and White-winged Wood Duck. Habitat, feeding and breeding habit and particularly genetic susceptibility are suggested to explain the different mortalities of avian tuberculosis in the different tribes. Furthermore environmental factors and the exposure to stress are considered to influence the development of disease.

There was no sex predilection or differences in the occurrence of deaths due to avian tuberculosis in different seasons.

There was a significant increase in the number of birds dying from avian tuberculosis over the last few years. Most notably there was a dramatic increase in mortality from 1997 (24.4%) to 1998 (40.7%), followed by a further, smaller increase in 1999 (43.4%). The increase mortality was found both in the overall number of birds and in a break down of the data according to different tribes.

Finally, a management plan was proposed to support efforts to control avian tuberculosis, including general considerations and aspects specific for the WWT centre at Llanelli. These suggestions involve disinfection, sanitation, conversion programs of the enclosures with particular emphasis on the water course, husbandry of very susceptible species, avoiding contact with other birds, reducing stress, record keeping and most importantly quarantine and regular screening programs. These programmes should consist of clinical examinations and performing of an ELISA.

G. Erweiterte Zusammenfassung

Harriet Petra Zsivanovits

Entwicklung, Einsatz und Bewertung eines Enzyme-linked Immunosorbent Assay (ELISA) zur Kontrolle und Management aviärer Tuberkulose in dem Wassergeflügelbestand des Wildfowl and Wetlands Trust Zentrums in Llanelli, Großbritannien

In den Beständen des Wildfowl and Wetlands Trust, unter anderem in Slimbridge und Llanelli, hält sich eine große Anzahl Wasservögel auf. Diese spielen bei der Erhaltung und Wiedereinführung von bedrohten Tierarten eine entscheidende Rolle. Hierbei ist die Gesundheitssicherung mitsamt entsprechenden Gesundheitskontrollen von entscheidender Bedeutung. Viele Krankheiten, wie zum Beispiel die aviäre Tuberkulose, sind schwierig zu diagnostizieren, haben eine lange Inkubationszeit und es steht keine wirksame Behandlung zur Verfügung. Da sich derartige Infektionen leicht unentdeckt in einem Bestand verbreiten können, repräsentieren sie eine beachtliche Bedrohung für Populationen, die in Kontakt mit infizierten Vögeln gelangen können. Auf Grund der hohen Tenazität sind aviäre Mycobakterien in Beständen von gefangenen Vögeln besonders schwierig auszumerzen. *Mycobacterium avium* kann in der Umwelt mehrere Jahre überleben. Desweiteren unterstützen der unauffällige und langsame Verlauf der Erkrankung, die ausgeprägte Erregerausscheidung, welche klinischen Symptomen vorangeht, und die Fähigkeit der Mykobakterien, außerhalb des Wirtes zu überleben, die Ansammlung eines Infektionsreservoirs, welches aviäre Tuberkulose zu einem bedeutenden Problem macht.
Aus diesen Gründen ist es wichtig, verschiedene Diagnosetechniken zu überprüfen und einen Management-Plan zur Kontrolle der Erkrankung zu entwickeln. Diese epizoologische Studie wurde in dem Wildfowl and Wetlands Trust (WWT) Zentrum in Llanelli in Süd-Wales durchgeführt. Hier hatte sich nach Angaben der dort tätigen Betreuer die Mortalität an aviärer Tuberkulose bei erwachsenen Entenvögeln innerhalb der letzten sechs Jahren (1994 bis 1999) von 20% auf 43% erhöht.

Zuerst wurde die Technik eines Enzyme-linked Immunosorbent Assays (ELISA) anhand von Blutproben von Vögeln, welche durch Sektionen als positiv oder negativ für aviäre Tuberkulose diagnostiziert wurden, überprüft und der Cut-off point bestimmt. Dieser ELISA ist von CROMIE 1991 entwickelt worden. Der in dieser Studie eingesetzte ELISA wurde modifiziert, indem ein kommerziell erhältliches Kaninchen Anti-Ente Meerrettichperoxidase-Konjugat verwendet wurde. Dadurch erfüllt der ELISA die Ansprüche eines Screening Programms für größere Bestände, indem finanzielle Aspekte und Praktikabilität berücksichtigt werden. Weiterhin wurden fünf mykobakterielle Antigene verwendet, welche aus Vögeln isoliert wurden, die in dem WWT Zentren in Llanelli und Slimbridge an aviärer Tuberkulose gestorben waren. Hiermit wurde die Spezifität des ELISAs bezüglich der Erkrankung bei Vögeln aus dem WWT Zentrum in Llanelli erhöht. Blutproben, welche einen Absorbtionskoeffizienten oberhalb des Cut-off Punktes hatten, wurden als positiv angesehen und jene unterhalb des Cut-off Punktes waren negativ. Die Ergebnisse wurden folgendermaßen kategorisiert:

Aviäre Tuberkulose negativ:	Werte < 20% der angeglichenen positiven Kontrolle
Grenzwerte:	Werte zwischen 20-50% der angeglichenen positiven Kontrolle
Aviäre Tuberkulose positiv:	Werte > 50% der angeglichenen positiven Kontrolle

Der ELISA wurde an Blutproben von 418 Vögeln des Bestandes, einschließlich Enten, Gänsen und Schwänen, durchgeführt. 219 Vögel wurden während der Testphase von zwei Jahren sowohl im Frühjahr als auch im Herbst des ersten Jahres getestet.

Während des Untersuchungszeitraums starben 51 Vögel und wurden einer Sektion unterzogen. Anhand der Sektionsergebnisse wurden Sensibilität und Spezifität des ELISAs überprüft und mittles Chi-Quadrat Test statistisch untersucht. Die Ergebnisse wurden separat für den ersten ELISA im Frühjahr, den zweiten ELISA im Herbst, einen dritten ELISA im Herbst des zweiten Untersuchungsjahres und die zusammengefaßten Ergebnisse des ersten und zweiten ELISAs analysiert.

16 Vögel, die anhand des ersten ELISAs als positiv für aviäre Tuberkulose diagnostiziert wurden, und drei weitere Vögel als Kontrolltiere sind in der zweiten Phase der Studie getötet

und seziert worden. Der obengenannte dritte ELISA wurde an Blutproben dieser Tiere durchgeführt. Von den 19 Vögeln wurden exemplarisch Leber- und Milzgewebeproben gewonnen und histopathologisch mittels Ziehl-Neelsen Färbung untersucht. 13 der histopathologisch untersuchten Proben wurden ebenfalls mittels PCR untersucht.

Zur endgültigen Bewertung von Spezifität und Sensibilität wurde auf die Daten des ersten ELISAs - von während des Untersuchungszeitraum verstorbenen Vögeln - kombiniert mit den Ergebnissen der 19 getöteten Tiere zurückgegriffen (Gesamtanzahl von 40 Vögeln).

Die Ergebnisse der ELISAs der Vögel, die sowohl im Frühjahr als auch im Herbst getestet wurden, zeigten statistisch signifikante Unterschiede (Sign-rank Test) zwischen den beiden Testreihen. Die Blutproben der Vögel, die im Herbst gewonnen wurden, zeichneten sich durch höhere Antikörper-Titer aus. Das heißt, daß die Tiere im Herbst durchschnittlich stärker positiv reagierten.

Bei den 19 während dieser Studie getöteten Vögeln konnten nur teilweise Übereinstimmungen der Sektionsergebnisse und histopathologischen Untersuchungen festgestellt werden. 27.8% (n=5) der Sektionsergebnisse wurden durch die Histopathologie als fraglich klassifiziert. Bei 13 dieser insgesamt 19 Vögel ließen sich nach Ziehl-Neelsen Anfärbung keine eindeutigen Befunde erzielen. Daraufhin wurden Untersuchungen mittels PCR durchgeführt, die jedoch in keinem Fall mykobakterielle DNA nachwiesen.

Basierend auf Sektionsergebnissen von 51 Vögeln, welche während des Untersuchungszeitraums verstarben, beträgt die Spezifität des untersuchten ELISAs 55.6% und die Sensitivität 76.9%. Die Analyse der ELISA- und Sektionsergebnisse mittels Chi-Quadrat Test auf unterschiedliche taxonomische Ordnung der Wasservögel bezogen, zeigte keine signifikanten Unterschiede. Die Ergebnisse in den Ordnungen *Dendrocygnini*, *Anserini*, *Tadornini*, *Somateriini* und *Oxyurini* müssen wegen des hohen Prozentsatzes an falsch positiven und falsch negativen Ergebnissen mit großer Vorsicht interpretiert werden. Es muß berücksichtig werden, daß die Anzahl der zur Verfügung stehenden Vögel in den unterschiedlichen Ordnungen teilweise für eine zuverlässige statistische Analyse zu gering ist.

Sommer und Herbst repräsentieren eine Zeit mit maximalem Stress und hohen Energieausgaben wegen des Brutgeschehens und der Mauser. Dieses zeigt sich in einem Verlust an Kondition und einer Beeinträchtigung des aviären Immunsystems. Vögel mit einem beeinträchtigten Immunsystem sind besonders anfällig für Infektionen mit nachfolgendem Krankheitsgeschehen. Bei den nachgewiesenen Antikörpern kann es sich entweder um unspezifische Antikörper zu einer Vielfalt von Antigenen handeln oder um Antikörper gegen Antigene von ubiquitäre nicht pathogene Mykobakterien.

Mykobakterien sind insbesondere aus Brackwasser und Schlamm isoliert worden, wo sie über Monate überleben können. Das Gelände in dem WWT Zentrum in Llanelli gewährleistet ideale Bedingungen für das Überleben und die Ausbreitung von Mykobakterien. In der hochgradig kontaminierten Umgebung müssen die Vögel sich sehr wahrscheinlich mit unterschiedlichen Serotypen des Erregers auseinandersetzen und entwickeln hohe Antikörper-Titer zu Antigenen von nicht pathogenen Mykobakterien. Dieses resultiert in Kreuz-Reaktionen und falsch positiven ELISA Ergebnissen.

Wiederholtes Testen von Blutproben, zum Beispiel zweimal jährlich über einen Zeitraum von mindestens zwei Jahren, ist notwendig, um mögliche jahreszeitliche Schwankungen der Antikörper-Titer aufzuzeigen.

Eine mögliche Erklärung für die unterschiedlichen Ergebnisse in den verschiedenen Ordnungen ist die Verwendung eines Anti-Enten-Antikörpers, welcher mit Meerrettichperoxidase konjugiert ist, für die ELISA Untersuchung von Blutproben von Vögeln aus mehreren unterschiedlichen Ordnungen (Enten, Gänse und Schwäne). Es ist denkbar, daß dieser Anti-Enten Antikörper nicht optimal an Antikörper von Gänsen und Schwänen bindet.

Falsch positive Ergebnisse sind möglicherweise durch unspezifische Bindung zu Antikörpern verursacht, welche einer anderen, in der Sektion jedoch nicht auffälligen, Infektion zugrunde liegen. Auf der anderen Seite ist bewiesen, daß eines der vier mykobakteriellen Antigene (Gruppe i) von allen Mykobakterien isoliert werden kann (GRANGE et al. 1980) (see **chapter B 2.1.3.2.**, **Literature review**, **Immunodiffusion serotypes**). Daher können bei der

Verwendung eines komplexen (polyklonalen) ELISAs Antikörper zu anderen Mykobakterien mit den *Mycobacterium avium* Antigenen des ELISAs kreuzreagieren. Durch die Verwendung spezifischer Antigene von tuberkulösen Vögeln des WWT Zentrum in Llanilli wurde versucht, diesem Problem entgegen zu treten.

Als Erklärungsmöglichkeiten für den hohen Anteil an fraglichen Ergebnissen bei dem Vergleich von Sektionsdaten und Histopathologie werden Färbetechnik und das Auftreten von Demarkierung und Einschmelzung der säurefesten Mykobakterien in verkäsenden Granulomen zu einem späten Stadium der Erkrankung bei älteren Tieren diskutiert. Desweiteren sind Fehlinterpretationen der Sektionsbefunde möglich. Zum Beispiel weist die Coligranulomatose differentialdiagnostisch Ähnlichkeiten mit mykobakteriellen Granulomen auf.

Auf Grund der geringen Übereinstimmung von Sektionsdaten und histopathologischen Untersuchungen muß die Evaluierung von Sensibilität und Spezifität des ELISAs kritisch betrachtet werden. Eine endgültige Diagnose könnte die Identifizierung spezies-spezifischer mykobakterieller DNA Fragmente mittels molekularbiologischer Techniken wie PCR liefern. Es ist zu bedenken, daß DNA, welche von gefrorenen Gewebeproben gewonnen wurde, eine geringere Zerfallstendenz bei der Durchführung der PCR zeigt als DNA, welche von Gewebeproben stammt, die für histopathologische Untersuchungen vorbereitete wurde. Für diesen Zerfall werden die Fixierung in Formalin, das Einbetten in Paraffinwax und die Extraktion der DNA verantwortlich gemacht. Zudem erschwert die starre Zellwand der Mykobakterien den Zugang zu der DNA.

Der in dieser Studie eingesetzte ELISA als ante-mortem Screening-Verfahren zum Nachweis einer aviären Tuberkulose-Infektion bedarf weiterer Verbesserung in Hinblick auf Spezifität und Sensibilität. Ein Screening Test mit 23.1% falsch negativer und 44.4% falsch positiver Ergebnissen, wie in der vorliegenden Studie, ist als diagnostischer Test sowohl für größere Bestände als auch für Einzeltiere nicht geeignet. In diesem Fall muß auf spezifischere Techniken zurückgegriffen werden. Einige solcher diagnostischer Techniken sind im Literaturteil im Detail erläutert. Weitere Untersuchungen zur Erhöhung der Spezifität sind wünschenswert. Als mögliche Verbesserung der Diagnostik kann eine begleitende

haematologische Untersuchung erwogen werden. Die Evaluierung des ELISAs muß anhand von Probanden, welche eindeutig als für Tuberkulose positiv oder negativ diagnostiziert wurden, durchgeführt werden. Da eine pathologische Sektion diese Bedingung nicht vollständig erfüllt, müssen eventuell molekular biologische Diagnosetechniken, beispielweise PCR, erwogen werden.

In einem weiteren Teil in dieser Studie wurden die Sektionsdaten von Vögeln, die in den letzten zehn Jahre verstorben sind, analysiert. So wurden Verteilung, Prävalenz und Mortalität der aviären Tuberkulose in dem WWT Zentrum in Llanelli bezüglich Geschlecht, Jahreszeit, Alter, Ordnung und Gehege beschrieben.

Hierbei wurden drei Gehege identifiziert (Nord-Amerika Gehege (72.4%), Asien-Gehege (32.4%) und Smew-Gehege (44.5%)), in denen die statistisch signifikant höchsten Vorkommen gefunden wurden. Die Mortalität der Erkrankung schien von dem Aufbau des Geheges, insbesondere des Wasserflusses durch andere Gehege, sowie vom Kontakt zu anderen Vögeln des Bestandes und zu Wildvögeln beeinflußt zu sein. Zusätzlich können die frühere Nutzung des Geländes als Farmland sowie die Nähe zu einer Dosenfabrik, welche ihre zinkhaltigen Abwässer in den Fluß leitet, der das WWT Zentrum in Llanelli mit Frischwasser versorgt, als Einflußfaktoren in Betracht gezogen werden.

Die Ordnungen *Mergini*, *Cairinini* und *Aythynini* zeigten die höchste Mortalität der aviären Tuberkulose, unter ihnen sind die statistisch signifikant am stärksten betroffenen Arten Zwergtaucher (*Mergus albellus*), Kappentaucher (*Mergus cucullatus*) und Malaien Ente (*Cairina scutulata*). Lebensraum, Freß- und Brutverhalten sind unter anderem für die Anfälligkeit einer bestimmten Ordnung für aviäre Tuberkulose verantwortlich. Weiterhin spielen auch verschiedene Umweltfaktoren wie die Vegetation in den Gehegen und die Streßbelastung eine Rolle in der Entwicklung der Erkrankung. Streß kann durch eine ungünstige Verteilung von Futter- und Nistplätzen, ein ungünstiges Verhältnis von weiblichen und männlichen Tieren, aggressiven und scheuen Arten in einem Gehege und hohe Besucherzahlen ausgelöst werden und beeinträchtigt das aviäre Immunsystem, so daß die Vögel für Infektionen anfälliger werden. Betrachtungen der Anfälligkeit unterschiedlicher

Arten in Bezug auf die oben genannten Faktoren zeigten eine deutliche genetische Prädisposition der Meeresenten und Säger.

Das Vorkommen von aviärer Tuberkulose zeigte keine Unterschiede bezüglich des Geschlechtes und unterschiedlicher Jahreszeiten.

1994 ist in dem WWT Zentrum in Llanelli zum ersten Mal aviäre Tuberkulose diagnostiziert worden. Seitdem ist eine ständige Erhöhung der Mortalität zu verzeichnen. Hervorzuheben ist ein drastischer Anstieg der Mortalität von 1997 (24,4%) zu 1998 (40,7%), gefolgt von einem weiteren geringeren Anstieg in 1999 (43,4%). Eine Auswertung der Daten bezüglich der verschiedenen Ordnungen zeigte, daß diese erhöhte Mortalität in allen Ordnungen zu finden ist. Eine mögliche Ursache ist das fortgeschrittene Alter vieler Vögel, welche ihre durchschnittliche Lebenserwartung erreicht haben und deren Immunsystem altersbedingt geschwächt sein kann (VAN DER HEYDEN, 1997a; CROMIE et al., 2000; and CROMIE, personal communication). Desweiteren sind die Teiche in 1998 entschlammt worden. Durch diese Arbeit ist viel Schlamm aufgewühlt und die Ufer sind mit dem Erreger kontaminiert worden. Es ist denkbar, daß Vögel so verstärkt mit Mykobakterien in Kontakt kamen und erkrankten.

Abschließend wurde ein Management-Plan entworfen, um Bemühungen, die aviäre Tuberkulose zu kontrolieren, zu unterstützen. Der Plan umfaßt sowohl generelle Aspekte der Kontrolle von aviärer Tuberkulose als auch spezifische Maßnahmen für das WWT Zentrum in Llanelli. Genaue Anweisungen für Reinigung und Desinfektion mit phenolischen Lösungen, organischen Säuren oder Kresolen werden gegeben. Die Sanierung der Gehege beinhaltet die Entfernung von Bodensubstrat und die Schaffung von suboptimalen Lebensbedingungen für Mykobakterien in den Gehegen. Alle Gehege sollten rotationsmäßig gereinigt und saniert werden. Weiterhin muß Ungeziefer, welches zur Verbreitung von Erkrankungen beiträgt, kontrolliert werden. Das Verbreiten der Erkrankung von einem Gehege zum anderen muß durch Kompartimentierung des Geländes mit minimaler Interaktion eingeschränkt werden. In dieser Beziehung ist es besonders wichtig, den Kontakt zu anderern Vögeln des Bestandes und zu Wildvögeln zu minimieren. Besonders empfindliche Arten wie die Malaien Ente, Zwergsäger und Kappensäger sollten isoliert

gehalten werden. Die wichtigste Maßnahme ist eine Veränderung des Wasserflusses durch das Gelände. Zur Kontrolle von Erkrankungen muß die Wasserversorgung der einzelnen Gehege parallel geschaltet sein. Dadurch wird eine Kontaminierung und eine Anhäufung der Erreger in den Gehegen vermieden. Desweiteren werden Vorschläge zur Minimierung des Stresses, dem Vögel eines Bestandes zwangsläufig ausgesetzt sind, gemacht. Eine sorgfältig durchdachte Quarantäne ist notwendig, um das Einbringen von Erkrankungen wie aviäre Tuberkulose in Bestände zu kontrollieren. Alle Neuzugänge, aber auch alle anderen Vögel in einem Bestand, sollen routinemäßig ein Screening Programm durchlaufen. Dieses Programm besteht aus einer klinischen und haematologischen Untersuchung sowie der Durchführung des ELISAs. Das regelmäßige Merzen Tuberkulose-positiver Vögel kann die Ansammlung von Erregern und Kontaminierung der Gehege erheblich reduzieren. Auf Grund der hohen Tenazität des Erregers, des chronischen Charakters der Erkrankung und des Carrier-Status einiger Tiere ist die völlige Entfernung der aviären Tuberkulose aus einem Bestand unrealistisch. Vielmehr müssen Maßnahmen zur Kontrolle der Erkrankung in den Vordergrund gestellt werden.

H. REFERENCES

ACKERMANN, L.J., S.C. BENBROOK and B.C. WALTON (1974):
Mycobacterium tuberculosis infection in a parrot (*Amazona farinosa*).
American Review of Respiratory Diseases 109, 388-390

AIKEN, J. (1999):
Comparative binding capacities of staphylococcal protein A and commercially available anti-bird sera to exotic bird immunoglobulins.
B.Sc. Thesis, Intercalated B.Sc Medical Microbiology, University College London

ASH, N.J. and R.L. CROMIE (1998):
Predictive value of the ELISA for the diagnosis of avian tuberculosis.
Wildfowl and Wetlands Trust, Internal report

ARANAZ, A., E. LIÉBANA, A. MATEOS. and L. DOMINGUEZ (1997):
Laboratory Diagnosis of avian mycobacteriosis.
Seminars in Avian and Exotic Pet Medicine 6 (1), 9-17

ARLOING, S. (1898):
Agglutination de bacillede la tuberculose vrai.
Comptes Rendues de l'Academie de Sciences 126, 1398-1400
cited by: DANIEL, T.M. and S.M. DEBANNE (1987):
The serodiagnosis of tuberculosis and other mycobacterial diseases by enzyme-linked immunosorbent assay.
American Reviews of Respiratory Diseases 135, 1137-1151

ARSTILA, T.P. (1996):
T-cell subsets and the activation of γδ-T-cells.
In: Vainio O., B.A. Imhoff (eds.), Immunology and developmental biology of the chicken.
Berlin, Heidelberg, Springer-Verlag, 71-78
cited by: CROMIE, L.R., N.J. ASH, M.J. BROWN and J.L. STANFORD (2000):
Avian immune responses to *Mycobacterium avium*: the wildfowl example.
Developmental and Comparative Immunology 24 (2000), 169-185

BARNES, P.F., M.P. HAPP, A. DALLAS, E. PALMER, R. KUBO and W.K. BORN (1992):
γδ-T-lymphocytes in human tuberculosis.
Journal of Infectious Diseases 165, 506-512

BARTON, N.W.H. and D.C. HOUSTON (1994):
Morphological adaptation of the digestive tract in relation to feeding ecology of raptors.
Journal of Zoology, London 232, 133-150

BEEHLER, B.A. (1990):
Management of *Mycobacterium avium* in a mixed species aviary.
Proceedings American Association of Zoo Veterinarians, October 21st-26th, Padre Island, Texas, USA, 125-129

BENSON, C.A. and J.J. ELLNER (1993):
Mycobacterium avium complex infection and AIDS:Advances in theory and practice.
Clinical Infectious Diseases 17, 7-20

BERMUDEZ, L.E., P. KOLONOSKI and L.S. YOUNG (1990):
Natural killer cell activity and macrophage dependent inhibition of growth of killing of *Mycobacterium avium* complex in a mouse model.
Journal of Leukocyte Biology 47, 67-73

BERMUDEZ, L.E. and L.S. YOUNG (1991a):
Natural killer cell dependent mycobacteriostatic and mycobactericidal activity in human macrophages.
Journal of Immunity 146, 265-269

BERMUDEZ, L.E., L.S. YOUNG and H. ENGEL (1991b):
Interaction of *Mycobacterium avium* complex with human macrophages: Roles of membrane receptors and serum proteins.
Infection and Immunity 59, 1697-1702

BERMUDEZ, L.E., M. WU, H. ENGEL and L.S. YOUNG (1992a):
Interleukin-6 antogonize tumor necrosis factor-mediated mycobacteriostatic and mycobacterial activities in macrophages.
Journal of Immunology 60, 4245-4251

BERMUDEZ, L.E., M. PETROFSKI, P. KOLONOSKI and L.S. YOUNG (1992b):
An animal model of *Mycobacterium avium* complex disseminated infection after colonisation of the intestinal tract.
Journal of Infectious Diseases 165, 75-79

BERMUDEZ, L.E. (1993):
Production of transforming growth factor b by *Mycobacterium avium* infected macrophages is associated with unresponsivesness to interferon gamma.
Journal of Immunology 150, 1838-1845

BERMUDEZ, L.E. (1994):
Immunobiology of *Mycobacterium avium* infection.
European Journal of Clinical Microbiology and Infectious Diseases 13 (11), 1000-1006

BILLIINGTON, S. (2000):
Extraction and semi-quantification of environmental *Mycobacterium avium* at the Wildfowl and Wetlands Trust, Slimbridge, using polymerase chain reaction.
MSc Thesis, University of London

BLANCHARD, D.K., M.B. MICHELINE-NORRIS, H. FRIEDMANN and J.Y. DJEU (1989):
Lysis of mycobacteria-infected monocytes by IL-2 activated killer cells.
Cellular Immunology 119, 402-411

BLANCHARD, D.K., S. MCMILLEN, S.L. HOFFMANN and J.Y. DJEU (1992):
Mycobacterial induction of activated killer cells: possible role of tyrosine kinase activity in interleukine-2 receptor alpha expression.
Infection and Immunity 60, 2843-2849

BRENNAN, P.J. (1981):
Structures of the typing antigens of atypical mycobacteria: a brief review of present knowledge.
Reviews of Infectious Diseases, 3B, 905-913

BROWN, M.J. and S.P.C. PICKERING (1992):
The mortality of captive flamingos at Slimbridge (1975-89).
Wildfowl 43, 185-190

BROWN, M.J., E. LINTON and E.C. REES (1992):
Causes of mortality among wild swans in Britain.
Wildfowl 43, 70-79

BROWN, D. (1998):
A guide to pheasants and waterfowl, their management, care and breeding.
Nigel Steele-Boych (eds.), A.B.K. Publications, Australia

BROWNBACK, P.E. and W.W. BARROW (1988):
Modified lymphocyte response to mitogens after intraperitoneal injection of glycopeptidolipid antigens from *Mycobacterium avium* complex.
Infection and Immunity 56, 1044-1050

BUSH, M., R.J. MONTALI, B.S. SMITH and W.S. PERATINO (1978):
Clinical experience with tuberculosis in exotic birds.
In: Mycobacterial Infections of Zoo Animals, Montali R.J. (eds.), Washington D.C.:
Smithsonian Institution Press, 199-204

BUTLER, W.R., K.C. JOST and J.O. KILBRUN (1991):
Identification of mycobacteria by high-performance liquid chromatography.
Journal of Clinical Microbiology 29, 2468-2472

CHAMPSI, J.H., L.E. BERMUDEZ and L.S. YOUNG (1994):
The role of cytokines in mycobacterial infection.
Biotherapy 7, 187-193

CHANG, C.F. and P.B. HAMILTON (1979):
Refractory phagocytosis by chicken thrombocytes during aflatoxicosis.
Poultry Science 58, 559-561
cited by SANDER, I. (1995):
Untersuchungen zum Leukozytentransformationstest unter Verwendung von 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazoliumbromid (MTT) an peripheren Blutleukozyten des Huhnes (*Gallus gallus domesticus*).
Dissertation an der Tierärztlichen Hochschule Hannover

CHEVILLE, N.F. and W.D. RICHARD (1971):
The influence of thymic and bursal lymphoid systems in avian tuberculosis.
American Journal of Pathology, 97-122
cited by: MONTALI, R.J., M. BUSH, C.O. THOEN and E. SMITH (1976):
Tuberculosis in captive exotic birds.
Journal of the American Veterinary Medical Association 169, 920-927

CHEVILLE, N.F. (1979):
Environmental factors affecting the immune response of birds- A review.
Avian Diseases 23, 308-314

CLARK, S.L., M.T. COLLINS and J.I. PRICE (1995):
New methods for diagnosis of *Mycobacterium avium* infection in birds.
Proceedings of the joint conference of the Association of Avian and Zoological Veterinarians, August 12th-17th, East Lansing, Minchigen, USA, 151-154

COLLINS, F.M. and R.W. STOKES (1987):
Mycobacterium avium complex infection in normal and immunosuppressed mice.
Tubercle 68, 127-136

COOK, H.C. (1974):
Ziehl-Neelsen (ZN) technique for tubercle bacilli.
In: Butterworths and Co.(eds.), Manual of histological demonstration techniques.
Ltd. London, 123-125

CORNER, L.A., A.C. TRAJSTMAN and K. LUND (1995):
Determination of the optimum concentration of decontaminants for the primary isolation of *Mycobacterium bovis*.
New Zealand Veterinary Journal 43, 129-133

COWAN, D.F. (1968):
Avian Amyloidosis. I. General Incidence in Zoo Birds.
Veterinary Pathology 5, 51-58
cited by: MONTALI, R.J., M. BUSH, C.O. THOEN and E. SMITH (1976):
Tuberculosis in captive exotic birds.
Journal of the American Veterinary Medical Association 169, 920-927

CRISP, E. (1872):
Specimens and drawings showing the origin of tubercle in the lower animals and the microscopic appearance of the blood in some examples.
Transactions of the Pathological Society of London 19, 472
cited by MUTALIB, A.A. and C. RIDDLE (1988):
Epizootiology and pathology of avian tuberculosis in chickens in Saskatchewan.
Canadian Veterinary Journal 29 (10), 840-842

CRISSEY, S., E.F. STEVENS, F. CUSIMANO and S.R. DERRICKSON (1989):
A submersible diet for ducks to control diseases and cost.
Journal of Zoo and Wildlife Medicine 20, 68-72

CROMIE, R.L., J.L. STANFORD, M.J. BROWN and D.J. PRICE (1989):
The transport and culture conditions for optimum transformation response of wildfowl lymphocytes to mycobacterial antigens.
Journal of Immunological Methods 122 (2), 195-201

CROMIE, R.L. (1991):
Development of an avian tuberculosis vaccine for captive wildfowl.
Ph.D.thesis, University of London

CROMIE, R.L., M.J. BROWN, D.J. PRICE and J.L. STANFORD (1991):
Susceptibility of captive wildfowl to avian tuberculosis: the importance of genetic and environmental factors.
Tubercle 72, 105-109

CROMIE, R.L., M.J. BROWN and J.L. STANFORD (1992):
The epidemiology of avian tuberculosis in white-winged wood ducks (*Cairina scutulata*) at The Wildfowl and Wetlands Trust, Slimbridge Centre (1976-1991).
Wildfowl 43, 211-214

CROMIE, R.L., J.L. STANFORD, M.J. BROWN and D.J. PRICE (1993a):
Epizootiology of avian tuberculosis at the Wildfowl and Wetland Trust Centre in Slimbridge.
Proceedings of the Zoological Society of London, British Veteriany Zoological Society, Autumn Meeting 1990

CROMIE, R.L., M.J. BROWN, J.L. STANFORD and N.A. FORBES (1993b):
Avian tuberculosis in captive and free-living waterfowl.
British Ornithologist's Union-Proceedings, Autumn Scientific Meeting, Cambridge

CROMIE, R.L., M.J. BROWN, N.A. FORBES, J. MORGAN and J.L. STANFORD (1993c):
A comparison and evaluation of techniques for diagnosis of avian tuberculosis in wildfowl.
Avian Pathology 22 (3), 617-630

CROMIE, R.L., N.J. ASH, M.J. BROWN and J.L. STANFORD (2000):
Avian immune responses to *Mycobacterium avium*: the wildfowl example.
Developmental and Comparative Immunology 24 (2000), 169-185

CROWLE, A., R. DAHL, E. ROSS and M.H. MAY (1991):
Evidence that vesicles containing living, virulent *Mycobacterium tuberculosis* or *Mycobacterium avium* in cultured human macrophages are not acidic.
Infection and Immunity 59, 1823-1827

CZIFRA, G., M. NILSSON, D.J. ALEXANDER, R. MANVELL, S. KECSKEMÉTI and B.E. ENGSTRÖM (1996):
Detection of PMV-1 specific antibodies with a monoclonal antibody blocking enzyme-linked immunosorbent assay.
Avian Pathology 25, 691-703

DABORN, C.J. and J.M. GRANGE (1993):
HIV/AIDS and its implication for the control of animal tuberculosis.
British Veterinary Journal 149, 405-417

DANIEL, T.M. and S.M. DEBANNE (1987):
The serodiagnosis of tuberculosis and other mycobacterial diseases by enzyme-linked immunosorbent assay.
American Reviews of Respiratory Diseases 135, 1137-1151

DELACOUR, J. and E. MAYR (1945):
The family *Anatidae*.
Wilson Bulletin 57, 3-55

DENIS, O. and E.O. GREGG (1990):
Recombinant tumor necrosis factor-alpha decreases whereas recombinant interleukin-6 increases growth of a virulent strain of *Mycobacterium avium* in human macrophages.
Journal of Immunology 71, 25-29

DERRICKSON, S.R. and C. PICKETT (1991):
History of avian tuberculosis at the National Zoological Park: gaining control through management and husbandry.
Annual Conference Proceedings of the American Association of Zoological Parks and Aquariums, Autumn meeting, St. Augustine, Florida - sponsored by Marineland, 476-784

DUMONCEAUX, G. and G. HARRISON (1994):
Toxins.
In: Ritchie, B.W., Harrison, G.J. and Harrison L.R. (eds.): Avian Medicine: Principles and Application.
Wingers Publishing, Inc., Lake Worth, FL, 950-582

ENGVALL, E. and P. PERLMANN (1971):
Enzyme-linked immunosorbent assay.
Immunochemistry 8, 871-874

FALKINHAM III, J.O. (1996):
Epidemiology of infection by non-tuberculous mycobacteria.
Clinical Microbiological Reviews 9, 177-215

FORBES, N.A., R.L. CROMIE, M.J. BROWN, R.J. MONTALI, M. BUSH and J.L. STANFORD (1993):
Diagnosis of avian tuberculosis in waterfowl.
Proceedings of the annual conference of the Association of Avian Veterinarians, Houston, Texas, USA, 23rd – 4th September 1993, 182-186

FORBES, N.A. and T. RICHARDSON (1996):
Husbandry and Nutrition.
In: Forbes, N.A. and Beynon, P.H. (eds.), BSAVA Manual of Raptors, Pigeons and Waterfowl, British Small Animal Veterinary Association Limited, Cheltenham, 289-298

FORSTER, F. and H. GERLACH (1987):
Mycobacteria in psittaciformes.
Proceedings of annual conference of the Association of Avian Veterinarians, Honolulu, Hawaiii, USA, 6th – 11th September 1987, 39-56

FREEMAN, S. and W.M. JACKSON (1990):
Univariate metrics are not adequate to measure avian body size.
The Auk 107, 69-74

GERLACH, H. (1994a):
Bacteria.
In: Ritchie, B.W., G.J. Harrison and L.R. Harrison (eds.): Avian Medicine: Principles and Application.
Wingers Publishing, Inc., Lake Worth, FL, 950-582

GERLACH, H. (1994b):
Defence mechanisms of the avian host.
In: Ritchi, B.W., G.J. Harrison and L.R. Harrison (eds.): Avian Medicine: Principles and Application., Wingers Publishing, Inc., Lake Worth, Florida, 109-120

GÖTZ, K., F. GRIMM and J. KÖSTER (1986):
Aviäre Tuberkulose – Erfahrungen mit einem Bekämpfungsprogramm.
Der Praktische Tierarzt, 3, 200-202

GOLDMANN, T., E. RICHTER, M. DEUTSCHBEIN, J. GALLE and E. VOLLMER (1998):
PCR-based detection of mycobacteria in formalin-.fixed, paraffin-embedded tissues as a molecular tool for the pathologist.
Electronic Journal of Pathology and Histopathology 4 (3), 1-6

GRANGE, J.M. and M.D. YATES (1986):
Infections caused by the opportunistic mycobacteria: A review.
Journal of the Royal Society of Medicine 79, 226-229

GRANGE, J.M., M.D. YATES and E. BOUGHTON (1990):
A Review. The avian tubercle bacillus and its relatives.
Journal of Applied Bacteriology 68, 411-431

GRANGE. J.M. (1996):
The biology of the genus *Mycobacterium*.
Society of Applied Bacteriology Symposium-Series 25, 1-9

GREEN, A.J. (2000):
Mass/length residuals: Measures of body condition or generators of spurious results?
Department of Applied Biology, Estación Biológica de Doñana, Sevilla, Spain. 1-36
(submitted for publication)

GREUEL, R. (1988):
Untersuchungen zum parenteralen Immunglobulintransfer bei der Taube.
Veterinärmedizinische Dissertation, München

GREY, H.M. (1967):
Duck immunoglobulin I.:Structural studies on 5.7S and 7.8S immunoglobulin G.
Journal of Immunology 98, 811-819

GROSS, W.B., J.O. FALKINHAM III and J.B. PAYEUR (1989):
Effect of environmental-genetic interactions on *Mycobacterium avium* challenge infection.
Avian Diseases 33 (3), 411-415

GRUFT, H., A. LODER, M. OSTERHOUT, B.C. PARKER and J.O. FALKINHAM III (1979):
Postulated sources of *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum* infection: isolation of mycobacteria from estuaries and ocean waters.
American Review of Respiratory Diseases 120, 1385-1388

HAAGSM, J. and A. EGER (1990):
ELISA for diagnosis of tuberculosis and chemotherapy in zoo and wildlife animals.
Proceedings of American Association of Zoo Veterinarians, October 21st-26th, Padre Island, Texas, USA, 125-129

HÄDGE, D. and H. AMBROSIUS (1983):
Evolution of low molicular weight immunoglobulins – III. The immunoglobulin of chicken bile – not an IgA.
Molecular Immunology 20, 597-606

HAWKEY, C., R.A. KOCK, G.M. HENDERSON and R.N. CINDERY (1990):
Haematological changes in domestic fowl (G*allus gallus*) and cranes (G*ruiformes*) with *Mycobacterium avium* infection.
Avian Pathology 19, 223-234

HEJLICEK, K. and F. TREML (1993a):
Epidemiology and pathogenesis of avian mycobacteriosis in collared doves and turtle doves (*Streptopelia spp*.).
Veterinarni-Medicina 38 (10), 619-628

HEJLICEK, K. and F. TREML (1993b):
Epidemiology and pathogenesis of avian mycobacteriosis in house sparrow (*Passer domesticus*) and the tree sparrow (*Passer mantanus*).
Veterinarni-Medicina 38 (11), 667-685

HEJLICEK, K. and F. TREML (1993c):
Epidemiology and pathogenesis of avian mycobacteriosis in pheasants (*Phasianus colchicus*) and partridges (*Perdix perdix*).
Veterinarni-Medicina 38 (11), 687-701

HEJLICEK, K. and F. TREML (1994a):
Epidemiology and pathogenesis of avian mycobacteriosis in rooks (*Corvus frugilegus*).
Veterinarni-Medicina 39 (3), 337-344

HEJLICEK, K. and F. TREML (1994b):
Epidemiology and pathogenesis of *Mycobacterium avium* in domestic pigeons (*Columba livia f. domestica*).
Veterinarni-Medicina 39 (10), 615-624

HEJLICEK, K. and F. TREML (1994c):
Epidemiology and pathogenesis of avian mycobacterial infection in the black-headed gull (*Larus ridibundus*).
Veterinarni-Medicina 39 (5), 271-278

HIGGINS, D.A. and S.H. CHUNG (1986):
Duck lymphocytes I. Purification and preliminary observations on surface markers.
Journal of Immunological Methods 86, 231-238

HIGGINS, D.A. (1990):
Duck lymphocytes III, Transformation responses to some common mitogens.
Comparative Immunology and Microbiology of Infectious Diseases 13 (1), 13-23

HIGGINS, D.A. and G.W. WARR (1993):
Duck immunoglobulins: structure, function and molecular genetics.
Avian Pathology 22, 211-236

HIGGINS, D.A., R.L. CROMIE, S.S. LIU, K.E. MAGOR and G.W. WARR (1995):
Purification of duck immunoglobulins: an evaluation of protein A and protein G affinity chromatography.
Veterinary Immunology and Immunopathology 44, 169-180

HINES, M.E., J.M. KREEGER and A.J. HERRON (1995):
Mycobacterial infections of animals: pathology and pathogenesis.
Laboratory Animal Science 45 (4), 334-351

HOFFNER S.E. (1988):
Improved detection of *Mycobacterium avium* complex with the BACTEC radiometric system.
Diagnosis and Microbiology of Infectious Diseases 10, 1-6

HOFFNER, S.E., M. KRATZ, B. OLSSON-LILJEQUIST, S.B. SVENSON and
G. KALLENIUS (1989):
In-vitro syergistic activity between ethambutol and fluorinated quinolones against *Mycobacterium avium* complex.
Journal of Antimicrobial Chemotherapy 24, 317-324

HOOP, R.K., E.C. BOTTGER and G.E. PFYFFER (1996):
Etiological agents of mycobacteriosis in pet birds between 1986 and 1995.
Journal of Clinical Microbiology 34, 991-992

HOOP, R.K. (1997):
Public Heath Implications of Exotic Pet Mycobacteriosis.
Seminar in Avian and Exotic Pet Medicine 6 (1), 3-8

HUBBARD, R.D., C.M. FLORY and F.M. COLLINS (1992):
T-cell immune response in *Mycobacterium avium*-infected mice.
Infection and Immunity 60, 150-153

INDERLIED, C.B., C.A. KEMPER and L.E.M. BERMUDEZ (1993):
The *Mycobacterium avium* complex.
Clinical Microbiology Reviews 6 (3), 266-310

JOHNSGARD, P.A. (1978):
Ducks, geese and swans of the world.
Lincoln, London: University of Nebraska Press, 17-33

KÄLLENIUS, G., S.G. SVENSON and S.E. HOFFNER (1989):
Ethambutol: a key for *Mycobacterium avium* complex chemotherapy.
American Review of Respiratory Diseases 140, 264

KEMPER, C.A., T. MENG and J. NUSSBAUM (1992):
Treatment of *Mycobacterium avium* complex bacteriemia in AIDS with a four drug oral regiment rifampin, ethambutol, clofazamine, and ciprofloxacin.
Annals of Internal Medicine 116, 466-472

KEMPER, C.A., D. HAVLIER and D. HAGHIGHAT (1994):
The individual microbiologic effects of the three antimycobacterial agents clofazamine, ethambutol and rifampin on *Mycobacterium avium* complex bacteraemia in patients with AIDS.
Journal of Infectious Diseases 170, 157-164

KIRIHANA, J.M., S.L. HILLIER and M.B. COYLE (1985):
Improved detection times for *Mycobacterium avium* and *Mycobacterium tuberculosis* with the BACTEC radiometric system.
Journal of Clinical Microbiology 22, 841-845

KIRSCHNER, R.A., B.C. PARKER and J.O. FALKINHAM III (1992):
Epidemiolgy of infection by non-tuberculous mycobacteria.
American Journal of Respiratory Diseases 145, 271-275

KIRSCHNER, R.A., B.C. PARKER and J.O. FALKINHAM III (1999):
Humic and fulvic acids stimulate the growth of *Mycobacterium avium*.
Federation of European Microbiological Societies (FEMS) Microbiology Ecology 30, 327-332

KÖHLER, H. (1991):
Vögel-Geflügeltuberkulose.
In: Schulz. L. (eds.): Pathologie der Haustiere, Teil II, Krankheiten und Syndrome
Gustav Fischer Verlag Jena, Stuttgart, 265-266

KOVACIC, H. and B. TUNKL (1978):
Avian tuberculosis in swine. Sparrows as carriers of *Mycobacterium avium*.
Veterinarski-Glasnik 32:8, 651-654

KOVACIC, H., M. LACKOVIC, S. MAJSTOROVIC and N. BRANEZAC (1981):
Importance of rooks (*Corvus frugilegus*) in the occurrence of *Mycobacterium avium* infection on pig farms.
Veterinarski-Glasnik 39:12, 1325-1330

KRISTENSEN, F., B. KRISTENSEN and S. LAZARY (1982):
The lymphocytes transformation test in veterinary immunology.
Veterinary Immunology and Immunopathology 3, 203-277

KUZE, F. and UCHIHIRA, F. (1984):
Various colony - formers of *Mycobacterium avium – intracellulare.*
European Journal of Respiratory Diseases 65, 402-410

KYARI, A.D. (1995):
Investigations into the vertical transmission of *Mycobacterium avium* through the eggs of wildfowl.
MSc Thesis, University of London, pp. 58

LATIMER, K.S. and P.M. RAKICH (1994):
Necropsy examination.
In: Ritchie, B.W., G.J. Harrison and L.R. Harrison (eds.): Avian Medicine: Principles and Application.
Wingers Publishing, Inc., Lake Worth, FL, 950-582

LUMEIJ, J.T. (1994):
Hepatology.
In: Ritchi, B.W., G.J. Harrison and L.R. Harrison (eds.): Avian Medicine: Principles and Application., Wingers Publishing, Inc., Lake Worth, Florida, 522-537

LYASHCHENKO, K.P., J.P. POLLOCK, R. COLANGELI and M.L. GENNARO (1998):
Diversity of antigen recognition by serum antibodies in experimental bovine tuberculosis.
Infection and Immunity 66 (11), 5344-5349

MANDELL, G.L. and W.A. PETRI (1996):
Drugs used in the chemotherapy of tuberculosis, *Mycobacterium avium* complex diseases and leprosy.
In: GOODMAN, L.S. (eds.): Goodman and Gilman´s The Pharmacologic Basis of Therapeutics. New York, NY, McGraw Hill. 1155-1166

MATTHEWS, P.R., A. McDIARMID, P. COLLINS and A. BROWN (1977):
The dependence of some strains of *Mycobacterium avium* on mycobactin for initial and subsequent growth.
Journal of Medical Microbiology 11, 53-57

MAYER, B.K. and J.O. FALKINHAM III (1986):
Superoxide dismutase activity of *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum*.
Infection and Immunity 56, 631-635

McCARTHY, C. and P. ASHBAUGH (1981):
Factors that affect the cell cycle of *Mycobacterium avium*.
Review of Infectious Diseases 3, 914-925

McINTYRE, G. and J.L. STANFORD (1986):
The relationship between immunodiffusion and agglutination serotypes of *Mycobacterium avium* and *Mycobacterium intracellulare*.
European Journal of Respiratory Diseases 69, 146-152

MEISNER P.S. and J.O. FALKINGHAM III (1986):
Plasmid DNA profiles as epidemiological markers for clinical and environmental isolates of *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum*.
Journal of Infectious Diseases 153, 325-331

MIDDLEBROOK, G., Z. REGGIARDO and W.D. TIGERTT (1977):
Automatable radiometric detection of growth of *Mycobacterium tuberculosis* in selective media.
American Review of Respiratory Diseases 155, 1067-1069

MIZUGUCHI, Y., M. FUKUNAGA and H. TANIGUCHI (1981):
Plasmid deoxyribonuclease and translucent-to-opaque variation in *Mycobacterium intracellulare*.
Journal of Bacteriology 146, 264-267

MONTALI, R.J., M. BUSH, C.O. THOEN and E. SMITH (1976):
Tuberculosis in captive exotic birds.
Journal of the American Veterinary Medical Association 169, 920-927

MONTALI, R.J., M. BUSH and R.A. FREEMEN (1983):
Effect of sanitation and antemortem screening on the incidence of tuberculosis in a zoo aviary.
Proceedings of the 25th International Symposium on Diseases in Zoo Animals, 95-99

MONTALI, R.J., D.K. NICHOLS, M. BUSH, S.R. DERRICKSON, C. PICKETT, R. IPPEN and H.D. SCHRÖDER (1992):
Control of avian tuberculosis in zoological parks: a perspective.
Erkrankungen der Zootiere, Verhandlungsbericht des 34. Internationalen Symposiums über Erkrankungen der Zoo- und Wildtiere, Santander- Spain, 71-76

MIYAMATOTO, M., Y. YAMAGUCHI and M. SASATSU (2000):
Disinfectant effects of hot water, ultraviolet light, silver ions and chlorine on strains of *Legionella* and non-tuberculous mycobacteria.
Microbios 101, 7-13

NESIC, D., D. RADOSAVLJEVIC and S. ROMANIC (1992):
Pigeons as vectors of tuberculosis in pigs.
Veterinarski-Glasnik 46:1, 51-53

NEUMANN, U. and E.F. KALETA (1992):
Immunsystem und Immunreaktionen.
In: Heider, G. and G. Montreal (eds.): Krankheiten des Wirtschaftsgeflügels, Gustav Fischer Verlag Jena, Stuttgart, Bd. I, 159-185.

O'BRIEN, S.J. and J.F. EVERMAN (1989):
Interactive influences of infectious diseases and genetic diversity in natural populations.
Trends in Ecology and Evolution 3, 254-259

PAINTER, K.S. (1995):
An investigation into the epizootiology and control of an outbreak of avian tuberculosis in captive wildfowl.
MSc Thesis, University of Kent

PAINTER, K.S. (1997):
Avian tuberculosis caused by *Mycobacterium avium* serotype 3 in captive wildfowl.
Veterinary Record 140 (17), 457-458

PALLASKE, G. (1960):
Entzündungen
In: Pallaske, G. (eds.): Pathologische Histologie, Leitfaden der Histopathologie für Studierende der Vet.-Medizin und Tierärzte, Gustav Fischer Verlag Jena, Stuttgart, 220-224

PANIGRAHY, B., F.D. CLARK and C.F. HALL (1983):
Mycobacteriosis in psittacine birds.
Avian Diseases 27, 1166-1168

PARKER, B.C., M.A. FORD, H. GRUFT and J.O. FALKINGHAM III (1983):
Epidemiology of infection by nontuberculous mycobacteria. IV. Preferential aerolisation of *Mycobacterium intracellulare* from natural waters.
American Review of Respiratory Diseases 128, 652-656

PAO, C.G., S.S. LIN and S.Y. WU (1988):
The detection of samples with cloned *Mycobacterium tuberculosis* DNA as probes.
Tubercle 69, 27-36

PAVLAS, M., V. PATLOKOVÁ and J. ZAJÍEK (1983):
Morphological changes in geese after experimental and natural infection with *Mycobacterium avium* serotype 2.
Acta Veterinaria Brno 52, 163-167

PAYNE, L.N. (1971):
The lymphoid system.
In: Phyiology and Biochemistry of the Domestic Fowl. Vol.2, D. J. Bell and B. M. Freeman (eds.), Academic Press, New York, 1024-1030

PIECHOCKI, R., R. IPPEN and H.D. SCHRÖDER (1981):
Tuberculosis in free-living hawks (*Falconiformes*) and owls (*Strigiformes*).
Erkrankungen der Zootiere,Verhandlungsbericht des XXIII. Internationalen Symposiums über die Erkrankungen der Zootiere, 24.-28. Juni, Halle-Saale, 55-66

PLIKAYTIS, B.B., B.D. PLIKAYTIS and M.A. YAKRUS (1992):
Differentiation of slow growing *Mycobacterium* species, including *Mycobacterium tuberculosis*, by gene amplification analysis and restriction fragment length polymorphism analysis.
Journal of Clinical Microbiology 30, 1815-1822

PORTAELS, F., L. REALINI, L. BAUWENS, B. HIRSCHEL, W.M. MEYERS and W. DE MEURICHY (1996):
Mycobacteriosis caused by *Mycobacterium genavense* in birds kept in a zoo:11-year survey.
Journal of Clinical Microbiology 34 (2), 319-323

RAO, S.P., K. OGATA and A. CATANZARO (1993):
Mycobacterium avium-Mycobacterium intracellulare binds to the integrin receptor (νβ3) on human monocytes and monocyte-derived macrophages.
Infection and Immunity 61, 663-670

REMPLE, J.D. and A. AL-ASHBAL (1993):
Raptor bumblefoot: another look at histopathology and pathogenesis.
In: Redig, P.T., J.E. Cooper, J.D. Remple and D.B. Hunter (eds.), Raptor Biomedicine.
Minneapolis, Minnesota: University of Minnesota Press, 92-98

RICHTER, E., C. SCHLÜTER, M. DUCHROW, M. HAHN, S. RÜSCH-GERDES, J. GALLE, H.D. FLAD and J. GERDES (1995):
An improved method for the species-specific assessment of mycobacteria in routinely formalin-fixed and paraffin-embedded tissues.
Journal of Pathology 175, 85-92

ROCKE, T.E., S.R. SMITH and S.W. NASHOLD (1998):
Preliminary evaluation of a simple *in vitro* test for the diagnosis of type C botulism in wild birds.
Journal of Wildlife Diseases 34 (4), 744-751

ROSSI, L. (1974):
Immunizing potency of inactivated and living *Mycobacterium avium* and *Mycobacterium intracellulare* vaccines against tuberculosis in domestic fowls.
Acta Veterinaria Brno 43 (2), 133–138

ROSSKOPF, W.J., R.W. WOERPEL and R. LANE (1989):
Avian tuberculosis in the grey-cheeked parakeet and other related brotogeris parakeets – another clinical syndrome: Incidence, clinical characteristics, clinical pathology, lesions and possible treatment.
Proceedings of the Association of Avian Medicine, Miami, FL, 219-236
cited by: VAN DER HEYDEN, N. (1997):
New strategies in the treatment of avian mycobacteriosis.
Seminars in Avian and Exotic Pet Medicine 6 (1), 25-33

ROSSKOPF, W.J., R.W. WOERPEL and R. ASTERINO (1991):
Successful treatment of avian tuberculosis in pet psittacines.
Proceedings of the annual conference of the Association of Avian Veterinarians, Chicogo, Illinois, USA, 23rd – 28th September, 238-251

RUNYON, E.H. (1967):
Mycobacterium intracellulare.
American Review of Respiratory Diseases 6, 251-255

SAITO, H. and H. TOMOIKA (1988):
Comparative *in vitro* and *in vivo* activity of rifabutin and rifampicin against *Mycobacterium avium* complex.
Tubercle 69, 178-192

SANDER, I. (1995):
Untersuchungen zum Leukozytentransformationstest unter Verwendung von 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazoliumbromid (MTT) an peripheren Blutleukozyten des Huhnes (Gallus gallus domesticus).
Dissertation an der Tierärztlichen Hochschule Hannover

SCHÄFER, W.B., J.V. BEER, N:A. WOOD, E. BOUGHTON, P.A. JENKINS and J. MARKS (1973):
A bacteriological study of endemic tuberculosis in birds.
Journal of Hygiene 71 (3), 549-557

SCHÄFER, W.B., C.L. DAVIS and M.L. COHN (1970):
Pathogenicity of transparent, opaque and rough variants of *Mycobacterium avium* in chicken and mice.
American Review of Respiratory Diseases 102, 499-506

SCHULZ, W. (1992):
Geflügeltuberkulose
In: Heider, G., G. Monreal and J. Mészáros (eds.): Krankheiten des Wirtschaftsgeflügels, ein Handbuch fr Wissenschaft und Praxis, Band II-spezieller Teil 2
Gustav Fischer Verlag Jena, Stuttgard, 157-164

SHAHEEN, A.A., A. KDAMEL, A.M. AMMAR, Y. ABDEL-GALIL, W. AWAD and E. AMIN (1997):
Untersuchungen über die Zuverlässigkeit der Diagnose atypischer mykobakterieller Infektionen mittels ELISA-Technik.
Fleischwirtschaft 77 (6), 568-570

SHARMA, J.M. and I. TIZARD (1984):
Avian cellular immune effector mechanism- a review.
Avian Pathology 13, 357-376.

SIBLEY, C.G. and B.L. MONROE Jr. (1990):
Distribution and taxonomy of birds of the world.
New Haven, London: Yale University Press

SIBLEY, C.G. and B.L. MONROE Jr. (1993):
A supplement to distribution and taxonomy of birds of the world.
New Haven, London: Yale University Press

SLAGLE, K.M. and S.J. GHOSN (1996):
Immunoassays, tools for sensitive, specific, and accurate test results.
Laboratory Medicine 27 (3), 177-183

SNIDER, D.E., P.C. HOPEWELL and J. MILL (1987):
Mycobacteriosis and the acquired immunodeficiency syndrome.
American Review of Respiratory Diseases 136, 492-496

SONGER, J.G. (1980):
Environmental sources of *Mycobacterium avium* for infection of animals and man.
Proceedings of the United States Animal Health Association 84, 528-535

SOOTHILL, E. and P. WHITEHEAD (1978):
Wildfowl of the world.
Fakenham Press Ltd, Fakenham, Norfolk

SPANGLER, E., S. BECH-NIELSEN, L.E. HEIDER and R. DORN (1992):
Interpretation of an enzyme-linked immunosorbent test using different cut-offs between positive and negative samples for diagnosis of paratuberculosis.
Preventive Veterinary Medicine 13 (3), 197-204

STANFORD, J.L. and J.M. GRANGE (1974):
The meaning and structure of species as applied to mycobacteria.
Tubercle 55, 143-152

STANFORD, J.L., W.D.L. REWILL, W.J. GUNTHORPE and J.M. GRANGE (1975):
The production and preliminary investigation of Burulin, a new skin test reagent for *Mycobacterium ulcerans* infection.
Journal of Hygiene, Cambridge, 74, 7-16

STORMER, R.S. and J.O. FALKINHAM III (1989):
Differences in antimicrobial susceptibility of pigmented and unpigmented colonial variants of *Mycobacterium avium*.
Journal of Clinical Microbiology 27, 2459-2465

SUGDEN, E.A., K. STILWELL, E.B. ROHONCZY and P. MARTINEAU (1997):
Competitive and indirect enzyme-linked immunosorbent assays for *Mycobacterium bovis* infections based on MPB70 and lipoarabinomannan antigens.
Canadian Journal of Veterinary Research 61, 8-14

TASSELL, S.K., M. POUSCHEFIE, E.L. WRIGHT, M.G. RICHMOND and
W.W. BORROW (1992):
Modified lymphocyte response to mitogens induced by lipopeptide fragment derived from *Mycobacterium avium* serovar-specific glycopeptidolipids.
Infection and Immunity 60 (2), 706-711

THOEN, C.O., M. RICHARDS and J.L. JARNAGIN (1977a):
Mycobacteria isolated from exotic animals.
Journal of the American Veterinary Medicine Association 170, 987-990

THOEN, C.O., E.M. HIMES and R.E. BARRET (1977b):
Mycobacterium avium serotype 1 infection in a sandhill crane (*Grus canadensis*).
Journal of Wildlife Diseases 13 (1), 40-42

THOEN, C.O., W.G. EARCRET and E.M. HIMES (1978):
An enzyme-labelled antibody test for detecting antibodies in chickens infected with *Mycobacterium avium* serotype 2.
Avian Diseases 22, 162-166

THOEN, C.O., A.L. ARMBRUST and M.P. HOPKINS (1979):
Enzyme-linked immunosorbent assay for detecting antibodies in swine infected with *Mycobacterium avium.*
American Journal of Veterinary Research 40, 1096-1099

THOEN, C.O., K. MILLS, M.P. HOPKINS (1980):
Enzyme-linked protein A: an enzyme-linked immunosorbent assay for detecting antibodies in tuberculous exotic animals.
American Journal of Veterinary Research 41 (5), 833-835

THOEN C.O. (1993):
Tuberculosis and other mycobacterial diseases of captive wild animals.
In: Zoo and Wild Animal Medicine, Fowler, M.E. (eds.), W.B. Saunders Co.,
Philadelphia, PA, 45-49

THOEN, C.O. (1994):
Mycobacterium avium infections in animals.
Research in Microbiology 145 (3), 173-175

TIZARD, I. (1979):
Avian immune responses: A brief review.
Avian Diseases 23, 290-298.

TORO, H, E.F. PAVÉZ and T. REDMANN (1996):
Virus serological diagnosis in two avian species based on binding of human C1q to avian antibody.
Avian Pathology 25, 155-160

VAN DER HEYDEN, N. (1989):
Avian tuberculosis: diagnosis and attempted treatment.
Proceedings of the annual conference of the Association of Avian Veterinarians, Seattle, Washington, USA, 11th – 16th September, 203-214

VAN DER HEYDEN, N. (1994):
Update of avian tuberculosis.
Proceedings of the annual conference of the Association of Avian Veterinarians, Reno Nevada, USA, 28th – 30th September, 53-61

VAN DER HEYDEN, N. (1997a):
Clinical manifestation of mycobacteriosis in pet birds.
Seminars in Avian and Exotic Pet Medicine 6 (1), 18-24

VAN DER HEYDEN, N. (1997b):
New strategies in the treatment of avian mycobacteriosis.
Seminars in Avian and Exotic Pet Medicine 6 (1), 25-33

VAN WEEMEN, B.K. and A.H.W.M. SCHUURS (1971):
Immunoassay using antigen-enzyme conjugates.
Federation of European Biochemical Societies Letters 15, 232-234

VOLLER, A. and D.E. BIDWELL (1988):
Immunoassays with special reference to ELISA methods.
Zoological Symposium, Society of London 60, 151-158

VOLNER, Z. (1978):
Mycobacteria in pigs and the importance of wild birds in tuberculosis transmission.
Veterinarski-Arhiv 48 (2), 107-110

VOLNER, Z., Z. HORVAT and B. TUNKL (1981):
Significance of *Mycobacterium avium* infection of eggs and chicks in the epidemiology of tuberculosis in fowls.
Veterinaria 30 (3-4), 377-382

VON KOLLIAS, G. (1986):
Relationships of avian immune structure and function to infectious disease.
In: Harrison, G.J. and L.R. Harrison (eds.), Clinical Avian Medicine and Surgery including aviculture, W.B. Saunders Company, Philadelphia, PA, 313-318

WANG, J. (1998):
Untersuchungen zur Entwicklung zellulärer und humoraler Immunreaktionen bei Enten (*Anas platyrhynchos*, L., 1758).
Dissertation an der Tierärztlichen Hochschule Hannover

WAYNE, L.G. and H.A. SRAMEK (1992):
Agents of newly recognised or infrequently encountered mycobacterial diseases.
Clinical Microbiology Reviews 5, 1-25

WENDT, S.L., K.L. GEORGE, B.C. PARKER, H. GRUFT and J.O. FALKINHAM III (1980):
Epidemiology of infection by non-tuberculous mycobacteria.III. Isolation of potentially pathogenic mycobacteria from aerosols.
American Review of Respiratory Diseases 122, 259-263

WOODLEY, L.G. and H.L. DAVID (1976):
Effect of temperature on the rate of the transparent to opaque colony transition in *Mycobacterium avium.*
Antimicrobacterial Agents Chemotherapy 9, 113-119

ZIMMERMANN, B., N. SHALATIN and H.M. GREY (1971):
Structural studies on the duck 5.7S and 7.8S immunoglobulins.
Biochemistry 10, 482-495

ZHOU, E.M., M. CHAN, R.A. HECKERT, J. RIVA and M.F. CANTIN (1998):
Evaluation of a competitive ELISA for detection of antibodies against avian influenza virus nucleoprotein.
Avian Diseases 42, 517-522

I. Appendices

Appendix 1: Tables with comprehensive data regarding the graphics in the chapter 'Results'

Table I: Number of birds tested in ELISA 1 (n=299) compared to the number of birds tested in ELISA 2 (n=322), as a proportion of the total number of birds tested, in the different diagnostic categories.

ELISA	Negative/ no. tested (%)	Borderline/ no. tested (%)	Positive/ no. tested (%)
1	91/299 (30.4%)	155/299 (51.8%)	53/299 (17.7%)
2	30/322 (9.3%)	176/322 (54.7%)	116/322 (36.0%)

Table II: Number of birds tested by both ELISA 1 and ELISA 2, as a proportion of the total number of birds tested, in the different diagnostic categories (n=203).

ELISA	Negative/ no. tested (%)	Borderline/ no. tested (%)	Positive/ no. tested (%)
1	63/203 (31.0%)	108/203 (53.2%)	32/203 (15.8%)
2	20/203 (9.9%)	121/203 (59.6%)	62/203 (30.5%)

Table III: The shift of diagnostic categories (initially negative results n=63, initially borderline results n=108, initially positive results n=32) from ELISA 1 to ELISA 2, as a proportion of the total number of individual birds tested (n=203).

Diagnostic Category	Number of birds tested	Percentage
Negative remains negative:	*8*	*(3.9%)*
Negative becomes borderline:	46	(22.7%)
Negative becomes positive:	9	(4.4%)
Borderline remains borderline:	*58*	*(28.6%)*
Borderline becomes positive:	42	(20.7%)
Borderline becomes negative:	8	(3.9%)
Positive remains positive:	*11*	*(5.4%)*
Positive becomes borderline:	17	(8.4%)
Positive becomes negative:	4	(2.0%)

Table IV: Number of birds with post mortem evidence of avian tuberculosis from 1989 to 1999, as a proportion of the total number of birds in each group (n=330).

Years	Total deaths	Non-ATb deaths	Percentage	ATb deaths	Percentage
1989	2	2	100	0	0
1990	5	5	100	0	0
1991	18	18	100	0	0
1992	27	27	100	0	0
1993	35	35	100	0	0
1994	26	23	88.5	3	11.1
1995	25	18	72	7	28
1996	40	34	85	6	15
1997	45	34	75.6	11	24.4
1998	54	32	59.6	22	40.7
1999	53	30	56.6	23	43.4
Total	330	258	78.2	72	21.8

Table V: Number of birds dying with post mortem evidence of avian tuberculosis by pen, as a proportion of the total number of birds in each group (n=330).

Pen	Total deaths	Non-ATb deaths	Percent.	ATb deaths	Percent.
NA	29	8	27.6	21	72.4
SPB	2	1	50	1	50
SMW	18	10	55.6	8	44.5
SPC	3	2	66.7	1	33.3
AS	34	23	67.6	11	32.4
FLM	18	13	72.2	5	27.8
AUS	11	8	72.7	3	27.3
LAG	8	6	75	2	25
ISL	19	15	79	4	21.1
ST	5	4	80	1	20
SPA	6	5	83.3	1	16.7
TOP	62	55	88.7	7	11.3
SA	28	25	89.3	3	10.7
EUR	38	35	92.1	3	7.9
GRD	32	31	96.9	1	3.1
EUS	2	2	100	0	0
HOS	4	4	100	0	0
OD	6	6	100	0	0
SAS	5	5	100	0	0
Total	330	258	78.2	72	21.8

Table VI: Number of birds dying showing post mortem evidence of avian tuberculosis according to tribe, as a proportion of the total number of birds in each group (n=330).

Tribe	Total deaths	non-ATb deaths	Percentage	ATb deaths	Percentage
Mergini (11)	31	16	51.6	15	48.4
Aythini (9)	28	18	90	10	35.7
Anseronatini (1)	3	2	66.7	1	33.3
Anserini (3)	30	21	70	9	30
Cairinini (10)	37	27	90	10	27.0
Tadornini (4)	8	7	87.5	1	25
Oxyurini (12)	11	9	81.8	2	18.2
Dendrocygnini (2)	47	39	83.0	8	17.0
Anatini (6)	122	106	86.9	16	13.1
Somateriini (8)	10	10	100	0	0
Phoenicopteridae (13)	3	3	100	0	0
Total	330	258	78.12	72	21.8

Table VII: Average age of the birds dying from avian tuberculosis or other non-tuberculous diseases, summarised by tribe.

Tribe	ATb deaths		Non-ATb deaths	
	Average age (years)	Number of birds	Average age (years)	Number of birds
Tadornini	9	1	5.7	8
Anserini	7.6	9	6.6	30
Aythini	7.6	10	5.1	28
Dendrocygnini	7.5	8	5	47
Anatini	7.1	16	4.5	122
Cairinini	6.9	10	4.9	37
Mergini	6.7	15	4.6	31
Oxyurini	4.5	2	5.4	11
Anseranatini	1	1	7.5	3
Somateriini	0	0	4.4	10

Table VIII: Number of birds dying with post mortem evidence of avian tuberculosis according to sex as a proportion of the total number of birds in each group (n=330).

Sex	Total deaths	Non-ATb deaths	Percentage	ATb deaths	Percentage
Female	186	151	81.2	35	18.8
Male	144	107	74.3	37	25.7
Total	330	258	78.2	72	21.8

Table IX: Number of birds dying with post mortem evidence of avian tuberculosis, according to season of death, as a proportion of the total number of birds in each group (n=330).

Season	Total deaths	Non-ATb deaths	Percentage	ATb deaths	Percentage
Spring	87	66	75.9	21	24.1
Summer	106	87	82.1	19	21.8
Autumn	71	57	80.3	14	24.6
Winter	66	48	72.7	18	27.3
Total	330	258	78.2	72	21.8

Table X: Number of birds dying with post mortem evidence of avian tuberculosis grouped by sex and season, as a proportion of the total number of birds in each group (n=330).

Season	Male deaths		Female deaths	
	ATb deaths/Total deaths	Percentage	ATb deaths/Total deaths	Percentage
Spring	10/41	24.4	11/46	23.9
Summer	10/46	21.7	9/60	15
Autumn	7/25	28	7/46	15.2
Winter	10/32	31.3	8/34	23.5

Appendix 2: Recipes for buffers used in the ELISA (PAINTER, 1995)

Coating buffer: 0.05 M Carbonate / Bicarbonate Buffer pH 9.6

0.795 g sodium carbonate ($Na_2 CO_3$, Sigma S-2127)
1.465 g sodium bicarbonate ($NaHCO_3$, BDH 10247)
The above are dissolved in 500 ml of distilled water.

Washing / Incubation Buffer: pH 7.4

40.00 g sodium chloride (NaCL. BDH 10241)
1.00 g potassium di-hydrogen phosphate (KH_2PO_4, Sigma-5379)
5.68 g di-sodium hydrogen phosphate (Na_2HPO_4, BDH 30158)
1.00 g potassium chloride (KCL, BDH 10198)
2.5 ml Tween 20 (polyoxyethylene sorbitan monolaurate, Sigma P-1379)
The above are dissolved in 5 litres of distilled water.

Citrate Phosphate Buffer: 0.1 M, pH 4.3

6.303 g citrate acid (Sigma C-2916) is dissolved in 300 ml distilled water, to give a 0.1 M solution (21.01 g/l).
10.68 g di-sodium hydrogen phosphate ($Na_2HPO_42H_2O$, BDH 30157) is dissolved in 300 ml distilled water, to give a 0.2 M solution (35.6 g/l).
272 ml of the first solution are added to 204 ml of the second and the pH is adjusted to 4.3.

Substrate: ABTS solution

25 mg ABTS (2,2 azino-di-(3 ethyl benthiazoline sullphonic acid), Sigma A1888) are dissolved in 50 ml of citrate phosphate buffer to which 17.5 µl of 20 volume hydrogen peroxide (H_2O_2, BDH 10127) has been added.

Equipment

Antigens:
Mycobacterium avium serotype 1 and 3 were isolated from bacterial cultures by Dr. R. Cromie, WWT centre at Slimbridge, England
Mycobacterium vaccae (R877R), Glaxo BCG, *Mycobacterium fortuitum* are taken from NTCC collection

HPR conjugate:
(RADu/IgG(H+L)/Po) from Nordic, Immological Laboratories, Netherlands

Microtitre plates:
Nunc Immuno Plates Maxisorb (442404), 96 flat bottomed welled ELISA plates, Nalge Nunc International, London, United Kingdom

Colorimetrical ELISA reader:
Dynatech Laboratories MR 7000 ELISA reader, Dynex Technologies Ltd, West Sussex, United Kingdom

Appendix 3: Detailed ELISA results of all birds which were tested in this study

The ELISA results are expressed as percentage of the adjusted positive control

Bird Identification	M.av.1238			RBG			Goosander			Yellow Billed			Mallard			Pen	Species	Species number	Sex	Age in years
	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3					
LC0284	23,83			30,38			26,79			14,5			20,18			SPE	SPOTTED WHISTL. D	2	F	7
LC0287	8,78			18,95			9,05			6,82			9,38			SPE	SPOTTED WHISTL. D	2	M	7
LC0288	18,98			25,11			22,2			23,99			23,11			SPE	SPOTTED WHISTL. D	2	M	7
LC0289	24,37			20,95			21,08			22,73			21,14			SPE	SPOTTED WHISTL. D	2	F	7
LC0291	15,93			12,43			11,58			13,69			15,05			SPE	SPOTTED WHISTL. D	2	M	7
LC0294	11,32			13,17			2,01			9,75			15,25			SPE	SPOTTED WHISTL. D	2	M	7
LC0295	22,15			20,09			16,33			15,77			20,4			SPE	SPOTTED WHISTL. D	2	M	7
LC0106	21,48			15,87			13,59			15,75			14,82			AUS	EYTON'S WHISTL. D	3	F	9
LC0108	36,42			39,94			32,35			34,81			24,97			AUS	EYTON'S WHISTL. D	3	M	9
LC0142	75,51			71,47			86,93			90,84			78,84			SMW	EYTON'S WHISTL. D	3	M	9
LC0238	42,28			30,38			36,86			30,39			26,96			AUS	EYTON'S WHISTL. D	3	F	8
LC0240	86,64		9,82	74,53		23,3	86,97		15,12	82,82		23,44	84,43		18,09	AUS	EYTON'S WHISTL. D	3	F	8
SC3173	5,46			8,45			1,15			7,69			10,12			SMW	EYTON'S WHISTL. D	3	F	10
SC3175	20,1	28,54		9,63	40,27		4,18	26,19		10,12	27,6		12,28	32,93		SMW	EYTON'S WHISTL. D	3	M	10
LC0071		5,9			6,94			8,32			4,12			3,82		AS	WANDER. WHISTL. D	4	M	9
LC0481		42,48			33,78			32,99			33,79			37,93		AS	WANDER. WHISTL. D	4	M	1
LC0483		31,86			28,79			25,22			23,64			25,07		AS	WANDER. WHISTL. D	4	M	1
LC0484		26,28			38,56			31,39			34,18			33,79		AS	WANDER. WHISTL. D	4	F	1
LC0485		19,18			25			26,1			27,24			27,15		AS	WANDER. WHISTL. D	4	M	1
LC0487		20,46			15,07			27,23			19,59			22,22		AS	WANDER. WHISTL. D	4	M	1
LC0488		18,57			34,95			19,79			21,71			24,2		AS	WANDER. WHISTL. D	4	F	1
LC0230	99,33		6,75	89,57		6,8	63,06		6,88	90,39		5,94	80,8		3,75	SA	FULVOUS WHISTL. D	7	F	8
LD0031	31,26			43,49			37,46			35,5			32,69			SA	FULVOUS WHISTL. D	7	M	7
LD0037	11,56			18,13			14,82			14,55			21,37			SA	FULVOUS WHISTL. D	7	F	7
LD0040	15,88	12,16		21,73	24,37		14,99	19,56		16,14	12,94		15,26	23,66		SA	FULVOUS WHISTL. D	7	M	7
LD0041	25,49	67,8		28,18	89,51		21,18	73,97		17,54	56,37		24,46	59,43		SA	FULVOUS WHISTL. D	7	F	7
SD2205	46,87	15,75		52,91	31,18		49,52	19,77		45,1	17,59		58,39	30,36		SA	FULVOUS WHISTL. D	7	F	10
SD2231	18,07	17,92		20,12	26,53		24,48	19,48		19,41	14,02		28,86	16,2		SA	FULVOUS WHISTL. D	7	F	10
SD2232		31,15			41,48			42,41			26,35			26,49		SA	FULVOUS WHISTL. D	7	M	10

Bird Iden-	M.av.1238			RBG			Goosander			Yellow Billed			Mallard			Pen	Species	Species	Sex	Age in
tification	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3			number		years
SD2244	43,37	28,59		64,72	36,18		42,27	26,94		38,03	19,31		38,17	32,9		SA	FULVOUS WHISTL. D	7	F	9
SD2246	14,94	27,25		16,66	36,38		9,36	26,41		11,34	13,93		12,81	29,45		SA	FULVOUS WHISTL. D	7	M	9
SD2248	32,77	32,13		47,92	46,12		27,19	34,25		29,12	23,19		25,7	29,17		SA	FULVOUS WHISTL. D	7	M	9
SD2250		35			37,83			39,64			41,42			38,03		AS	FULVOUS WHISTL. D	7	M	9
SD2252	31,89			44,89			30,88			36,13			34,15			SA	FULVOUS WHISTL. D	7	F	9
SD2256	6,2			8,41			0,24			0			3,04			AUS	FULVOUS WHISTL. D	7	M	9
SD2258	18,81	22,68		26,98	40,97		20,17	31,44		23,07	25,01		26,28	27,25		SA	FULVOUS WHISTL. D	7	F	9
SD2260	24,49			37,41			26,47			28,68			31,15			SA	FULVOUS WHISTL. D	7	F	9
SD2292	13,99			19,49			5,46			16,48			13,8			SA	FULVOUS WHISTL. D	7	M	10
LD0009	26,81			37,78			30,71			20,82			28,06			TOP	CUBAN WHISTL. D	8	M	7
LD0061		57,75			54,43			43,74			43,55			48,58		TOP	CUBAN WHISTL. D	8	M	4
LB0323	37,6			49,71			32,87			37,86			37,18			TOP	WHI.FACE.WHISTL. D	10	F	7
LB0470		13,56			17,61			20,61			20,37			11,74		TOP	WHI.FACE.WHISTL. D	10	F	3
LC0134	0			1,46			2,01			0			5,43			TOP	WHI.FACE.WHISTL. D	10	M	9
LC0136	54,59		9,2	75		16,18	44,76		13,06	56,7		14,06	57,68		12,63	TOP	WHI.FACE.WHISTL. D	10	M	9
LC0139	7,38	20,31		0,32	25,42		0,11	21,53		6,9	10,58		0	19,43		TOP	WHI.FACE.WHISTL. D	10	F	9
LC0247	10,27	28,52		9,5	24,1		9,44	36,31		13,9	29,8		8,85	28,94		TOP	WHI.FACE.WHISTL. D	10	F	8
LC0271		18,54			27,87			23,51			23,19			26,83		TOP	WHI.FACE.WHISTL. D	10	M	7
LC0273	11,03	6,79		27,14	16,48		20,87	7,95		15,44	5,38		20,92	12,79		TOP	WHI.FACE.WHISTL. D	10	F	7
LC0275	7,2			7,75			5,49			9,92			0,99			TOP	WHI.FACE.WHISTL. D	10	F	7
LC0278		72,45			70,35			66,69			69,4			70,85		TOP	WHI.FACE.WHISTL. D	10	F	7
LC0281	14,64	13,31		19,88	27,61		20,49	16,79		21,92	17,73		28,33	18,4		TOP	WHI.FACE.WHISTL. D	10	M	7
LC0316	33,67		47,24	47,91		37,86	41,65		33,33	53,55		41,25	51,98		42,32	TOP	WHI.FACE.WHISTL. D	10	F	7
LC0317	3,06			7,58			9,02			2,83			5,27			TOP	WHI.FACE.WHISTL. D	10	M	7
LC0321	37,13	6,61		41	3,98		32,08	13,43		33,85	11,5		37,16	8,31		TOP	WHI.FACE.WHISTL. D	10	M	7
LC0322		32,34			51,02			30,7			36,49			33,74		TOP	WHI.FACE.WHISTL. D	10	F	7
LC0323		34,62			45,84			38,59			35,48			37,9		TOP	WHI.FACE.WHISTL. D	10	M	7
LC0364	14,09	46,28		24,93	57,9		17,16	59,11		14,29	64,3		19,55	56,29		TOP	WHI.FACE.WHISTL. D	10	M	6
LC0421	53,37			59,04			52,18			58,8			48,69			TOP	WHI.FACE.WHISTL. D	10	F	3
LC0430	3,55			28,4			17,84			21,74			21,86			TOP	WHI.FACE.WHISTL. D	10	M	4
LC0431		10,73			19,76			8,02			0			15,33		TOP	WHI.FACE.WHISTL. D	10	M	4
LC0468	14,78	23,3		9,32	31,95		8,46	31,03		10,07	32,39		13,59	25,73		TOP	WHI.FACE.WHISTL. D	10	M	3
LC0469	20,39	57,9		48,05	59,92		21,35	48,56		30,79	62,83		20,82	47,54		TOP	WHI.FACE.WHISTL. D	10	F	3
LC0470	31,87			33,23			28,16			31,66			24,13			TOP	WHI.FACE.WHISTL. D	10	F	3

Bird Identification	M.av.1238			RBG			Goosander			Yellow Billed			Mallard			Pen	Species	Species number	Sex	Age in years
	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3					
LC0471	0	49,94		9,69	64,69		5,74	44,24		2,56	50,5		5,09	50,02		TOP	WHI.FACE.WHISTL. D	10	F	3
LC0472		52,73			50,93			53,82			51,48			49,24		TOP	WHI.FACE.WHISTL. D	10	F	3
SC3264	20,24	29,11		19,4	45,37		12,96	37,25		18,42	27,39		17,17	35,7		TOP	WHI.FACE.WHISTL. D	10	M	10
SC3289		12,45			15,78			7,86			17,42			10,04		TOP	WHI.FACE.WHISTL. D	10	M	10
SC3290	12,16	4,02		20,02	4,79		11,45	2,09		14,16	9,45		16	12,25		TOP	WHI.FACE.WHISTL. D	10	M	10
L0068	21,64	24,28		27,51	28,31		15,96	13,8		20,98	14,75		19,42	20,67		TOP	BLACK NECKED SWAN	16	M	1
L0069	17,64	35,15		20,59	51,53		24,61	34,27		25,33	39,57		28,57	38,82		TOP	BLACK NECKED SWAN	16	F	1
L0070	14,57	35,77		9,13	44,74		5,29	41,85		18,75	38,45		21,43	37,48		TOP	BLACK NECKED SWAN	16	M	2
LK0003	44,9	67,73		54,1	64,15		50,57	69,21		49,58	78,04		47,28	68,67		TOP	BLACK NECKED SWAN	16	M	3
LK0004		34,63			57,67			51,42			35,39			39,74		TOP	BLACK NECKED SWAN	16	F	3
LK0005		35,21			45,35			35,43			30,75			33,22		TOP	BLACK NECKED SWAN	16	M	3
PO0635	19,81	22,85		21,81	32,5		14,99	22,27		20,3	24,28		17,48	23,66		TOP	BLACK NECKED SWAN	16	F	10
PO0637	47,06	41,03		48,71	54,98		40,4	48,26		40,52	46,26		44,38	52,05		TOP	BLACK NECKED SWAN	16	F	10
PO0638	59,09	53,32		50,64	74,23		44,98	56,11		43,76	46,47		51,54	52,36		TOP	BLACK NECKED SWAN	16	M	10
PO0639	24,51	49,28		37,19	60,33		29,82	70,67		34,52	57,44		25,35	53,72		TOP	BLACK NECKED SWAN	16	F	10
L0003		28,27			45,28			40,31			39,32			39,64		AS	SWAN G	22	M	7
L0005	21,68	27,41		34,33	40,11		22,14	34,92		29,44	41,71		27,46	43,68		AS	SWAN G	22	F	7
L0042	12,22	48,63		15,58	54,39		4,16	47,32		12,33	54,94		12,41	52,83		AS	SWAN G	22	F	6
L0058	11,85	38,43		18,97	57,19		19,86	54,15		16,14	57,43		20,12	57,6		AS	SWAN G	22	M	6
PO0614		24,88			36,03			35,24			43,08			37,89		AS	SWAN G	22	F	10
PO0618	13,75	55,54		16,98	58,33		5,7	51,2		14,96	54,58		9,29	51,45		AS	SWAN G	22	M	10
PO0622	25,28	43,22		32,07	58,21		32,34	45,03		36,63	40,1		35,13	34,41		AS	SWAN G	22	F	10
L0052	25,09	28,85		22,13	44,21		20,18	40,95		19,47	41,03		16,56	39,73		AS	PINKFOOTED G	28	M	6
L0053	30,4	76,79		27,86	76,55		19,09	80,63		30,23	77,23		25,69	78,48		NA	PINKFOOTED G	28	F	6
L0092		60,92			58,54			71,02			75,11			69,49		AS	PINKFOOTED G	28	F	1
PO0607	19,5	53,21		20,17	56,66		20,2	64,45		16,37	50,44		16,55	59,03		NA	PINKFOOTED G	28	M	11
PO0609	34,51	54,71		44,49	62,66		39,38	64,24		29,44	46,42		30,85	56,75		NA	PINKFOOTED G	28	M	11
S2056	54,8			21,28			27,71			31,13			24,24			NA	PINKFOOTED G	28	F	11
S2077	48,25			54,19			32,66			22,81			31,85			NA	PINKFOOTED G	28	F	10
L0051	35,55	52,23		36,88	55,66		27,39	62,46		33,94	56,19		28,09	60,73		AS	GR. WHI. FRONTED G	31	F	6
S2079	46,46	50,52		61,16	48,49		46,45	51,94		48,27	48,28		53,32	44,5		AS	GR. WHI. FRONTED G	31	F	10
S2081	46,73	51,63		36,14	53,97		33,44	55,33		38,5	49,73		43,65	59,23		NA	GR. WHI. FRONTED G	31	F	10
S2083	35,81	62,18		42,28	77,99		43,96	65,07		35,23	53,1		41,65	56,09		NA	GR. WHI. FRONTED G	31	F	10
S2084	27,13	48,99		20,24	58,1		16,08	41,11		22,74	58,34		21,78	56,28		NA	GR. WHI. FRONTED G	31	F	10

Bird Identification	M.av.1238			RBG			Goosander			Yellow Billed			Mallard			Pen	Species	Species number	Sex	Age in years
	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3					
S2431	46,09			39,77			38,19			27,6			28,66			NA	GR. WHI. FRONTED G	31	M	9
S2432	37,64	41,72		45,21	49,33		34,72	54,57		27,56	37,63		27,76	49,96		NA	GR. WHI. FRONTED G	31	M	9
S2433	49,12	39,44		61,65	42,35		52,88	48,07		35,94	37,55		39,23	45,51		NA	GR. WHI. FRONTED G	31	M	9
S2435	33,91	42,72		51,65	38,8		48,59	36,83		4,08	33,55		38,95	45,44		AS	GR. WHI. FRONTED G	31	M	9
S2436	40,26	37,18		58,33	42,77		39,5	45,03		31,71	36,95		30,92	43,2		NA	GR. WHI. FRONTED G	31	M	9
S2437	55,35	46,53		59,7	47,31		43,2	55,72		30,75	43,67		34,11	48,73		NA	GR. WHI. FRONTED G	31	F	9
S2438	41,32	59,07		32,38	66,71		39,51	68,06		40,97	71,96		35,94	77,03		NA	GR. WHI. FRONTED G	31	F	9
LD0057	12,18	44,81		35,64	53,55		27,33	41,51		32,13	30,52		34,57	33,46		TOP	LES.WHITE.FRONT. G	33	M	2
SD2181	37,57	26,49	41,1	67,86	40,33	48,87	58,04	28,69	43,64	50,93	36,59	46,88	49,3	34,06	39,59	TOP	LES.WHITE.FRONT. G	33	M	10
SD2185	5,97	37,36		25,87	49,38		11,09	28,35		17,47	22,5		24,64	26,46		TOP	LES.WHITE.FRONT. G	33	M	10
SD2186	59,81	49,16	8,28	74,32	56,95	0,65	69,43	50,02	2,06	67,72	53,17	3,75	63,71	51,32	13,31	TOP	LES.WHITE.FRONT. G	33	F	10
SD2187	32,19	54,63		42,76	65,23		35,91	51,98		41,74	39,97		41,36	37,99		TOP	LES.WHITE.FRONT. G	33	F	10
SD2188	1,33	34,71		16,13	50,43		16,5	38,57		8,49	33,17		18,31	31,57		TOP	LES.WHITE.FRONT. G	33	F	10
SD2190	14,87	23,47		21,44	43,91		15,54	30,74		21,03	25,6		18,79	27,7		TOP	LES.WHITE.FRONT. G	33	F	10
SD2195	37,71	49,16		39,99	43,22		33,44	52,22		35,67	42,22		39,18	43,33		TOP	LES.WHITE.FRONT. G	33	M	10
L0043	54,43	52,19		40,71	56,63		31,13	52,59		28,37	55,83		36,58	47,86		AS	BARHEADED G	36	F	6
L0045		111,6			86,59			91,03			94,58			85,9		AS	BARHEADED G	36	M	6
L0060	24,12	51,55		29,55	40,49		25,14	45,45		24,92	38,49		21,73	42,7		AS	BARHEADED G	36	M	6
S2098	15,64	52,72		18,94	67,7		6,39	58,81		16,62	60,21		11,7	60,5		AS	BARHEADED G	36	F	10
S2102	23,7	65,34		20,14	69		19,5	79,37		28,78	89,93		25,48	78,02		AS	BARHEADED G	36	F	10
S2104	9,94	41,87		19,38	43,69		13,88	43,36		14,39	43,59		11,8	37,15		AS	BARHEADED G	36	M	10
S2105	14,7	23,64		20,32	24,6		15,63	19,75		9,01	23,86		16,03	20,12		AS	BARHEADED G	36	M	10
S2106	107,8	33,8	45,71	110,2	36,94	45,95	112,8	38,68	44,32	98,59	36,63	44,69	98,76	35,84	50,85	AS	BARHEADED G	36	M	10
L0054	47,53	29,89		56,22	38,37		41,95	36,48		40,12	33,38		38,05	40,67		NA	EMPEROR G	37	M	6
L0055	64,28	49,86		56,02	58,35		42,41	46,87		43,28	60,19		54,58	62,94		NA	EMPEROR G	37	M	6
L0093		45			36,59			42,56			33,05			46,13		NA	EMPERER G	37	F	1
S2092	35,03	51,66		51,32	60,44		31,84	68,61		29,19	35,95		30,75	59,74		NA	EMPEROR G	37	F	10
S2093	3,98	74,72		37,72	69,06		33,82	72,41		33,45	72,54		28	66,22		NA	EMPEROR G	37	M	10
S2094	29,22	35,05		14,1	31,84		7,91	40,3		14,75	36,8		9,26	39,45		NA	EMPEROR G	37	F	10
S2095	39,46	42,23		32,14	40,1		15,25	41,31		24,38	38,1		17,46	47,64		NA	EMPEROR G	37	F	10
S2110	40,93	56,47		32,45	58,99		23,89	71,7		33,55	81,38		38,02	74,72		NA	EMPEROR G	37	M	10
S2085	52,09			52,71			40,57			31,54			32,17			NA	GREATER SNOW G	38	M	10
S2086		28,49			50,38			52,56			30,15			42,36		NA	GREATER SNOW G	39	F	10
S2087	29,58	48,76		27,06	60,36		23,76	69,12		17,15	53,45		20,1	57,6		NA	GREATER SNOW G	39	M	10

Bird Iden-	M.av.1238			RBG			Goosander			Yellow Billed			Mallard			Pen	Species	Species	Sex	Age in
tification	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3			number		years
LD0052	13,58	21,24		20,89	33,62		6,79	29,02		16,22	21,36		14,09	30,28		NA	ROSS'S G	40	M	4
LD0053		33,21			40,53			39,26			28,84			38,06		NA	ROSS'S G	40	M	4
LD0054	18,9	22,61		17,11	36,54		7,84	35,34		8,83	29,4		7,02	40,45		NA	ROSS'S G	40	F	4
LD0055	29,31	45,62		43,55	42,66		34,34	44,91		28,99	37,02		29,26	38,29		NA	ROSS'S G	40	F	4
SD2237	29,11	34,57		38,89	41		20,2	40,33		22	32,12		21,28	37,75		NA	ROSS'S G	40	M	9
SD2238	43,69	53,77		40,95	56,11		25,34	48,47		23,99	45,42		18,2	53,52		NA	ROSS'S G	40	F	9
SD2239	10,96	15,23		10,24	22,24		5,04	21,06		12,59	16,17		12,27	21,74		NA	ROSS'S G	40	F	9
SD2243	9,94	32,92		12,43	45,11		6,79	48,08		14,01	31,35		14,46	41,05		NA	ROSS'S G	40	M	9
SD2199		62,36			66,91			68,2			67,42			81,34		AS	ALEUTIAN CANADA G	49	M	10
L0047 ACA		43,3			35,98			35,12			33,1			33,17		AS	HAWAIIAN G	53	M	5
L0061	18,05			16,26			15,21			17,62			11,96			AS	HAWAIIAN G	53	F	10
S2072		23,8			28,83			23,1			28,65			34,43		AS	HAWAIIAN G	53	M	10
LC0121	8,75	32,63		2,57	36,23		0	44,61		11,71	34,54		6,59	39,85		NA	BLACK BRANT G	58	F	9
LC0123	36,02	57,02		21,19	55,29		17,52	63,44		23,66	52,49		28,7	65,29		NA	BLACK BRANT G	58	M	9
LC0129	27,95	26,67		31,93	28,21		30,64	32,55		21,52	24,72		20,28	31,77		NA	BLACK BRANT G	58	F	9
LC0388	55,94	12,94		60,48	32,19		36,58	17,87		40,84	16,85		37,62	17,69		TOP	RED BREASTED G	59	M	5
LC0390	19,98	50,97		19,51	50,57		17,27	43,09		17,41	37,4		20,32	43,6		TOP	RED BREASTED G	59	F	5
LC0392	4,24	32,2		18,15	47,97		14,98	39,72		8,98	37,39		17,21	32,57		TOP	RED BREASTED G	59	M	5
LC0435	9,9	96,45		30,5	83,39		19,32	90,65		19,74	82,58		20,23	92,83		TOP	RED BREASTED G	59	M	2
LC3198		14,39			36,46			22,95			25,43			24,05		TOP	RED BREASTED G	59	M	10
LD0058	17,15	20,89		31,46	21,87		23,73	12,75		27,98	12,25		14,09	17,23		TOP	RED BREASTED G	59	M	2
SC3172	26,77	12,08		35,68	25,9		34,75	18,39		31,97	15,5		38,08	20,84		TOP	RED BREASTED G	59	F	10
SC3177	24,74	21,37		38,39	39,45		29,32	20,2		34,65	14,88		30,93	23,6		TOP	RED BREASTED G	59	F	10
SC3191	37,6	19,24		31	31,48		14,35	25,17		27,6	20,6		28,4	25,22		TOP	RED BREASTES G	59	F	10
SC3193	56,48	51,32	27,3	67,25	57,45	28,16	67,99	45,97	27,15	64,34	48,72	30,63	63,16	42,81	32,08	TOP	RED BREASTED G	59	F	10
SC3197	67,8	20,1	15,64	50,47	42,37	24,92	48,84	28,65	3,09	54,41	33,31	11,88	60,34	32,73	4,44	TOP	RED BREASTED G	59	M	10
SC3198	58,83		2,76	89,52		18,45	82,12		6,87	79,32		17,5	70,19		13,65	TOP	RED BREASTED G	59	M	10
SC3200	14,68	61,07		40,78	50,13		25,36	47,33		29,82	36,82		21,86	42,31		TOP	RED BREASTED G	59	M	10
SC3226	19,83	33,01		27,53	50,37		21,99	26,38		29,63	33,83		22,68	32,17		TOP	RED BREASTED G	59	F	10
SC3228	42,85	8,9		45,94	19,91		42,01	15,58		42,42	7,43		39,3	17,16		TOP	RED BREASTED G	59	M	10
SC3229	2,04	45,3		21,22	47,79		13,03	39,84		13,69	34,87		16,62	38,71		TOP	RED BREASTED G	59	F	10
SD2217	16,32	15,32		23,99	31,39		22,41	19,82		20,3	20,86		26,44	22,73		TOP	RED BREASTED G	59	M	10
LC0035		23,11			23,43			8,67			8,42			25,35		AS	RUDDY SHELDUCK	61	M	9
LC0036		45,38			59,22			56,68			49,08			53,25		AS	RUDDY SHELDUCK	61	F	9

Bird Iden-	M.av.1238			RBG			Goosander			Yellow Billed			Mallard			Pen	Species	Species	Sex	Age in
tification	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3			number		years
LC0037		41,45			43,09			58,53			60,48			53,64		AS	RUDDY SHELDUCK	61	F	9
LC0041	40,61			45,42			52,9			52,11			50,63			TOP	RUDDY SHELDUCK	61	M	9
LC0043	27,79			27,34			20,34			26,95			20,42			AS	RUDDY SHELDUCK	61	M	9
LC0044	23,38			31,27			19,99			15,83			15,36			AS	RUDDY SHELDUCK	61	M	9
LC0393		48,52			35,39			34,01			28,71			29,33		AS	RUDDY SHELDUCK	61	F	6
LD0048	48,28			57,32			49,13			60,01			55,92			TOP	RUDDY SHELDUCK	61	F	6
LC0115	17,03			19,7			13,08			15,97			10,44			SA	RADJAH SHELDUCK	65	F	9
LC0116	22,18			22,93			14,15			17,87			17,67			SA	RADJAH SHELDUCK	65	M	9
LC0117		25,78			34,67			17,86			17,44			20,26		TOP	RADJAH SHELDUCK	65	F	9
LC0145	37,58	25,88		56,58	26,11		39,65	18,4		42,34	22,17		40,18	25,07		TOP	COMMON SHELDUCK	67	M	8
LC0152	5,39	79,95		14,78	91,63		13,66	69,87		12,03	75,98		19,74	68,07		TOP	COMMON SHELDUCK	67	F	8
LC0474	16,32	11,16		23,05	9,17		19,08	6,1		23,52	10,18		23,89	14,42		TOP	COMMON SHELDUCK	67	F	2
SD2106	18,56	10,06		36,16	12,89		22,79	10,65		18,15	5,35		22,07	6,55		TOP	COMMON SHELDUCK	67	F	11
SD2128	37,1	11,39	44,79	56,55	24,53	47,25	48,53	13,48	43,99	51,38	17,74	45	50,72	12,18	52,22	TOP	COMMON SHELDUCK	67	F	11
SD2134	44,71	44,41	38,34	65,68	47,91	24,92	54,27	38,99	11	55,44	34,23	17,5	49,69	39,92	16,38	TOP	COMMON SHELDUCK	67	M	11
PO0653	12,77			22,49			4,35			12,47			11,79			SPB	ANDEAN G	71	M	8
S2410	45,64			26,1			28,69			31,31			33,4			SPB	ANDEAN G	71	F	8
LD0059	39,91			39,18			30,25			25,62			19,32			SA	RUDDY HEADED G	73	M	2
SD2197	14,38	34,45		21,41	47,1		12,63	36,91		14,38	40,44		11,21	36,5		SA	RUDDY HEADED G	73	F	10
SD2198		48,72			44,25			43,52			48,76			37,85		SA	RUDDY HEADED G	73	M	10
SD2242	34,85			56,43			31,99			40,53			34,73			SA	RUDDY HEADED G	73	M	10
PO0682		81,55			73,39			65,78			73,77			75		AS	CEREOPSIS	78	F	7
LA0006		79,11			87,16			86,97			89,76			89,77		AS	MARBLED TEAL	86	F	9
LA0120		45,71			43,09			41,29			41,16			37,36		AS	MARBLED TEAL	86	F	1
SB4465		55,39			63,52			75,56			64,14			61,98		AS	MARBLED TEAL	86	F	10
LA0051		23,97			32,36			26,34			28,69			28,02		TOP	CAPE TEAL	87	M	7
LB0278		68,51			49,03			53,18			49,58			43,26		TOP	CAPE TEAL	87	F	8
LB0279	66,99	60,11		42,95	63,48		32,51	71,65		37,75	52,55		38,84	49,91		TOP	CAPE TEAL	87	M	8
LB0320		29,94			24,83			34,48			34,83			25,8		TOP	CAPE TEAL	87	M	7
LB0325	15,2	27,32		9,24	46,28		9,11	25,24		10,12	24,87		7,47	32,03		TOP	CAPE TEAL	87	M	7
LB0326	21,2	37,59		32,55	59,29		20,41	48,12		20,79	48,61		24,25	48,73		TOP	CAPE TEAL	87	F	7
LB0474	27,59	45,97		38,85	53,32		28,79	46,31		24,21	44,79		32,63	46,79		TOP	CAPE TEAL	87	F	4
SB4444	36,82	34,48		40,92	25,76		33,74	21,97		36,12	25,33		41,5	20,36		TOP	CAPE TEAL	87	F	10
SB4447	25,78	14,82		54,71	12,95		40,72	16,83		49,28	19,69		42,85	15,68		TOP	CAPE TEAL	87	M	10

Bird Iden-	M.av.1238			RBG			Goosander			Yellow Billed			Mallard			Pen	Species	Species	Sex	Age in
tification	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3			number		years
SB4448	26,62	37,21		34,46	56,52		15,02	32,7		40,85	42,52		23,58	41,41		TOP	CAPE TEAL	87	M	10
SB4449		11,97			9,5			6,21			0,05			8,18		TOP	CAPE TEAL	87	M	10
SB4457	12,71	24,71		20,32	28,32		19,22	27,1		21,15	25,09		16,39	22,5		TOP	CAPE TEAL	87	F	10
SB4459	16,58			17,74			14,37			14,61			13,78			TOP	CAPE TEAL	87	M	10
SB4460	16,37	44,6		34,73	45,24		15,23	37,38		18,63	30,51		25,58	27,28		TOP	CAPE TEAL	87	M	10
SB4461	23,66	36,42		21,04	30,81		20,58	45,86		16,82	32,13		15,88	34,38		TOP	CAPE TEAL	87	F	10
LA0087	26,59			32,99			21,72			34,54			35,01			SPC	HOTTENTOT TEAL	88	F	5
LA0107	49,25			49,85			36,55			43,47			46,21			SPC	HOTTENTOT TEAL	88	F	2
LA0108	26,48			28,41			14,03			14,92			22,92			SPC	HOTTENTOT TEAL	88	F	2
LB0513	18,38	19,62		19,79	23,5		14,04	17,64		31,75	8,03		18,01	22,82		SA	PUNA TEAL	91	F	2
LB0514	18,48			19,74			14,69			5,97			6,78			SA	PUNA TEAL	91	F	2
LB0515	12,53			18,36			15,2			12,28			14,04			SA	PUNA TEAL	91	F	2
LB0516	18,77			26,79			21,84			15,26			26,16			SA	PUNA TEAL	91	F	2
SB3999	37,75	21,75		44,03	38,68		21,03	27,45		38,02	23,58		32,53	20,33		SA	PUNA TEAL	91	M	10
SB4000	12,36			19,25			11,89			10,39			9,26			SA	PUNA TEAL	91	F	10
SB4403	14,85			30,17			22,22			7,25			19,76			AUS	PUNA TEAL	91	F	10
TB0754		57,69			46,99			47,61			57,75			40,57		SA	PUNA TEAL	91	F	1
TB0756		21,37			22,57			20,46			9,62			19,12		SA	PUNA TEAL	91	M	1
LB0011	38,68			44,28			43,04			40,84			36,53			SAS	S. GEORG. PINTAIL	96	M	10
LB0179	26,37			36,55			31,56			20,83			27,22			SAS	S. GEORG. PINTAIL	96	F	9
LB0539	23,75			23,23			21,22			15,7			11,3			SAS	S. GEORG. PINTAIL	96	M	2
LB0295	42,87			38,48			44,87			33,81			29,57			SA	SHARP WINGED TEAL	103	F	8
LB0465	34,11	19,91		48,24	17,65		38,65	20,45		32,87	17,33		30,5	18,06		SA	SHARP WINGED TEAL	103	M	4
LB0466	57,32		26,99	64,5		41,75	59,47		39,18	53,96		33,75	52,35		34,81	SA	SHARP WINGED TEAL	103	M	4
LB0517	20,35			22,62			12,35			22,67			12,24			SA	SHARP WINGED TEAL	103	M	2
LB0518	20,15	16,92		36,37	32,22		22,03	19,85		30,48	21,02		15,09	22,5		SA	SHARP WINGED TEAL	103	M	2
LB0519	12,01	22,62		12,51	22,14		9,06	16,41		14,88	18,05		4,84	20,23		SA	SHARP WINGED TEAL	103	F	2
LB0520	18,89			19,91			10,67			1,73			14,57			SA	SHARP WINGED TEAL	103	F	2
SA1064	35,74	30,1		30,38	32,15		26,55	35,34		32,53	35,68		38,36	33,05		SA	SHARP WINGED TEAL	103	M	9
SB3964	11,69			17,12			12,17			8,45			9,24			SA	SHARP WINGED TEAL	103	F	10
SB3965	0	18,3	23,93	8,21	33,18	34,63	6,13	23,46	27,15	0	13,93	33,13	9,98	12,93	31,06	SA	SHARP WINGED TEAL	103	F	10
TB0752		30,04			46,04			37,76			29,5			36,95		SA	SHARP WINGED TEAL	103	M	1
TB0753		35,53			34,43			20,39			22,63			23,04		SA	SHARP WINGED TEAL	103	F	1
LB0006		49,77			37,42			56,89			41,59			35,46		AS	FALCATED TEAL	110	M	10

Bird Identification	M.av.1238			RBG			Goosander			Yellow Billed			Mallard			Pen	Species	Species number	Sex	Age in years
	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3					
LB0012		104,5			76,77			72,65			81,55			92,9		AS	FALCATED TEAL	110	M	10
LB0259		21,81			22,39			15,05			14,07			14,12		AS	FALCATED TEAL	110	M	8
LB0398		28,42			36,53			30,94			33,78			46,03		AS	FALCATED TEAL	110	F	6
LB0430		92,66			103			75,32			74,61			72,7		AS	FALCATED TEAL	110	F	5
LB0468		25,75			28,97			33,94			29,27			30,9		AS	FALCATED TEAL	110	M	5
LB0482		14,66			8,72			6,9			13,01			12,74		AS	FALCATED TEAL	110	F	4
LB0544	26,35	77,93		31,41	97,18		27,43	73,03		30,19	64,76		23,41	66,64		TOP	CHESTNUT TEAL	116	F	1
SB4408	12,41			9,65			12,01			9,39			21,11			SA	CHESTNUT TEAL	116	F	10
SC3237		53,62			54,61			60,22			65,08			60,94		AS	CHIN.SPOTBILL	130	F	10
LC0055	37,77	44,36		32,68	64,5		35,69	50,33		38,88	51,89		35,56	52,84		TOP	YELLOW BILLED D	136	F	9
LC0206	24,36			40,42			21,79			16,99			21,62			TOP	YELLOW BILLED D	136	M	8
LC0207	6,64	36,88		4,46	47,83		1,39	38,91		6,1	31,97		3,05	41,02		TOP	YELLOW BILLED D	136	M	8
LC0336	15,57	40,67		46,29	51,82		18,84	35,23		24,28	42,03		30,7	49,45		TOP	YELLOW BILLED D	136	F	6
LC0337	16,98	34,22		19,44	35,79		21,08	36,72		14,48	39,58		13,84	35,99		TOP	YELLOW BILLED D	136	F	6
LC0339	53,42	31,68		79,32	39,2		50,42	24,54		37,24	26,54		47,94	31,36		TOP	YELLOW BILLED D	136	F	6
LC0340		37,78			71,26			50,32			45,17			47,05		TOP	YELLOW BILLED D	136	F	6
LC0397	28,28	0,04		61,09	12,62		21,52	0		38,24	0		39,82	8,3		TOP	YELLOW BILLED D	136	F	5
SC3142	5,03	20,95		2,12	51,09		0,89	38,72		5,86	42,47		2,43	40,88		TOP	YELLOW BILLED D	136	M	10
SC3143	15,59	20,84		19,22	36,54		9,61	17,34		13,1	22,19		19,07	22,95		TOP	YELLOW BILLED D	136	M	10
SC3144	0	16,61		10,11	33,02		5,94	15,23		5,46	26,67		11,27	27,06		TOP	YELLOW BILLED D	136	M	10
SC3145	19,02			33,66			32,6			34,01			20,05			TOP	YELLOW BILLED D	136	F	10
SC3146	114,4			104,2			122			101,8			120,5			TOP	YELLOW BILLED D	136	M	10
SC3201	20,44	49,06		14,22	39,39		12,25	28,25		7,79	37,05		11,31	35,66		TOP	YELLOW BILLED D	136	M	10
SC3203	13,23	34,17		11,57	35,96		8,57	32,7		14,43	29		8,78	27,52		TOP	YELLOW BILLED D	136	M	10
LC0328	58,06	31,57		77,15	22,91		65,33	30,18		77,66	25,51		73,13	27,98		TOP	AFRIC. BLACK D	138	F	6
LC0330	40,05	33,23		38,38	56,3		33,16	38,98		38,5	45,03		29,47	32,96		TOP	AFRIC. BLACK D	138	M	6
LC0444	20,79	22,82		16,13	30,12		26,32	17,09		30,16	20,61		28,16	17,52		TOP	AFRIC. BLACK D	138	M	2
LC0445	57,34	36,96		55,63	44,9		65,18	35,83		52,29	41,1		47,57	32,47		TOP	AFRIC. BLACK D	138	F	2
LC0446	8,8			12,37			8,09			16,65			2,64			TOP	AFRIC. BLACK D	138	F	2
LC0446		6,99			10,32			1,17			9,39			14,94		TOP	AFRIC. BLACK D	138	F	2
LC0447	18,07	17,74		32,47	30,11		24,12	13,73		31,3	12,04		17,92	21,33		TOP	AFRIC. BLACK D	138	M	2
LC0449	67,66	44,54		62,36	43,67		62,13	55,5		50,87	46,4		54,24	38,78		TOP	AFRIC. BLACK D	138	F	2
LC0450	10,75	36,47		7,88	31,04		12,97	22,82		23,59	29,45		10,74	29,42		TOP	AFRIC. BLACK D	138	F	2
LB0089	23,41			20,78			19,35			18,17			13,51			SA	CHILOE WIGEON	145	M	9

Bird Identification	M.av.1238			RBG			Goosander			Yellow Billed			Mallard			Pen	Species	Species number	Sex	Age in years
	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3					
LB0091	35,51	28,75		42,8	39,81		27,12	25,24		44,45	18,99		39,16	26,8		SA	CHILOE WIGEON	145	M	9
LB0093	17,53	29,88		20,21	27,66		22,54	28,9		23,08	25,6		12,17	24,96		SA	CHILOE WIGEON	145	F	9
LB0094	94,67			68,34			66,18			70,25			65,71			SA	CHILOE WIGEON	145	M	9
LB0462	19,48	22,56		28,7	28,22		23,09	24,11		23,03	25,76		18,4	18,99		SA	CHILOE WIGEON	145	M	4
LB0463	16,06	40,89		27,91	38,34		12,37	37,98		10,07	38,31		12,04	33,03		SA	CHILOE WIGEON	145	F	4
LB0527	57,44	13,89		64,91	21,37		48,67	10,11		46,63	10,56		36,05	8,63		SA	CHILOE WIGEON	145	F	2
SB4438	12,91	20,52		15,16	27,66		4,55	24,36		11,41	12,44		9,16	21,38		SA	CHILOE WIGEON	145	F	10
SB4439	13,1			19,61			19,12			10,16			7,96			SA	CHILOE WIGEON	145	F	10
SB4443	12,55	9,57		20,99	19,18		11,39	9,85		16,31	4,56		15,26	16,91		SA	CHILOE WIGEON	145	F	10
SC3196		49,16			56,67			46,24			42,19			39,04		SA	CHILOE WIGEON	145	M	10
LA0063	26,22			30,42			32,96			29,43			30,13			SAS	GARGANEY	153	M	6
LA0068	67,65			66,4			65,39			69,51			52,57			SAS	GARGANEY	153	F	6
LA0069	51,5			48,02			65,5			56,44			50,33			SAS	GARGANEY	153	M	6
LB0008	24,59			27,55			31,15			23,93			28,66			SAS	GARGANEY	153	F	10
LB0178	31,43			31,31			23,46			25,97			30,71			SAS	GARGANEY	153	M	9
LB0494	46,31			49,31			50,55			44,44			45,82			SAS	GARGANEY	153	M	4
LB0538	24,83			35			28,63			22,46			23,09			SAS	GARGANEY	153	F	2
LB0541	14,38			16,63			17,2			14,68			11,19			SAS	GARGANEY	153	M	2
LB0264	27,02	26,65		48,47	43,38		36,14	24,32		32,16	28,81		30,88	25,4		SA	ARG. RED SHOVELER	154	M	8
LB0266	13,19	20,11		15,34	25,09		16,84	21,06		17,62	21,08		22,84	22,74		SA	ARG. RED SHOVELER	154	M	8
LB0267	9,85			16,58			14,94			11,67			14,33			SA	ARG. RED SHOVELER	154	M	8
LB0268	20,97	39,03		31,37	52,64		24,67	38,55		13,81	25,95		21,44	29,44		SA	ARG. RED SHOVELER	154	F	8
LB0369	24,99			30,04			30,2			22,29			28,4			SA	ARG. RED SHOVELER	154	M	7
LB0523	21,88	17,35		27,75	28,52		20,43	24,56		21,59	11,18		23,66	17,82		SA	ARG. RED SHOVELER	154	F	2
LB0524	28,28	10,29		27,27	30,06		21,52	18,28		22,77	19,1		26,46	16,03		SA	ARG. RED SHOVELER	154	F	2
LB0525	88,13	80,58	14,72	80,4	62,72	26,54	89,7	46,11	16,15	64,84	41,56	19,69	69,2	45,97	24,91	SA	ARG. RED SHOVELER	154	F	2
LB0526	19,11			29,65			26,61			21,38			26,71			SA	ARG. RED SHOVELER	154	F	2
LB0528		29,91			45,57			36,52			37,05			35,04		SA	ARG. RED SHOVELER	154	M	2
LC0231			74,85			73,46			86,25			68,13			72,01	SA	ARG. RED SHOVELER	154	F	2
LA0038	56,42	61,85		75,05	52,21		44,18	68,77		41,05	66,88		43,57			TOP	RINGED TEAL	159	M	7
LA0052	7,39	39,82		26,16	44,92		12,35	45,06		19,25	33,15		15,54	46,53		TOP	RINGED TEAL	159	M	7
LA0054	43,21	42,99		42,47	30,88		41,13	39,17		51,87	34,24		60,39	38,81		TOP	RINGED TEAL	159	F	7
LA0057		78,01			78,58			63,44			59,26			66,1		TOP	RINGED TEAL	159	M	7
LA0058		45,05			59,51			39,89			43,16			44,82		TOP	RINGED TEAL	159	M	7

Bird Iden-	M.av.1238			RBG			Goosander			Yellow Billed			Mallard			Pen	Species	Species	Sex	Age in
tification	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3			number		years
LA0059		22,31			38,63			18,05			20,34			19,22		TOP	RINGED TEAL	159	F	7
LA0073		22,32			35,58			22,38			15,99			19,33		TOP	RINGED TEAL	159	M	6
LA0090		72,08			77,08			75,64			56,84			70,35		TOP	RINGED TEAL	159	M	3
LA0100		2,64			30,87			19,31			17,09			21,42		TOP	RINGED TEAL	159	F	3
LA0109	8	30,54		24,54	10,63		17,07	20,66		20,12	22,4		23,74	17,52		TOP	RINGED TEAL	159	M	2
LA0110	12,59	25,59		30,24	36,39		24,02	30,02		4,09	17,81		19,53	21,04		TOP	RINGED TEAL	159	M	2
LA0111	7,09	22,53		16,6	24,23		14,25	24,59		16,73	19,91		22,5	20,67		TOP	RINGED TEAL	159	M	2
LA0112		41,2			53,29			49,47			51,21			48,23		TOP	RINGED TEAL	159	F	2
LA0113	14,3	21,6		6,97	34,66		9,42	23,23		35,56	15,58		15,39	24,02		TOP	RINGED TEAL	159	F	2
LA0114	18,04	45,71		17,23	50,07		18,28	49,41		15,47	36,63		15,07	40		TOP	RINGED TEAL	159	F	2
SA1023	36,83	52,01		51,16	49,37		42,59	52,39		30,18	57,37		35,93	51,19		SA	RINGED TEAL	159	M	10
SA1024	27,99	10,13		35,17	24,17		28,6	13,97		19,02	9,98		25,35	16,9		SA	RINGED TEAL	159	F	10
SA1026	24,49	20,83		34,23	26,92		25,59	17,03		20,11	11,8		24,93	18,08		SA	RINGED TEAL	159	M	10
SA1040	23,97			30,86			18,14			39,46			21,5			SA	RINGEL TEAL	159	M	10
SA1041	43,68	45,04		64,86	45,08		38,07	40,81		38,69	45,57		40,47	47,53		TOP	RINGED TEAL	159	M	10
SA1053	5,94	15,76		6,01	15,9		7,85	14,83		6,86	15,36		3,3	14,16		TOP	RINGED TEAL	159	M	10
SA1055	32,02	48,14		37,33	19,2		21,04	34,97		33,46	22,35		29,91	34,17		SA	RINGED TEAL	159	M	10
SB4445	13,91	23,87		27,28	27,44		22,98	30		17,99	26,32		22,2	24,63		TOP	RINGED TEAL	159	M	10
no ring			17,79			28,8			16,84			17,19			10,92	SA	RINGED TEAL	159	M	
LD0044	27,35	63,46		51,51	81,29		33,81	57,96		42,74	52,79		36,58	53,94		TOP	EUROP. EIDER	170	M	7
LD0062	9,16	32,29		28,01	43,72		28,87	48,09		24,85	43,32		31,5	35,9		TOP	EUROP. EIDER	170	F	4
LD0063	25,99	27,68		34,39	39,14		31,19	23,39		27,05	25,13		37,23	33,49		TOP	EUROP. EIDER	170	M	4
LD0064	27,7	73,73		24,59	74,86		32,47	62,64		37,65	80,6		35,66	72,11		TOP	EUROP. EIDER	170	M	4
LD0065		69,49			66,7			66,78			71,27			57,76		TOP	EUROP. EIDER	170	M	4
LD0066	24,46	78,98		38,84	87,72		31,64	67,77		29,32	61,11		40,35	60,26		TOP	EUROP. EIDER	170	M	4
SD2145	57,38	47,95	62,57	57,6	59,34	77,67	61,07	52,62	69,07	58,16	38,54	69,69	48,24	41,91	64,16	TOP	EUROP. EIDER	170	M	11
SD2169	72,8	64,84	71,78	89,6	81,73	73,14	68,28	75,24	63,57	77,26	57,74	61,88	84,54	56,83	58,02	TOP	EUROP. EIDER	170	F	10
SD2236	14,6	61,03		17,63	84,02		12,52	66,84		24,6	43,62		16,03	55,68		TOP	EUROP. EIDER	170	M	9
LC0226		66,4			62,1			62,81			58,67			54,5		AS	RED CREST. POCHARD	178	M	8
LC0227		39,01			40,8			26,45			33,03			32,63		AS	RED CREST. POCHARD	178	F	8
LC0228		39,84			30,1			46,18			30,88			38,37		AS	RED CREST. POCHARD	178	M	8
LC0229		45,72			50,87			36,55			40,09			41,95		AS	RED CREST. POCHARD	178	M	8
LC0385		52,51			38,08			44,37			40,55			43,36		AS	RED CREST. POCHARD	178	M	7
SC3134		47,39			62,56			57,72			55,24			53,26		AS	RED CREST. POCHARD	178	F	10

Bird Identification	M.av.1238 elisa1	elisa2	elisa3	RBG elisa1	elisa2	elisa3	Goosander elisa1	elisa2	elisa3	Yellow Billed elisa1	elisa2	elisa3	Mallard elisa1	elisa2	elisa3	Pen	Species	Species number	Sex	Age in years
SC3136		37,79			30,42			24,46			34,13			35,12		AS	RED CREST. POCHARD	178	M	10
SC3137		53,49			52,88			53,82			51,07			42,2		AS	RED CREST. POCHARD	178	F	10
SC3139		47,39			52,59			55,2			60,52			51,04		AS	RED CREST. POCHARD	178	M	10
SC3222		38,62			52,26			49,03			53,9			49,67		AS	RED CREST. POCHARD	178	F	10
SC3225		42,02			47,63			38,38			48,59			44,02		AS	RED CREST. POCHARD	178	F	10
LC0079	22,69	24,03		37,23	30,4		24,09	21,68		14,19	10,14		16,59	16,3		SA	ROSYBILL	179	M	9
LC0243	14,33	83,47		18,53	74,87		18,06	70,97		20,99	75,55		14,14	66,56		SA	ROSYBILL	179	M	8
LC0244	35,04	42,96		43,01	39,94		21,84	45,41		23,01	39,19		26,74	40,57		SA	ROSYBILL	179	F	8
LC0386	19,12	13,06		34,79	28,9		30,49	20,91		32,39	18,47		33,39	22,08		SA	ROSYBILL	179	M	5
LC0387	54,24	35,5		66,16	51,54		38,06	40,01		37,14	31,17		42,81	34,08		SA	ROSYBILL	179	F	5
LD0039	12,95	17,16		16,72	21,52		20,54	10,6		7,74	7,02		23,94	19,31		SA	ROSYBILL	179	M	7
SC3115	26,7	24,62		35,01	22,27		24,17	23,8		28,27	14,75		28,93	15,13		SA	ROSYBILL	179	M	11
SC3164	9,52	18,95		6,84	30,67		4,76	24,92		2,36	13,65		4,7	18,33		SA	ROSYBILL	179	M	10
SC3165	40,44	15,09		46,01	31,55		32,86	17,51		41,07	7,06		40,02	16,18		SA	ROSYBILL	179	F	10
SC3166	26,72	31,3		35,98	38,36		31,9	35,66		27,11	35,69		26,15	40,8		SA	ROSYBILL	179	M	10
SC3167	10,01	20,53		18,44	31,92		9,21	22,76		3,48	20,51		7,28	23,39		SA	ROSYBILL	179	F	10
SC3169	18,81	35,76		18,28	25,06		14,56	33,71		11,07	29,78		14,43	26,85		SA	ROSYBILL	179	M	10
SC3170	52,09			36,99			27,27			43,05			59,14			SA	ROSYBILL	179	F	10
SC3171	38,72			39,37			28,76			38,49			36,77			SA	ROSYBILL	179	F	10
LC0102		45,4			56,21			69,11			57,68			52,6		NA	CANVASBACK	182	F	9
LC0104		36,27			47,62			38,79			54,9			48,03		NA	CANVASBACK	182	M	9
LC0105		63,19			55,16			63,19			47,45			60,31		NA	CANVASBACK	182	M	9
LC0214		57,07			60,78			64,62			51,09			53,74		AS	CANVASBACK	182	M	8
LC0215		35,11			44,55			38,89			53,97			50,09		NA	CANVASBACK	182	M	8
LC0216		121,3			121,5			164,8			144,9			140,7		AS	CANVASBACK	182	F	8
LC0218		52,64			63,48			78,57			76,36			71,93		NA	CANVASBACK	182	F	8
LC0333		51,78			46,2			28,05			37,82			29,99		NA	CANVASBACK	182	F	6
LC0335		36,86			34,04			31,31			29,73			27,68		NA	CANVASBACK	182	F	6
LB0366		52,42			49,86			56,89			58,83			48,69		AS	BAERS POCHARD	187	F	7
LB0408		83,49			59,72			71,42			77,53			75,1		AS	BAERS POCHARD	187	M	10
LB0175		59,51			66,94			71,45			77,61			74,01		NA	LESSER SCAUP	193	M	9
LB0449		55,45			68,26			57,28			61,91			64,28		NA	LESSER SCAUP	193	M	8
SB4486		59,77			51,29			56,01			50,9			61,43		AS	LESSER SCAUP	193	F	10
SB4490		120			115,2			110,3			111			112,7		AS	LESSER SCAUP	193	M	10

Bird Iden-	M.av.1238			RBG			Goosander			Yellow Billed			Mallard			Pen	Species	Species	Sex	Age in
tification	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3			number		years
SB4491		39,55			36,08			27,52			32,98			39,16		NA	LESSER SCAUP	193	M	10
SB4492		104,8			114			118,6			125,3			117,2		NA	LESSER SCAUP	193	M	10
LB0185	29,01	56,1		36,69	52,96		24,65	53,83		35,41	55,23		30,82	50,28		SA	GR. BRAZIL. TEAL	197	M	9
LB0186	33,27	35,63		40,98	37,02		33,09	37,28		43,41	36,25		30,99	31,76		SA	GR. BRAZIL. TEAL	197	M	9
LB0521	14,33	44,74		25,32	37,02		14,3	51,59		13,73	41,17		16,22	45,09		SA	GR. BRAZIL. TEAL	197	M	2
LC0112	29,03			37,09			38,38			41,72			33,97			TOP	MANED G	198	F	9
LC0434	46,32			50,33			52,72			60,05			55,08			TOP	MANED G	198	M	3
LB0188		24,85			18,69			17,51			17,27			24,07		SA	MANDARIN D	199	M	9
LB0419		22,54			34,63			20,53			16,13			25,76		SA	MANDARIN D	199	M	5
LB0435	65,43	10,71		8,34	7,04		88,5	10,8		65,33	12,58		56,56	16,4		TOP	MANDARIN D	199	M	5
LB0437	18,78	14,5		18,79	14,29		16,88	11,74		16,22	13,22		19,2	7,42		TOP	MANDARIN D	199	M	5
LB0475		61,85			58,65			60,12			61,07			56,18		TOP	MANDARIN D	199	M	4
LB0477	1,92			12,73			9,48			12,25			18,14			TOP	MANDARIN D	199	M	4
LB0479	19,12	22,03		34,61	28,33		17,24	19,28		27,73	23,43		24,86	24,65		TOP	MANDARIN D	199	F	4
LB0505		41,14			39,79			43,99			47,9			38,75		TOP	MANDARIN D	199	M	3
LB0506	17,91	27,96		22,07	32,7		14,02	25,7		38,78	25,01		15,51	36,8		TOP	MANDARIN D	199	M	3
LB0507	14,17	16,01		22,74	17,53		12,41	13,39		14,15	16		9,88	15,79		TOP	MANDARIN D	199	M	3
LB0545		28,33			48,88			37,95			26,12			36,44		TOP	MANDARIN D	199	F	1
SB3861	2,17	10,16		11,85	9,69		7,26	11,1		4,53	3,91		12,63	6,98		TOP	MANDARIN D	199	F	11
SB3862	16,93			20,89			14,66			16,09			7,49			TOP	MANDARIN D	199	M	11
TB0757		63,92			58,24			60,22			68,07			66,19		TOP	MANDARIN D	199	M	1
S2131	25,56			27,23			10,83			23,88			27,5			SMW	WHITE.WING.WOOD D	209	F	9
S2402	92,98			75,51			64,02			77,26			88,54			SMW	WHITE.WING.WOOD D	209	F	9
LB0403		12,03			12,96			10,02			11,49			9,82		TOP	BUFFLEHEAD	227	F	6
LB0404	8,47	32,97		14,7	31,6		10,52	34,42		7,83	34,09		10,17	36,2		TOP	BUFFLEHEAD	227	M	6
LB0405	56,4	54,61		65,58	51,59		53,34	41,39		59,64	42,94		57,2	44,34		TOP	BUFFLEHEAD	227	F	6
LB0492		35,55			38,13			51,38			41,72			40,21		TOP	BUFFLEHEAD	227	F	3
LB0536	10,88	27		7,8	47,45		7,51	37,92		10,53	41,9		9,31	41,44		TOP	BUFFLEHEAD	227	F	2
LB0537	7,22	14,83		6,37	15,34		5,44	15,68		10,25	11,41		8,82	11,84		TOP	BUFFLEHEAD	227	F	2
SA1048		23,22			39,06			32,06			33,16			31,7		TOP	BUFFLEHEAD	227	F	9
SB4476		86,21			81,87			79,22			74,67			67,78		TOP	BUFFLEHEAD	227	M	10
SB4478		38,26			38,23			29,21			34,5			38,43		TOP	BUFFLEHEAD	227	M	10
SB4480		30,23			34,14			30,54			32,79			32,58		TOP	BUFFLEHEAD	227	M	10
SP4476	7,27			8,5			4,64			6,07			0			TOP	BUFFLEHEAD	227	M	10

Bird Identification	M.av.1238			RBG			Goosander			Yellow Billed			Mallard			Pen	Species	Species number	Sex	Age in years
	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3					
LB0314	22,07			57,58			26,78			25,31			29,88			SMW	SMEW	228	F	7
LB0354		32,26			41,92			34,41			42,66			37,1		AS	HOODED MERGANSER	229	F	7
LB0532		31,01			35,39			34,02			31,38			39,7		AS	HOODED MERGANSER	229	M	2
LB0533		32			39,42			31,07			31,86			31,99		NA	HOODED MERGANSER	229	M	2
LB0534		92,88			100			100			86,03			80,83		NA	HOODED MERGANSER	229	F	2
LB0535		85,12			79,89			101,9			73,17			81,36		NA	HOODED MERGANSER	229	M	2
LB0543		12,17			13,53			11,88			10,88			15,14		NA	HOODED MERGANSER	229	M	2
LB0546		55,93			59,75			81,8			66,53			84,6		AS	HOODED MERGANSER	229	F	1
LB0547		48,5			63,29			57,59			61,24			60,91		AS	HOODED MERGANSER	229	M	1
LB0548		69,85			57,72			62,54			60,43			63,58		NA	HOODED MERGANSER	229	M	1
LB0549		54,2			55,31			58,04			55,06			55,62		AS	HOODED MERGANSER	229	F	1
LC0341	32,56			43,21			27,52			38,22			35,85			SPC	MACCOA D	245	M	6
LC0342	32			35,75			15,71			32,15			33,85			SPC	MACCOA D	245	F	6
LC0428	25,69	59,48		24,59	57,18		17,63	56,54		16,33	57,63		15,27	53,8		SPC	MACCOA D	245	F	2
LC0914		15,45			14,39			13,7			12,13			10,29		TOP	MACCOA D	245	F	1
SC3297	17,82	13,44		14,9	10,34		9,49	15,34		8,89	13,97		7,86	11,42		TOP	MACCOA D	245	F	10
SC3300	12,8	48,01		19,69	58,78		1,42	43,65		0	43,69		7,54	42,13		TOP	MACCOA D	245	F	10
LB0425	17,96			30,43			19,42			17,93			19,74			SAS	BLACK HEADED D	249	M	5

Appendix 4: Detailed ELISA and post mortem results of birds which were tested and have died during this study

Bird Iden-	M.av.1238			RBG			Goosander			Yellow Billed			Mallard			PM	Histo.	Pen	Species	Species	Sex	Age in
tification	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	Diagno.	Diagno.			number		years
LC0289	24,37			20,95			21,08			22,73			21,14			NTB		SPE	SPOTTED WHISTL. D	2	F	7
LC0240	86,64		9,82	74,53		23,3	86,97		15,12	82,82		23,44	84,43		18,09	NTB	NTB	AUS	EYTON'S WHISTL. D	3	F	8
LC0485		19,18			25			26,1			27,24			27,15		NTB		AS	WANDER. WHISTL. D	4	M	1
LC0364	14,09	46,28		24,93	57,9		17,16	59,11		14,29	64,3		19,55	56,29		NTB		TOP	WHI.FACE.WHISTL. D	10	M	6
LK0004		34,63			57,67			51,42			35,39			39,74		NTB		TOP	BLACK NECKED SWAN	16	F	3
S2431	46,09			39,77			38,19			27,6			28,66			NTB		NA	LES.WHITE.FRONT. G	31	M	9
SD2181	37,57	26,49	41,1	67,86	40,33	48,87	58,04	28,69	43,64	50,93	36,59	46,88	49,3	34,06	39,59	NTB	NTB	TOP	LES.WHITE.FRONT. G	33	M	10
S2106	107,8	33,8	45,71	110,2	36,94	45,95	112,8	38,68	44,32	98,59	36,63	44,69	98,76	35,84	50,85	NTB	NTB	AS	BARHEADED G	36	M	10
S2110	40,93	56,47		32,45	58,99		23,89	71,7		33,55	81,38		38,02	74,72		NTB		NA	EMPEROR G	37	M	10
SC3193	56,48	51,32	27,3	67,25	57,45	28,16	67,99	45,97	27,15	64,34	48,72	30,63	63,16	42,81	32,08	NTB	NTB	TOP	RED BREASTED G	59	F	10
SC3197	67,8	20,1	15,64	50,47	42,37	24,92	48,84	28,65	3,09	54,41	33,31	11,88	60,34	32,73	4,44	NTB	NTB	TOP	RED BREASTED G	59	M	10
SC3198	58,83		2,76	89,52		18,45	82,12		6,87	79,32		17,5	70,19		13,65	NTB	NTB	TOP	RED BREASTED G	59	M	10
SD2217	16,32	15,32		23,99	31,39		22,41	19,82		20,3	20,86		26,44	22,73		NTB		TOP	RED BREASTED G	59	M	10
LC0036		45,38			59,22			56,68			49,08			53,25		NTB		AS	RUDDY SHELDUCK	61	F	9
SD2128	37,1	11,39	44,79	56,55	24,53	47,25	48,53	13,48	43,99	51,38	17,74	45	50,72	12,18	52,22	NTB	NTB	TOP	COMMON SHELDUCK	67	F	11
SD2134	44,71	44,41	38,34	65,68	47,91	24,92	54,27	38,99	11	55,44	34,23	17,5	49,69	39,92	16,38	NTB	NTB	TOP	COMMON SHELDUCK	67	M	11
LA0107	49,25			49,85			36,55			43,47			46,21			NTB		SPC	HOTTENTOT TEAL	88	F	2
LB0515	12,53			18,36			15,2			12,28			14,04			NTB		SA	PUNA TEAL	91	F	2
LB0295	42,87			38,48			44,87			33,81			29,57			NTB		SA	SHARP WINGED TEAL	103	F	8
LB0466	57,32		26,99	64,5		41,75	59,47		39,18	53,96		33,75	52,35		34,81	NTB	NTB	SA	SHARP WINGED TEAL	103	M	4
SB3965	0	18,3	23,93	8,21	33,18	34,63	6,13	23,46	27,15	0	13,93	33,13	9,98	12,93	31,06	NTB	NTB	SA	SHARP WINGED TEAL	103	F	10
LC0339	53,42	31,68		79,32	39,2		50,42	24,54		37,24	26,54		47,94	31,36		NTB		TOP	YELLOW BILLED D	136	F	6
LB0093	17,53	29,88		20,21	27,66		22,54	28,9		23,08	25,6		12,17	24,96		NTB		SA	CHILOE WIGEON	145	F	9
SB4439	13,1			19,61			19,12			10,16			7,96			NTB		SA	CHILOE WIGEON	145	F	10
LB0369	24,99			30,04			30,2			22,29			28,4			NTB		SA	ARG. RED SHEVOLER	154	M	7
LB0525	88,13	80,58	14,72	80,4	62,72	26,54	89,7	46,11	16,15	64,84	41,56	19,69	69,2	45,97	24,91	NTB	NTB	SA	ARG. RED SHEVOLER	154	F	2
no ring			17,79			28,8			16,84			17,19			10,92	NTB	NTB	SA	RINGED TEAL	159	M	
SD2145	57,38	47,95	62,57	57,6	59,34	77,67	61,07	52,62	69,07	58,16	38,54	69,69	48,24	41,91	64,16	NTB	NTB	TOP	EUROP. EIDER	170	M	11
S2131	25,56			27,23			10,83			23,88			27,5			NTB		SMW	WHITE.WING.WOOD D	209	F	9

Bird Identification	M.av.1238			RBG			Goosander			Yellow Billed			Mallard			PM Diagno.	Histo. Diagno.	Pen	Species	Species number	Sex	Age in years
	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3	elisa1	elisa2	elisa3							
SB4476		86,21			81,87			79,22			74,67			67,78		NTB		TOP	BUFFLEHEAD	227	M	10
LC0342	32			35,75			15,71			32,15			33,85			NTB		SPC	MACCOA D	245	F	6
SC3300	12,8	48,01		19,69	58,78		1,42	43,65		0	43,69		7,54	42,13		NTB		TOP	MACCOA D	245	F	10
LC0230	99,33		6,75	89,57		6,8	63,06		6,88	90,39		5,94	80,8		3,75	TB	NTB	SA	FULVOUS WHISTL. D	7	F	8
LC0136	54,59		9,2	75		16,18	44,76		13,06	56,7		14,06	57,68		12,63	TB	NTB	TOP	WHI.FACE.WHISTL. D	10	M	9
LC0316	33,67		47,24	47,91		37,86	41,65		33,33	53,55		41,25	51,98		42,32	TB	TB	TOP	WHI.FACE.WHISTL. D	10	F	7
PO0622	25,28	43,22		32,07	58,21		32,34	45,03		36,63	40,1		35,13	34,41		TB		AS	SWAN G	22	F	10
L0053	30,4	76,79		27,86	76,55		19,09	80,63		30,23	77,23		25,69	78,48		TB		NA	PINKFOOTED G	28	F	6
S2056	54,8			21,28			27,71			31,13			24,24			TB		NA	PINKFOOTED G	28	F	11
SD2186	59,81	49,16	8,28	74,32	56,95	0,65	69,43	50,02	2,06	67,72	53,17	3,75	63,71	51,32	13,31	TB	NTB	TOP	LES.WHITE.FRONT. G	33	F	10
LC0041	40,61			45,42			52,9			52,11			50,63			TB		TOP	RUDDY SHELDUCK	61	M	9
SB4403	14,85			30,17			22,22			7,25			19,76			TB		AUS	PUNA TEAL	91	F	10
LB0259		21,81			22,39			15,05			14,07			14,12		TB		AS	FALCATED TEAL	110	M	8
LB0094	94,67			68,34			66,18			70,25			65,71			TB		SA	CHILOE WIGEON	145	M	9
LC0231			74,85			73,46			86,25			68,13			72,01	TB	NTB	SA	ARG. RED SHEVOLER	154	F	
SD2169	72,8	64,84	71,78	89,6	81,73	73,14	68,28	75,24	63,57	77,26	57,74	61,88	84,54	56,83	58,02	TB	NTB	TOP	EUROP. EIDER	170	F	10
LC0105		63,19			55,16			63,19			47,45			60,31		TB		NA	CANVASBACK	182	M	9
SB4491		39,55			36,08			27,52			32,98			39,16		TB		NA	LESSER SCAUP	193	M	10
LB0435	65,43	10,71		8,34	7,04		88,5	10,8		65,33	12,58		56,56	16,4		TB		TOP	MANDARIN D	199	M	5
S2402	92,98			75,51			64,02			77,26			88,54			TB		SMW	WHITE.WING.WOOD D	209	F	9
LB0354		32,26			41,92			34,41			42,66			37,1		TB		AS	HOODED MERGANSER	229	F	7
LB0533		32			39,42			31,07			31,86			31,99		TB		NA	HOODED MERGANSER	229	M	2

Appendix 5: Detailed post mortem results of all birds which died from 1989 to 1999

Bird identification	Date	Season	Species number	Species	Tribe	Pen	Sex	Age in years	PM Diagnosis
	1.1.89	WINTER	93	BAHAMA PINTAIL	6	GRD	M	1	NTB
	1.11.89	WINTER	99	NORTHERN PINTAIL (WILD)	6	GRD	F	1	NTB
SB3936	1.24.90	WINTER	192	TUFTED DUCK	9	LAG	M	2	NTB
SC3112	3.26.90	SPRING	136	African Yellowbill	6	GRD	F	2	NTB
SB3872	5.8.90	SPRING	199	MANDARIN DUCK	10	FLM	F	2	NTB
SB3891	5.25.90	SPRING	143	EUROPEAN WIGEON	6	GRD	F	2	NTB
	11.10.90	AUTUMN	11	RED BILLED WHISTL. D	2	GRD	M	2	NTB
LB0021	4.13.91	SPRING	87	CAPE TEAL	6	TOP	M	2	NTB
FSD	5.4.91	SPRING	253	CARIB FLAMINGO	13	FLM	F		NTB
SB3879	5.11.91	SPRING	143	EUROPEAN WIGEON	6	EUR	F	3	NTB
SA1060	5.12.91	SPRING	153	GARGANEY	6	EUR	M	2	NTB
SB3904	5.12.91	SPRING	141	GADWALL	6	GRD	F	3	NTB
LB0019	5.13.91	SPRING	200	CAROLINA	10	GRD	F	2	NTB
SB3876	5.14.91	SPRING	199	MANDARIN DUCK	10	GRD	F	3	NTB
SC3151	5.20.91	SPRING	134	PHILIPPINE DUCK	6	GRD	M	2	NTB
SB3920	6.14.91	SUMMER	192	TUFTED DUCK	9	EUR	F	3	NTB
SA1008	6.21.91	SUMMER	146	BLUE WING TEAL	6	GRD	M	2	NTB
LB0010	7.3.91	SUMMER	96	SOUTH GEORGIAN PINTAIL	6	GRD	F	2	NTB
	7.26.91	SUMMER	159	RINGED TEAL	6	SA	M	2	NTB
LC0015	8.21.91	SUMMER	240	NORTH AMERIC. RUDDY DUCK	12	FLM	F	2	NTB
SB4412	9.5.91	AUTUMN	116	CHESTNUT-BREASTED TEAL	6	GRD	F	2	NTB
SA1086	10.30.91	AUTUMN	147	ATLANTIC BLUE-WIND. TEAL	6	ST	M	1	NTB
SC3283	11.20.91	AUTUMN	198	MANED GOOSE	10	AUS	M	2	NTB
SB4466	12.1.91	WINTER	87	CAPE TEAL	6	GRD	F	2	NTB
SA1029	12.28.91	WINTER	159	RINGED TEAL	6	TOP	M	2	NTB
LA0017	1.14.92	WINTER	106	EUROP. GREEN-WINGED TEAL	6	EUR	M	2	NTB
LA0001	1.15.92	WINTER	86	MARBLED TEAL	6	AS	M	2	NTB
SB4464	1.23.92	WINTER	87	CAPE TEAL	6	TOP	F	3	NTB

Bird identification	Date	Season	Species number	Species	Tribe	Pen	Sex	Age in years	PM Diagnosis
SB3982	1.27.92	WINTER	103	SHARP-WINGED TEAL	6	SA	M	3	NTB
S2405	2.1.92	WINTER	209	WHITE-WINGED WOOD DUCK	10	SMW	M	2	NTB
SC3284	2.18.92	WINTER	198	MANED GOOSE	10	GRD	F	3	NTB
LB0184	4.5.92	SPRING	197	GREATER BRAZILIAN TEAL	10	SA	M	2	NTB
SC3113	4.6.92	SPRING	136	AFRICAN YELLOWBILL	6	TOP	F	4	NTB
SB3856	4.8.92	SPRING	199	MANDARIN DUCK	10	TOP	M	3	NTB
	4.8.92	SPRING	67	COMMON SHELDUCK	4	GRD	M	4	NTB
SC3110	4.14.92	SPRING	136	AFRICAN YELLOWBILL	6	TOP	F	4	NTB
SA1075	4.27.92	SPRING	153	GARGANEY	6	SAS	M	2	NTB
LC0081	4.28.92	SPRING	130	CHINESE SPOTBILL	6	AS	M	2	NTB
LB0031	5.7.92	SPRING	126	HAWAIIAN DUCK	6	ISL	F	2	NTB
SC3138	5.10.92	SPRING	178	RED-CRESTED POCHARD	9	AS	F	3	NTB
S2100	5.26.92	SPRING	36	BAR-HEADED GOOSE	3	AS	F	3	NTB
LB0104	5.29.92	SPRING	200	CAROLINA	10	AS	M	2	NTB
	6.1.92	SUMMER	67	COMMON SHELDUCK	4	GRD	F	3	NTB
SC3263	6.11.92	SUMMER	10	WHITE-FACED WHISTL. D	2	TOP	M	3	NTB
SB4475	6.22.92	SUMMER	187	BAERS POCHARD	9	AS	F	3	NTB
LB0071	6.27.92	SUMMER	158	COMMON SHOVELER	6	EUR	M	2	NTB
SC3288	7.10.92	SUMMER	10	WHITE-FACED WHISTL. D	2	SA	F	3	NTB
SA1083	8.3.92	SUMMER	88	HOTTENTOT TEAL	6	EURS	M	2	NTB
SC3120	10.6.92	AUTUMN	225	EUROPEAN GOLDENEYE	11	GRD	F	4	NTB
S000311	11.2.92	AUTUMN	15	MUTE SWAN	3	GRD	F	3	NTB
SD2257	11.14.92	AUTUMN	7	FULVOUS WHISTLING DUCK	2	ISL	F	2	NTB
SB3869	12.20.92	WINTER	199	MANDARIN DUCK	10	ISL	F	4	NTB
	1.14.93	WINTER	170	EUROPEAN EIDER	8	TOP	M	2	NTB
SA1037	2.12.93	WINTER	152	NORTHERN CINNAMON TEAL	6	FLM	F	4	NTB
SD2155	3.7.93	SPRING	63	AUSTRALIAN SHELDUCK	4	AUS	F	4	NTB
SB3914	3.16.93	SPRING	199	MANDARIN DUCK	10	TOP	F	5	NTB
SC3188	3.16.93	SPRING	12	SOUTH. RED BILLED WHISTL. D	2	FLM	F	4	NTB
FAY	3.21.93	SPRING	253	CARIBBEAN FLAMINGO	13	FLM	F	?	NTB
S000337	4.2.93	SPRING	14	BLACK SWAN	3	GRD	M	4	NTB

Bird identification	Date	Season	Species number	Species	Tribe	Pen	Sex	Age in years	PM Diagnosis
SC3202	4.6.93	SPRING	136	AFRICAN YELLOWBILL	6	TOP	F	4	NTB
S000323	5.1.93	SPRING	20	WHOOPER SWAN	3	GRD	M	4	NTB
SB4413	5.3.93	SPRING	229	HOODED MERGANSER	11	NA	M	4	NTB
LC0162	5.15.93	SPRING	183	EUROPEAN POCHARD	9	EUR	F	2	NTB
SA1028	5.28.93	SPRING	159	RINGED TEAL	6	SA	M	4	NTB
SC3298	7.1.93	SUMMER	245	AFRICAN MACCOA DUCK	12	TP	F	4	NTB
SA1098	7.2.93	SUMMER	88	HOTTENTOT TEAL	6	EURS	F	3	NTB
SB4458	7.12.93	SUMMER	87	CAPE TEAL	6	TOP	M	4	NTB
	7.24.93	SUMMER	193	LESSER SCAUP	9	NA	F	4	NTB
	7.24.93	SUMMER	193	LESSER SCAUP	9	NA	F	4	NTB
LB0171	8.1.93	SUMMER	91	PUNA TEAL	6	SA	F	3	NTB
LB0171	8.1.93	SUMMER	91	PUNA TEAL	6	SA	F	3	NTB
SA1022	8.11.93	SUMMER	159	RINGED TEAL	6	SA	F	4	NTB
SA1022	8.11.93	SUMMER	159	RINGED TEAL	6	SA	F	4	NTB
SD2147	8.24.93	SUMMER	170	EUROPEAN EIDER	8	EUR	F	5	NTB
SD2147	8.24.93	SUMMER	170	EUROPEAN EIDER	8	EUR	F	5	NTB
SC3224	9.12.93	AUTUMN	178	RED-CRESTED POCHARD	9	GRD	M	4	NTB
SC3224	9.12.93	AUTUMN	178	RED-CRESTED POCHARD	9	GRD	M	4	NTB
SD2172	10.5.93	AUTUMN	235	GOOSANDER	11	EUR	M	4	NTB
SD2172	10.5.93	AUTUMN	235	GOOSANDER	11	EUR	M	4	NTB
LC0135	11.25.93	AUTUMN	10	WHITE-FACED WHISTL. D	2	TOP	M	3	NTB
LC0135	11.25.93	AUTUMN	10	WHITE-FACED WHISTL. D	2	TOP	M	3	NTB
SB3996	11.30.93	AUTUMN	158	COMMON SHOVELER	6	EUR	M	4	NTB
SB3996	11.30.93	AUTUMN	158	COMMON SHOVELER	6	EUR	M	4	NTB
SD2159	12.9.93	WINTER	170	EUROPEAN EIDER	8	TOP	M	4	NTB
SD2159	12.9.93	WINTER	170	EUROPEAN EIDER	8	TOP	M	4	NTB
LB0161	12.12.93	WINTER	91	PUNA TEAL	6	SA	F	3	NTB
LB0161	12.12.93	WINTER	91	PUNA TEAL	6	SA	F	3	NTB
LC0103	1.31.94	WINTER	153	GARGANEY	6	SAS	M	4	NTB
LD0029	2.8.94	WINTER	73	RUDDY-HEADED GOOSE	4	SA	F	2	NTB
SA1001	2.20.94	WINTER	153	GARGANEY	6	SAS	M	5	NTB

Bird identification	Date	Season	Species number	Species	Tribe	Pen	Sex	Age in years	PM Diagnosis
SC3293	2.28.94	WINTER	170	EUROPEAN EIDER	8	TOP	M	5	NTB
SA1039	3.9.94	SPRING	10	WHITE-FACED WHISTL. D	2	ZO	F	5	TB
LC0250	3.13.94	SPRING	245	AFRICAN MACCOA DUCK	12	AUS	F	3	TB
LC0133	3.28.94	SPRING	126	HAWAIIAN DUCK	6	ISL	M	4	NTB
LC0246	3.28.94	SPRING	3	EYTON'S WHISTLING DUCK	2	AUS	F	3	NTB
LB0066	3.29.94	SPRING	4	E. IND. WANDER. WHISTL. D	2	AS	F	4	NTB
SD2234	4.12.94	SPRING	136	AFRICAN YELLOWBILL	6	SA	F	4	NTB
SC3268	4.15.94	SPRING	8	CUBAN WHISTLING DUCK	2	ISL	M	5	NTB
SD2219	4.22.94	SPRING	182	CANVASBACK	9	NA	F	5	NTB
	4.25.94	SPRING	198	MANED GOOSE	10	AUS	F	4	NTB
LB0276	5.4.94	SPRING	199	MANDARIN DUCK	10	TOP	F	4	NTB
LC0245	6.4.94	SUMMER	170	EUROPEAN EIDER	8	TOP	F	3	NTB
SA1069	6.7.94	SUMMER	10	WHITE-FACED WHISTL. D	2	TOP	F	4	NTB
LA0022	6.11.94	SUMMER	141	GADWALL	6	EUR	F	4	NTB
SB3939	6.14.94	SUMMER	86	MARBLED TEAL	6	AS	F	5	TB
LA0020	7.1.94	SUMMER	187	BAER'S POCHARD	9	AS	F	3	NTB
S2411	8.6.94	SUMMER	10	WHITE-FACED WHISTL. D	2	GRD	F	3	NTB
LC0208	8.11.94	SUMMER	4	E. IND. WANDER. WHISTL. D	2	AS	F	3	NTB
LC0076	9.13.94	AUTUMN	99	NORTHERN PINTAIL	6	EUR	F	4	NTB
SD2168	9.22.94	AUTUMN	159	RINGED TEAL	6	TOP	F	5	NTB
LC0109	9.23.94	AUTUMN	209	WHITE-WINGED WOOD DUCK	10	FLM	F	4	NTB
SB4474	9.24.94	AUTUMN	170	EUROPEAN EIDER	8	TOP	F	5	NTB
LB0358	11.2.94	AUTUMN	106	EUROP. GREEN-WINGED TEAL	6	EUR	F	2	NTB
LC0270	12.1.94	WINTER	10	WHITE-FACED WHISTL. D	2	GRD	F	2	NTB
S2415	1.18.95	WINTER	209	WHITE-WINGED WOOD DUCK	10	SMW	F	4	TB
SA1081	1.23.95	WINTER	86	MARBLED TEAL	6	AS	M	5	NTB
SB4402	1.25.95	WINTER	91	PUNA TEAL	6	SA	M	5	NTB
LB0069	2.12.95	WINTER	158	COMMON SHOVELER	6	EUR	F	4	NTB
P00647	2.24.95	WINTER	22	SWAN GOOSE	3	AS	M	6	NTB
	3.4.95	SPRING	2	SPOTTED WHISTLING DUCK	2	SPA	F	3	NTB
LB0101	3.25.95	SPRING	200	CAROLINA	10	TOP	F	5	NTB

Bird identification	Date	Season	Species number	Species	Tribe	Pen	Sex	Age in years	PM Diagnosis
LB0402	3.25.95	SPRING	227	BUFFLEHEAD	11	AS	F	2	NTB
SC3239	3.30.95	SPRING	134	PHILIPPINE DUCK	6	TOP	M	6	TB
SA1080	4.7.95	SPRING	152	NORTHERN CINNAMON TEAL	6	FLM	M	5	NTB
LC0072	4.18.95	SPRING	4	E. IND. WANDER. WHISTL. D	2	AS	M	5	TB
SA1071	5.4.95	SPRING	147	ATLANTIC BLUE-WIND. TEAL	6	ST	M	5	NTB
SC3296	5.11.95	SPRING	245	AFRICAN MACCOA DUCK	12	TOP	F	6	NTB
LB0296	5.16.95	SPRING	103	SHARP-WINGED TEAL	6	SA	M	4	NTB
LA0036	5.25.95	SPRING	159	RINGED TEAL	6	SA	F	4	TB
LB0378	6.6.95	SUMMER	127	LAYSAN TEAL	6	ISL	F	2	NTB
SC3287	6.20.95	SUMMER	10	WHITE-FACED WHISTL. D	2	TOP	F	5	NTB
LA0016	8.25.95	SUMMER	106	EUROP. GREEN-WINGED TEAL	6	EUR	M	5	NTB
LC0279	10.9.95	AUTUMN	10	WHITE-FACED WHISTL. D	2	TOP	M	3	NTB
SB4462	10.15.95	AUTUMN	87	CAPE TEAL	6	TOP	F	6	NTB
LA0024	12.17.95	WINTER	152	NORTHERN CINNAMON TEAL	6	FLM	F	4	TB
S2413	12.30.95	WINTER	209	WHITE-WINGED WOOD DUCK	10	FLM	M	4	TB
S2413	12.30.95	WINTER	209	WHITE-WINGED WOOD DUCK	10	FLM	M	4	TB
SC3176	12.31.95	WINTER	3	EYTON'S WHISTLING DUCK	2	SMW	M	6	NTB
LBO172	1.22.96	WINTER	91	PUNA TEAL	6	SA	F	6	NTB
LD0045	1.25.96	WINTER	170	EUROPEAN EIDER	8	TOP	F	4	NTB
SC3268	1.25.96	WINTER	10	WHITE-FACED WHISTL. D	2	TOP	M	7	NTB
S2403	1.27.96	WINTER	209	WHITE-WINGED WOOD DUCK	10	SMW	F	6	TB
SA1038	1.28.96	WINTER	152	NORTHERN CINNAMON TEAL	6	FLM	F	7	NTB
LC0068	2.1.96	WINTER	4	E. IND. WANDER. WHISTL. D	2	AS	M	6	NTB
LB0392	2.6.96	WINTER	229	HOODED MERGANSER	11	NA	M	3	TB
P00688	2.27.96	WINTER	75	GREATER MAGELLAN GOOSE	4	SA	F	1	NTB
LC0138	3.3.96	SPRING	10	WHITE-FACED WHISTL. D	2	TOP	M	6	NTB
LB0401	3.15.96	SPRING	227	BUFFLEHEAD	11	TOP	M	3	NTB
SD2107	3.15.96	SPRING	67	COMMON SHELDUCK	4	TOP	M	8	NTB
LC0320	3.24.96	SPRING	10	WHITE-FACED WHISTL. D	2	TOP	M	4	NTB
LB0381	3.29.96	SPRING	199	MANDARIN DUCK	10	TOP	F	3	NTB
LC0205	3.29.96	SPRING	239	AFRICAN YELLOWBILL	12	TOP	F	5	NTB

Bird identification	Date	Season	Species number	Species	Tribe	Pen	Sex	Age in years	PM Diagnosis
	11.21.96	AUTUMN	16	BLACK-NECKED SWAN	3	TOP	M	1	NTB
LB0097	4.2.96	SPRING	145	CHILOE WIGEON	6	SA	M	6	NTB
LA0061	4.16.96	SPRING	159	RINGED TEAL	6	TOP	F	4	NTB
SB3912	4.23.96	SPRING	199	MANDARIN DUCK	10	TOP	F	8	NTB
LC0031	4.24.96	SPRING	183	EUROPEAN POCHARD	9	EUR	M	6	NTB
LA0085	5.2.96	SPRING	159	RINGED TEAL	6	TOP	F	3	NTB
SD2173	5.9.96	SPRING	235	GOOSANDER	11	EUR	F	7	NTB
LB0308	5.16.96	SPRING	143	EUROPEAN WIGEON	6	EUR	M	4	NTB
LA0021	5.20.96	SPRING	86	MARBLED TEAL	6	AS	M	6	NTB
SB3919	5.24.96	SPRING	192	TUFTED DUCK	9	EUR	F	8	NTB
S2090	6.6.96	SUMMER	37	EMPEROR GOOSE	3	NA	M	7	NTB
SA1031	6.10.96	SUMMER	159	RINGED TEAL	6	ZO	M	7	NTB
LC0292	6.26.96	SUMMER	2	SPOTTED WHISTLING DUCK	2	SPA	F	4	NTB
SD2230	7.9.96	SUMMER	8	CUBAN WHISTLING DUCK	2	ISL	F	7	NTB
SA1045	8.18.96	SUMMER	227	BUFFLEHEAD	11	TOP	F	6	TB
S2055	8.30.96	SUMMER	28	PINK-FOOTED GOOSE	3	NA	F	7	TB
LD0051	9.3.96	AUTUMN	8	CUBAN WHISTLING DUCK	2	ISL	F	1	NTB
SB3955	10.8.96	AUTUMN	200	CAROLINA	10	AS	F	7	NTB
SD2167	10.24.96	AUTUMN	190	NEW ZEALAND SCAUP	9	TOP	F	7	NTB
LC0209	11.10.96	AUTUMN	239	WHITE-HEADED DUCK	12	ST	F	5	NTB
SD2153	11.21.96	AUTUMN	190	NEW ZEALAND SCAUP	9	EUR	M	8	TB
LC0242	11.25.96	AUTUMN	125	NORTH AMERIC. BLACK DUCK	6	NA	F	5	TB
LB0391	12.9.96	WINTER	158	COMMON SHOVELER	6	EUR	F	3	NTB
LA0066	12.12.96	WINTER	153	GARGANEY	6	GRD	F	3	NTB
LC0365	12.15.96	WINTER	10	WHITE-FACED WHISTL. D	2	TOP	F	3	NTB
SC3176	12.31.96	WINTER	3	EYTON'S WHISTLING DUCK	2	SMW	M	7	NTB
LC0023	1.6.97	WINTER	2	SPOTTED WHISTLING DUCK	2	SPA	F	8	NTB
LC0382	1.7.97	WINTER	130	CHINESE SPOTBILL	6	AS	M	5	NTB
SC3280	1.9.97	WINTER	239	WHITE-HEADED DUCK	12	ST	F	8	NTB
LC0137	1.10.97	WINTER	10	WHITE-FACED WHISTL. D	2	LAG	M	7	NTB
SD2118	1.10.97	WINTER	67	COMMON SHELDUCK	4	TOP	F	9	NTB

Bird identification	Date	Season	Species number	Species	Tribe	Pen	Sex	Age in years	PM Diagnosis
LC 0022	1.13.97	WINTER	2	SPOTTED WHISTLING DUCK	2	SPA	M	8	NTB
LC0286	1.13.97	WINTER	2	SPOTTED WHISTLING DUCK	2	SPB	F	5	NTB
SA1021	1.23.97	WINTER	159	RINGED TEAL	6	GRD	M	8	NTB
SB3868	1.24.97	WINTER	199	MANDARIN DUCK	10	TOP	F	9	NTB
SC3135	2.1.97	WINTER	178	RED-CRESTED POCHARD	9	AS	F	8	TB
SC3275	2.7.97	WINTER	239	WHITE-HEADED DUCK	12	SMW	F	8	NTB
S2071/ZF	2.15.97	WINTER	53	HAWAIIAN GOOSE	3	ISL	F	8	NTB
LC0021	2.27.97	WINTER	2	SPOTTED WHISTLING DUCK	2	SPA	M	8	TB
LC0338	3.3.97	SPRING	136	AFRICAN YELLOWBILL	6	TOP	M	4	NTB
LB0407	4.7.97	SPRING	99	NORTHERN PINTAIL	6	EUR	F	5	NTB
LA0079	4.11.97	SPRING	106	EUROP. GREEN-WINGED TEAL	6	GRD	F	4	NTB
LA0009	4.12.97	SPRING	86	MARBLED TEAL	6	AS	F	7	NTB
LC0237	5.9.97	SPRING	130	CHINESE SPOTBILL	6	AS	M	6	NTB
LB0303	5.14.97	SPRING	99	NORHTERN PINTAIL	6	EUR	F	5	NTB
LB0302	5.15.97	SPRING	99	NORTHERN PINTAIL	6	EUR	F	5	NTB
LB0087	5.23.97	SPRING	191	RING-NECKED DUCK	9	SMW	M	7	TB
LB0301	6.21.97	SUMMER	99	NORTHERN PINTAIL	6	EUR	F	5	NTB
SC3174	6.24.97	SUMMER	3	EYTON'S WHISTLING DUCK	2	SMW	M	8	NTB
LB0068	7.1.97	SUMMER	158	COMMON SHOVELER	6	EUR	F	7	NTB
NO RING	7.10.97	SUMMER	13	COSCOROBA SWAN	3	ISL	M	2	NTB
LB0493	7.20.97	SUMMER	227	BUFFLEHEAD	11	GRD	F	2	NTB
P00636	7.20.97	SUMMER	16	BLACK NECKED SWAN	3	GRD	F	8	NTB
S2139	7.20.97	SUMMER	209	WHITE WINGED WOOD DUCK	10	GRD	M	7	TB
SB4415	9.11.97	AUTUMN	229	HOODED MERGANSER	11	NA	M	8	TB
LB0393	9.28.97	AUTUMN	229	HOODED MERGANSER	11	HOS	F	4	NTB
SA1047	10.1.97	AUTUMN	227	BUFFLEHEAD	11	TOP	F	8	NTB
SC3158	10.3.97	AUTUMN	134	PHILIPPINE DUCK	6	ISL	M	8	NTB
LC0024	10.20.97	AUTUMN	2	SPOTTED WHISTLING DUCK	2	SPB	F	8	TB
FAX	11.8.97	AUTUMN	253	CARIBBEAN FLAMINGO	13	HOS	F	?	NTB
SB3983	11.8.97	AUTUMN	103	SHARP-WINGED TEAL	6	FLM	F	8	NTB
LB0124	11.14.97	AUTUMN	193	LESSER SCAUP	9	SMW	M	7	TB

Bird identification	Date	Season	Species number	Species	Tribe	Pen	Sex	Age in years	PM Diagnosis
LB0277	11.14.97	AUTUMN	191	RING-NECKED DUCK	9	NA	M	6	TB
S2416	11.14.97	AUTUMN	209	WHITE WINGED WOOD DUCK	10	FLM	F	6	NTB
LC0122	11.18.97	AUTUMN	58	BLACK BRANT GOOSE	3	EUR	M	7	TB
SB3987	11.18.97	AUTUMN	99	NORHTERN PINTAIL	6	NA	M	8	NTB
SB4418	11.22.97	AUTUMN	229	HOODED MERGANSER	11	NA	F	8	TB
SB3986	11.30.97	AUTUMN	103	SHARP -WINGED TEAL	6	HP	F	8	NTB
SC3111	11.30.97	AUTUMN	136	AFRICAN YEELOWBILL	6	TOP	M	8	TB
LC0090	12.7.97	WINTER	12	SOUTH. RED BILLED WHISTL. D	2	FLM	F	7	NTB
SB4477	12.26.97	WINTER	227	BUFFLEHEAD	11	TOP	M	8	NTB
LD0024	2.1.98	WINTER	170	EUROPEAN EIDER	8	EUR	F	7	NTB
LA0086	2.2.98	WINTER	88	HOTTENTOT TEAL	6	SPC	M	4	TB
LB0118	2.9.98	WINTER	193	LESSER SCAUP	9	NA	F	8	TB
LB0189	2.12.98	WINTER	199	MANDARIN DUCK	10	TOP	F	8	NTB
LC0258	2.15.98	WINTER	239	WHITE-HEADED DUCK	12	FLM	M	6	TB
S2118	2.15.98	WINTER	209	WHITE-WINGED WOOD DUCK	10	ST	F	9	NTB
LB0326	2.26.98	WINTER	87	CAPE TEAL	6	TOP	F	4	NTB
SB4419	2.28.98	WINTER	191	RING-NECKED DUCK	9	SMW	M	9	TB
LC0073	3.13.98	SPRING	4	E. IND. WANDER. WHISTL. D	2	AS	F	8	NTB
SD2189	3.26.98	SPRING	235	GOOSANDER	11	EUR	M	9	TB
LB0486	4.9.98	SPRING	109	BAIKAL TEAL	6	SPC	M	3	NTB
SA1087	4.9.98	SPRING	88	HOTTENTOT TEAL	6	SPC	F	8	NTB
LB0028	4.11.98	SPRING	99	NORTHERN PINTAIL	6	HOS	M	8	NTB
LB0105	4.12.98	SPRING	200	CAROLINA	10	AS	M	8	TB
LB0480	4.14.98	SPRING	200	CAROLINA	10	FLM	F	3	NTB
SA1076	4.14.98	SPRING	152	NORTHERN CINNAMON TEAL	6	GRD	F	8	TB
SD2240	4.14.98	SPRING	40	ROSS'S GOOSE	3	NA	M	8	NTB
LA0075	4.22.98	SPRING	159	RINGED TEAL	6	TOP	F	5	NTB
	4.26.98	SPRING	136	AFRICAN YELLOWBILL	6	OD	F	2	NTB
LB0316	5.16.98	SPRING	228	SMEW	11	SMW	M	6	TB
SB3963	5.18.98	SPRING	103	SHARP WINGED TEAL	6	SA	M	9	NTB
LB0415	5.20.98	SPRING	199	MANDARIN DUCK	10	TOP	M	4	NTB

Bird identification	Date	Season	Species number	Species	Tribe	Pen	Sex	Age in years	PM Diagnosis
LD0038	5.21.98	SPRING	7	FULVOUS WHISTLING DUCK	2	SA	F	6	NTB
S000321	6.1.98	SUMMER	20	WHOOPER SWAN	3	LAG	M	9	NTB
S000322	6.1.98	SUMMER	20	WHOOPER SWAN	3	LAG	M	9	TB
S000325	6.1.98	SUMMER	20	WHOOPER SWAN	3	LAG	F	9	NTB
S000326	6.1.98	SUMMER	20	WHOOPER SWAN	3	LAG	F	9	TB
SB4421	6.1.98	SUMMER	191	RING-NECKED DUCK	9	SMW	F	9	NTB
LB0176	6.8.98	SUMMER	193	LESSER SCAUP	9	NA	M	8	TB
LB0428	6.8.98	SUMMER	190	NEW ZEALAND SCAUP	9	ISL	M	6	NTB
LA0104	6.22.98	SUMMER	153	GARGANEY	6	OD	M	3	NTB
LB0280	7.2.98	SUMMER	87	CAPE TEAL	6	OT	F	7	TB
	7.7.98	SUMMER	160	NEW ZEALAND BLUE DUCK	6	OD	F	9	NTB
LB0357	7.10.98	SUMMER	126	HAWAIIAN DUCK	6	ISL	M	6	TB
L30014 NZ	7.13.98	SUMMER	160	NEW ZEALAND BLUE DUCK	6	NA	M	4	NTB
S2086	7.13.98	SUMMER	39	GREATER SNOW GOOSE	3	OD	F	9	NTB
L0031	7.16.98	SUMMER	53	HAWAIIAN GOOSE	3	ISL	M	2	TB
SC3294	7.17.98	SUMMER	245	AFRICAN MACCOA DUCK	12	TOP	F	9	NTB
SA1046	9.7.98	AUTUMN	227	BUFFLEHEAD	11	TOP	F	9	TB
	9.15.98	AUTUMN	13	COSCOROBA SWAN	3	ISL	M	10	NTB
S2144	9.21.98	AUTUMN	209	WHITE-WINGED WOOD DUCK	10	FLM	M	8	TB
LC0366	9.25.98	AUTUMN	229	HOODED MERGANSER	11	AS	M	5	TB
SB3959	9.25.98	AUTUMN	200	CAROLINA	10	NA	F	9	TB
LB0117	9.29.98	AUTUMN	193	LESSER SCAUP	9	NA	F	8	TB
	10.1.98	AUTUMN	144	AMERICAN WIGEON	6	OD	M	3	NTB
P00623	11.1.98	AUTUMN	22	SWAN GOOSE	3	AS	F	9	NTB
S2082	11.6.98	AUTUMN	31	GREENL. WHITE-FRONTED G	3	NA	F	9	TB
SB3877	11.9.98	AUTUMN	143	EUROPEAN WIGEON	6	EUR	M	10	TB
LB0491	11.16.98	AUTUMN	249	BLACK-HEADED DUCK	12	SAS	F	2	NTB
S2076	11.20.98	AUTUMN	53	HAWAIIAN GOOSE	3	ISL	M	9	NTB
LB0009	11.21.98	AUTUMN	96	SOUTH GEORGIAN PINTAIL	6	SAS	F	9	NTB
	11.25.98	AUTUMN	20	WHOOPER SWAN	3	LAG	F	3	NTB
LC0026	12.1.98	WINTER	136	AFRICAN YELLOWBILL	6	OT	M	8	NTB

Bird identification	Date	Season	Species number	Species	Tribe	Pen	Sex	Age in years	PM Diagnosis
LB0318	12.3.98	WINTER	228	SMEW	11	SMW	M	6	TB
L0002	05.01.99	WINTER	1	MAGPIE GOOSE	1	AUS	F	8	TB
L0081	12.01.99	WINTER	1	MAGPIE GOOSE	1	OD	M	9	NTB
	15.01.99	WINTER	127	LAYSAN TEAL	6	NA	M	2	NTB
LC0217	22.01.99	WINTER	182	CANVASBACK	9	AUS	F	7	TB
P00655	23.01.99	WINTER	14	BLACK SWAN	3	AUS	F	7	NTB
L0001	28.01.99	WINTER	1	MAGPIE GOOSE	1	AUS	F	8	NTB
LB0300	11.02.99	WINTER	200	CAROLINA	10	AS	F	8	NTB
LC0052	13.02.99	WINTER	134	PHILIPPINE DUCK	6	ISL	M	9	NTB
S2402	25.02.99	WINTER	209	WHITE-WINGED WOOD DUCK	10	SMW	F	9	TB
LB0295	05.03.99	SPRING	103	SHARP-WINGED TEAL	6	SA	F	8	NTB
LC0067	12.03.99	SPRING	4	E. IND. WANDER. WHISTL. D	2	AS	M	9	TB
S2056	19.03.99	SPRING	28	PINK-FOOTED GOOSE	3	NA	F	11	TB
L0030/ACB	26.03.99	SPRING	53	HAWAIIAN GOOSE	3	ISL	F	4	TB
LC0364	26.03.99	SPRING	10	WHITE-FACED WHISTL. D	2	TOP	F	6	NTB
SD2227	03.04.99	SPRING	8	CUBAN WHISTLING DUCK	2	ISL	F	10	NTB
LB0369	09.04.99	SPRING	154	ARGENTINE RED SHOVELER	6	SA	M	7	NTB
LC0106	13.04.99	SPRING	3	EYTON'S WHISTLING DUCK	2	AUS	F	9	TB
SD2154	20.04.99	SPRING	63	AUSTRALIAN SHELDUCK	3	AUS	F	10	NTB
S2431	22.04.99	SPRING	31	GREENL. WHITE-FRONTED G	3	NA	M	9	NTB
LC0069	30.04.99	SPRING	4	E. IND. WANDER. WHISTL. D	2	AS	M	9	TB
LC0293	02.05.99	SPRING	2	SPOTTED WHISTLING DUCK	2	SPA	M	7	NTB
LB0020	10.05.99	SPRING	200	CAROLINA	10	AS	F	10	TB
LC0277	15.05.99	SPRING	10	WHITE-FACED WHISTL. D	2	TOP	M	7	TB
LB0191	17.05.99	SPRING	229	HOODED MERGANSER	11	NA	F	9	TB
LB0094	22.05.99	SPRING	145	CHILOE WIGEON	6	SA	M	9	TB
SB4439	05.06.99	SUMMER	145	CHILOE WIGEON	6	SA	F	10	NTB
LB0153	07.06.99	SUMMER	110	FALCATED TEAL	6	AS	F	9	NTB
LA0093	10.06.99	SUMMER	153	GARGANEY	6	GRD	M	4	NTB
LC0140	16.06.99	SUMMER	10	WHITE-FACED WHISTL. D	2	TOP	F	9	NTB
LC0041	19.06.99	SUMMER	61	RUDDY SHELDUCK	4	AS	M	9	TB

Bird identification	Date	Season	Species number	Species	Tribe	Pen	Sex	Age in years	PM Diagnosis
LB0226	23.06.99	SUMMER	99	NORTHERN PINTAIL	6	EUR	M	8	NTB
SB3977	23.06.99	SUMMER	99	NORTHERN PINTAIL	6	LAG	M	10	NTB
S2131	24.06.99	SUMMER	209	WHITE-WINGED WOOD DUCK	10	SMW	F	9	NTB
LB0496	09.07.99	SUMMER	229	HOODED MERGANSER	11	NA	M	3	TB
SB4414	20.07.99	SUMMER	229	HOODED MERGANSER	11	NA	F	10	TB
LB0049	16.08.99	SUMMER	126	HAWAIIAN DUCK	6	ISL	F	9	TB
SB4403	01.09.99	AUTUMN	91	PUNA TEAL	6	SA	F	10	TB
LD0067	14.09.99	AUTUMN	235	GOOSANDER	11	EUR	M	1	NTB
LD0068	26.09.99	AUTUMN	235	GOOSANDER	11	EUR	M	1	NTB
LB0017	30.09.99	AUTUMN	200	CAROLINA	10	AS	M	10	NTB
LD0069	30.09.99	AUTUMN	235	GOOSANDER	11	EUR	M	1	NTB
LC0179	15.10.99	AUTUMN	183	EUROPEAN POCHARD	9	SMW	F	8	NTB
SB3254	18.11.99	AUTUMN	125	NORTH AMERIC. BLACK DUCK	6	NA	F	10	TB
LB0001	30.11.99	AUTUMN	228	SMEW	11	SMW	F	10	NTB
LB0192	11.12.99	WINTER	229	HOODED MERGANSER	11	NA	M	9	TB
SC3130	11.12.99	WINTER	225	EUROPEAN GOLDENEYE.	11	EUR	M	11	NTB
LB0208	14.12.99	WINTER	158	COMMON SHOVELER	6	EUR	M	8	NTB
LB0259	15.12.99	WINTER	110	FALCATED TEAL	6	AS	M	8	TB
SB4420	20.12.99	WINTER	191	RING-NECKED DUCK	9	SMW	F	10	NTB
LB0515	24.12.99	WINTER	91	PUNA TEAL	6	SA	F	2	NTB
LB0533	29.12.99	WINTER	229	HOODED MERGANSER	11	NA	M	2	TB
P00622	29.12.99	WINTER	22	SWAN GOOSE	3	AS	F	10	TB
LB0354	30.12.99	WINTER	229	HOODED MERGANSER	11	NA	F	7	TB

Acknowledgement

Mein besonderer Dank gilt Herrn Prof. Dr. Neumann für die Überlassung des Themas sowie für die freundliche Unterstützung bei der Anfertigung dieser Arbeit.

I would like to thank Dr. Ruth Cromie for her great support and advise in working with the ELISA and in writing this thesis.

Thanks a lot to Mr Richard Edwards, director of the WWT centre at Llanelli, and his team for agreeing that the project was carried out at the WWT centre in Llanelli and for their valuable help in catching and bleeding the birds, and answering hundreds of questions.

Frau Dr. Ruesch-Gerdes und Frau Dr. Richter des Forschungsinstitutes in Borstel möchte ich herzlich für die Untersuchung der Gewebeproben mittels PCR Technik danken.

Vielen Dank auch an Herrn Dr. Rohn und Herrn Meier aus dem Institut für Statistik und Biometrie der Tierärztlichen Hochschule Hannover für die tatkräftige und flexible Unterstützung bei den Hürden der Statistik.

I would like to acknowledge Mr Neil Forbes who initiated the project and offered a lot of help and advise. Thank you a lot to Dr. Jenni Bass who found time to prove read my thesis in between preparing lots of bowls of tomato soup and moving to a different country. Für ihre Aufmunterungen und die unermüdliche Hilfe im Dunkeln der Computerwelt möchte ich mich herzlich bei Reimer, Clems und Nigel bedanken.

Meinen Eltern und meiner Schwester Karin möchte ich ein besonderes Dankschön aussprechen, daß sie immer für mich da waren, und mich auf all meinen Wegen mit viel Liebe unterstützen.