DEVELOPMENT OF AN AVIAN TUBERCULOSIS VACCINE

FOR CAPTIVE WILDFOWL.

by

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For the White-winged Wood Ducks

White-winged Wood Duck in freshwater swamp forest in Way Kambas National Park, Lampung Province, Sumatra. (Photo: Southampton University).

ABSTRACT.

The Wildfowl and Wetlands Trust maintain a large and comprehensive collection of captive wildfowl at Slimbridge in Gloucestershire. Avian tuberculosis caused by *Mycobacterium avium*, has been enzootic since 1948 and is now the main cause of death, accounting for over a third of post fledgling mortalities. There is a grave risk of spreading infection to the enormous number of wild birds that use the grounds.

The introductory chapters review the disease in both wild and captive wild birds. The particular susceptibility of the rarest duck in the world: the White-winged Wood Duck *Cairina scutulata*, and the effect of the disease on its captive breeding programme, are discussed. The disease situation at Slimbridge is assessed in an epizootiological study which is carried out on adult *post mortem* data of the years 1980-1989. This study shows both genetic and environmental factors greatly influencing incidence of the disease. However, it also suggests that there are definite levels of immunity to *M.avium* in some groups and individuals.

The main experiments in the thesis investigate the possibility of immunoprophylaxis in the form of a vaccine as a possible method of controlling the disease. Past attempts at vaccination are reviewed, as is the current knowledge of the wildfowl immune system.

Conditions for the immunological tests used to assess immune responses throughout the vaccine studies had to be optimised. These tests included: wildfowl lymphocyte transformation test (LTT) which has been made to work in the presence of antigen for the first time; a method for skin testing, previously not done in wildfowl; and an enzyme linked immunosorbence assay (ELISA).

The main study chapters report the use of intradermal BCG and *M.vaccae* as potential vaccines. Studies with Mallard *Anas platyrhynchos platyrhynchos* indicate killed *M.vaccae* administered at one day old as an immunopotentiating agent. Studies with Mandarin ducks *Aix galericulata* confirm the optimum age for vaccination to be one day old. An optimum dose of this vaccine has been elucidated in studies using Gadwall *Anas strepera* and Hawaiian Geese or Nenes *Branta sandvicensis*. No protection was afforded to White-winged Wood Ducks when vaccinated with killed *M.vaccae* in adult life.

A bacteriological study of strains of *M.avium* isolated from tuberculous birds at *post mortem* is also reported. Strains were isolated from birds from both Slimbridge and Arundel Wildfowl and Wetlands Trust centres, and also from several wild birds. These strains were subjected to lipid and DNA analysis, and studies were made of their protein patterns using SDS-polyacrylamide gel electrophoresis.

The current vaccination programme at Slimbridge and The Wildfowl and Wetlands Trust centre at Llanelli, South Wales, is discussed. Results from tuberculous birds in the vaccine trials indicate the potential for the development of a diagnostic test.

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THE MYCOBACTERIA AND AVIAN TUBERCULOSIS

IN WILD AND CAPTIVE BIRDS

CHAPTER ONE

THE MYCOBACTERIA AND AVIAN TUBERCULOSIS IN WILD AND CAPTIVE BIRDS

The Mycobacteria and Mycobacterial Infections.

For centuries the genus *Mycobacterium* has been responsible for infections in both man and animals. In man, the most infamous and dreaded are tuberculosis and leprosy, caused by *M.tuberculosis* and *M.leprae* respectively. In 1882, a breakthrough in the history of the mycobacteria was made when Robert Koch announced that he had managed to culture the tubercle bacillus (Collins and Grange, 1983). He also described a method to stain the bacilli, based on their characteristic acid fastness, this has since been modified and bears the name Ziehl Neelsen. Using this staining technique, preparations from ancient mummified bodies have shown organisms like today's mycobacteria. Acid-fast bacteria have been demonstrated in mammoths and woolly rhinoceroses, as well as mummified cats and ibises. Skeletons from Neolithic men have shown evidence of mycobacterial invasions of the bones and joints (Keers, 1978) and there is even a report that Christ suffered from pulmonary tuberculosis complicated with pleurisy (Keers, 1978).

Mycobacterium, meaning 'fungus-bacterium', was so named by Lehmann and Neumann in 1896, due to the mould-like appearance of the pellicles as they grew on liquid media. Although the major pathogens *M.tuberculosis* and *M.leprae* have received most scientific attention, they are only two species of about 50 'taxonomically approved' species (Skerman *et al*, 1980).

Thorough accounts of these species are given by Grange (1980; 1988) and Collins *et al* (1985a). Typically, mycobacteria are free-living saprophytes with few species dependent on a living host. They are to be found worldwide in many environments, however they are most commonly found in aquatic habitats, i.e. marshes, estuaries, ponds, rivers and sphagnum bogs. Due to their thick and waxy hydrophobic cell walls they are usually found at air-water interfaces, and for this reason Grange (1987) termed them the "ducks of the microbial world". In these environments they respire aerobically and obtain nutrients from decomposing plants in the water. These aquatic habitats now include domestic, industrial and hospial water supplies and hence human contact with environmental mycobacteria is very significant. Water, therefore, may form both the source and the vector by which many mycobacteria cause either immunological sensitisation or infection in both man and animals.

The obligate pathogenicity of *M.tuberculosis* and *M.leprae* has already been mentioned but many species of 'harmless saprophytic' mycobacteria become opportunist pathogens when conditions are right, as in the immunocompromised host. It would appear that few vertebrates are immune to mycobacterial infections, and indeed various species of mycobacteria are capable of infecting many different species of animals and may be passed interspecifically and even to man.

In Great Britain *M.kansasii*, found in some piped water supplies, was the most frequent cause of opportunist mycobacterial disease in man prior to the HIV epidemic. A similar species, *M.marinum*, causes an infection in man known as 'swimming-pool granuloma', and has been isolated from diseased fish. It enters the hosts through abrasions in the skin and causes soft warty lesions in the skin and rarely lesions

in the draining lymph nodes. The disease Buruli Ulcer is caused by *M.ulcerans* and is thought to be introduced to the host by spikes on vegetation, then causing necrosis and liquifaction of the host's subcutaneous fat, eventually the skin breaks down creating a deep ulcer. *M.gordonae*, *M.szulgai* and *M.xenopi* (isolated originally from skin granulomas of the toad *Xenopus laevis*) are all occasional pathogens in man, causing pulmonary infections.

Species isolated from animals and rarely or never pathogenic in man include *M.farcinogenes*, resonsible for farcy in African zebu cattle; *M.lepraemurium*, responsible for murine leprosy; and *M.simiae* and *M.asiaticum* isolated from macaques.

Of the rapidly growing mycobacteria only two species tend to be pathogenic to both man and animals, these are *M.chelonei* which was originally isolated from a turtle; and *M.fortuitum*, which has been isolated from lesions of several species including man, frogs and birds. Other rapidly growing mycobacteria tend to be non-pathogenic environmental saprophytes.

On a global scale the most widely distributed species of mycobacteria causing the most opportunist human and animal infections is *M.avium*. The taxonomy of the *M.avium* group is somewhat complicated as there are very many closely related strains. From immunodiffusion analysis (Stanford, 1983a) it is now recognised as containing *M.avium*, the pathogenic avian tubercle bacillus (serotypes 1-3), *M.avium brunense*, (serotypes 4-12 and 20-21) and the distinct but similar species *M.intracellulare* (serotypes 13-19). Also included in the *M.avium* group is the mycobactin dependent strain isolated from Woodpigeons *Columba palumbus*, and in the *M.avium brunense* group the mycobactin dependent strain causing Johne's disease (chronic hypertrophic enteritis) in

cattle, a subspecies known as *M.paratuberculosis* or *M.avium paratuberculosis* (Grange, 1980). Some workers also group *M.scrofulaceum*, responsible for tuberculous cervical adenitis in children, with this complex.

From this it can be seen that the mycobacteria are a very important group in terms of both human and animal disease. The disease avian tuberculosis caused mainly by *M.avium* serotypes 1-3 will be discussed more fully in this thesis.

AVIAN TUBERCULOSIS.

History of Avian Tuberculosis.

The first description of the contagious disease, avian tuberculosis was made by Crisp in 1868, long before the aetiology of the causative organism was established. Crisp gave detailed accounts of both signs and pathology of the disease in chickens and pheasants (1872; 1875).

At this time Koch maintained that tubercle bacilli were always the same, regardless of the species of the host. But the extensive work done by Maffucci (1890, 1892; quoted by Francis, 1958) showed that the bacilli differed in both cultural and pathogenic properties from those responsible for human tuberculosis. In the light of this, and other evidence (Rivolta, 1883; quoted by Francis, 1958) Koch finally abandoned his position in 1902 and admitted that there were indeed differences between the various types of tubercle bacilli that cause tuberculosis of man, cattle and poultry.

In the following years the disease was described by Crisp and others in different avian species (Sibley, 1890; quoted by Wilson, 1960), however avian tuberculosis in poultry flocks was of the greatest

economic importance.

At the turn of the Century poultry were generally kept in flocks in backyards or gardens. The birds were usually retained for many years after their economic period of egg production had passed. Such environments tended to be overcrowded and in continual use for many years.

With the advent of a specialised poultry industry after World War I, chickens were largely managed in semi-intensive systems. These new farms were often built in urban areas where available land was somewhat limited. The farms were never rested from birds and were frequently overstocked, allowing avian tuberculosis to become increasingly prevalent. Fresh blood was introduced to flocks by the purchase of cockerels from farms operating similar systems. As a consequence infection was often introduced and spread of the disease was inevitable.

At this time (1913-1933) avian tuberculosis was one of the commomest finding in birds sent for diagnosis in Great Britain and other countries with poultry industries. In the USA there was widespread infection in the corn belt where poultry were kept in dense populations. The problem was at its worst where birds were kept until three or four years old. Francis (1958) quotes Feldman's figures (1938) of infection existing in 80% of farms in USA with up to 75% of individual birds being diseased.

Sweeping changes in poultry keeping methods markedly reduced the incidence of avian tuberculosis. New intensive methods, especially the battery and deep litter systems were employed. In the larger commercial poultry flocks, birds were generally disposed of after 18

months, in this way egg production was maximised and as avian tuberculosis is a disease of older birds, the disease was practically eliminated (Johnson and Ranney, 1956; quoted by Wilson, 1960).

Distribution.

Avian tuberculosis is worldwide in distribution but reports are most frequent in the North Temperate zone (Feldman, 1959). It occurs in most European countries but cases are infrequent in Finland, Switzerland, Italy and Greece. The Balkan States have however, been seriously affected by outbreaks of the disease.

As has already been mentioned, the disease has occurred across the USA, most frequently where birds are confined during the winter months. Incidences in both Canada and Australia vary greatly from province to province.

Francis (1958) reports few cases from South America, but Thoen and Karlson (1978) report high incidences in Uruguay and Venezuela, several cases in Argentina and low incidence in Brazil. Incidence in South Africa, Kenya and Zimbabwe is fairly low (Thoen and Karlson, 1978). The distribution of the disease in other African countries is difficult to ascertain due to the limited bacteriological studies carried out.

Pathogenicity in Species other than Birds.

M.avium is capable of causing infections in many species of mammals, although development of pathological disease tends to be less progressive than that caused by mammalian tubercle bacilli.

Pigs.

The pathogenicity of *M.avium* in pigs has been widely reported and in a contaminated environment swine readily become infected. Figures of incidence of the disease in the UK range from 12% in the 1930's-1950's to about 1% more recently, with the greatest fall occurring between 1945-1955. This fall coincided with new poultry keeping methods. The serotypes responsible for avian tuberculosis in swine are those found within fowl, namely serotypes 1 and 2. Avian tuberculosis in swine is of great economic importance in The Developing Countries where pigs and fowl are often kept together and will remain an unneccessary burden until the disease is eliminated from poultry.

M.avium infection in pigs is usually manifested by gross lesions in lymph nodes and microscopic lesions in the lungs and kidneys. Other *M.avium* serotypes, often of the *M.avium brunense* subspecies, are responsible for pigs' heads and necks being condemned in slaughter houses.

Cattle.

Avian tubercle bacilli have limited pathogenicity for cattle. Reports of generalised disease are uncommon and infections usually manifest as 'open' cases, in particular as mastitis (Boughton, 1969). These infections, and sensitisation of cattle without visible disease, are of importance because they produce false positive skin test reactions to mammmalian tuberculin by cross-reaction. In Great Britain about 11% of tubercle bacilli isolated from reactor cattle in the 1950's and 1960's were identified as *M.avium* (Thoen and Karlson, 1978). In USA this figure has been as high as 10-20% (Thoen and Karlson, 1978). The mycobactin dependent variant of *M.avium*: *M.avium paratuberculosis*, is responsible for Johne's disease in cattle and a variety of other

ungulates including gnu, camels and llama (Grange, 1980).

Other Domestic Livestock.

The disease is of little significance in other livestock but cases have been reported in sheep, goats, horses and very occasionally in cats and dogs (Boughton, 1969; Thoen and Karlson, 1978).

Rabbits and Mink.

Both rabbits and mink are highly susceptible to *M.avium* infections and play a role in the infection of predatory and scavenging animals.

Mice and Rats.

Mice and rats are both relatively resistant to *M.avium* infections (Thoen and Karlson, 1978).

Man.

Prior to the epidemic of HIV, *M.avium* infections in man were few, although often devastating because of their natural resistance to antibacterial agents. However, with the decline in the incidence of tuberculosis in humans prior to HIV, an increasing number of infections with *M.avium* are being recognised, although the rather similar species *M.intracellulare* is a more common cause of disease.

Most infections in non-HIV infected humans involve the pulmonary system and in some cases have been associated with pulmonary silicosis. Involvement of the skin and lymph nodes have been seen. The relatively uncommon occurrence of avian tubercle bacilli infections, even where the disease is common in poultry, indicates that humans are extremely resistant to this infection and that poultry are of little importance in the epidemiology of tuberculosis in man. Although cases of infection leading to pathological disease are infrequent, sensitisation to *M.avium* may be common. Lind *et al* (1988) found that

25% of Swedish children gave positive skin test reactions to *M.avium* sensitins. This sensitisation correlated to the presence of pet birds in the children's homes.

M.avium infections are however common in immunocompromised patients and with the advent of AIDS, such infections have increased dramatically. AIDS patients are highly susceptible to a variety of unusual and oppurtunist viral, bacterial, fungal and protozoal infections, for example *M.avium* infection, manifesting itself as a disseminated disease. The bacilli may be isolated from lymph nodes, bone marrow, liver, spleen, blood, urine, faeces, lungs and skin (Macher and Reichert, 1984) and in the UK usually belong to serotypes 4 or 8 of the *M.avium brunense* subspecies. Clinically, the patients with disseminated disease present with fever, weight loss and debilitation. In advanced AIDS, splenic *M.avium* infection may cause the spleen to become firm and enlarged. Milliary pulmonary disease may occur secondary to either *M.avium* or *M.tuberculosis*. Intestinal disease caused by *M.avium* leads to enteritis with diarrhoea, abdominal cramps and bloating with normal sigmoidoscopy.

In USA *M.avium* has been isolated from blood and bone marrow of between 10-30% of AIDS patients (Greene *et al*, 1982). Such infections in African and Haitian AIDS patients are less common, with *M.tuberculosis* infections being more commonly encountered.

Table 1.1: Animal Susceptibility to *Mycobacterium avium* Infection.

Species	Susceptibility
Pigs	Readily infected
Rabbits and mink	" "
Cattle	Infection occurs - usually localised
Sheep	Moderately susceptibility
Horses and goats	Thought to be relatively resistant
Mice and rats	Relatively resistant
Cats and dogs	Highly resistant
Man	" " - except immunocompromised

Avian Tuberculosis in Wild Birds.

The situation of avian tuberculosis in free living wild birds is more difficult to ascertain than that in captive and domestic species as insufficient *post mortem* (PM) data are available; culling of large numbers of birds would generally be considered ethically unsound; and the disease is rarely recognised in shot game birds. J.E. Wilson (1960) suggests that the disease is relatively rare in the wild and infectious disease is usually following contact with infected poultry. It is therefore fairly unlikely that they provide a significant or permenant reservoir of infection for captive birds. However, avian tuberculosis has been reported in practically all groups of birds so it would seem that all have the potential for developing infection. But the disease is largely confined to gregarious and carnivorous species due to lifestyle, rather than genetic predisposition to infection (Wilson, 1960).

The majority of cases have been reported in house sparrows *Passer domesticus*, starlings *Sturnus vulgaris*, pigeons, gulls, ducks and waders. Hawks and owls account for most other reports. The disease is found quite frequently in pheasants in captivity, semi-domesticated or free living. These were considered to be responsible for sensitisation of cattle to avian tuberculin (Krogh, 1937).

In wild birds avian tuberculosis may be responsible for about 1% to 2.8% of mortalities (Wilson and MacDonald, 1965). The Central Veterinary Institute in Holland quotes similar figures of 2% to 3% of mortalities between 1976 and 1979 being due to tuberculosis (Lumeij *et al* 1980). However, these may be an under estimation as most reports are based on the presence of lesions rather than isolation of *M.avium* as in a study carried out by Plum (1942). This estimation is also the mean of the higher mortality rates in the gregarious and carnivorous species associated with agricultural premises and the lower rates in other bird species.

Other estimations of incidence vary, Hignett and Mackenzie (1940) found that 4.8% (12/252) of starlings were affected. These birds were associated with an outbreak of infection in poultry. Other investigations with starlings found 5.6% (7/125) to have tuberculous lesions (Bickford *et al*, 1966). *M.avium* was isolated from 5.3% to 40% of sparrows from flocks associated with pigs affected with avian tuberculosis. Thirty two other flocks not associated with the pigs were found to be free from tuberculosis. Matejka and Kubin (1967) isolated *M.avium* from 23.3% (7/30) of sparrows on a farm where cattle had become sensitised to avian tuberculin.

In other gregarious birds 3.3% (8/246) of crows were found to be infected (Harshfield and Roderick, 1934). A higher level of incidence

was recorded by Mitchell and Duthie (1950) who found 9.5% (25/263) of crows in Western Ontario to be tuberculous. Plum (1942) found lesions suggestive of tuberculosis in 5.9% (48/816) of gulls, however *M.avium* was isolated from only four of these and also from 28 of the birds without lesions.

Blackmore and Keymer (1969) describe a tuberculous infection associated with the carpel joint of the wing of an immature Herring gull *Larus argentatus*. Cases of tuberculosis have also been found in a Common Gull *L.canus* and Black-headed Gulls *L.ribibundas* (Garden, 1961; Jennings, 1959).

After the gregarious and scavenging species, avian tuberculosis is most commonly found in carnivorous predatory species, in fact tuberculosis is one of the most common bacterial infection in birds of prey (Lumeij *et al*, 1980). Tuberculosis in the order Falconiformes was first described in 1884 by Sutton and Gibbes. More recently, reports of avian tuberculosis have been quoted by MacDonald (1965) in a Buzzard *Buteo buteo*, Sparrowhawk *Accipiter nisus*, Barn Owls *Tyto alba* and a Tawny Owl *Strix aluco*. Another case of the disease in a wild Sparrowhawk was reported by Harrison in 1949. Harrison (1943) reported an unusual case in a Short-eared Owl *Asio flammeus* which had been partially predated.

The importance of applying histological techniques to lesions was demonstrated in the case of a Barn Owl which had enormous numbers of acid-fast bacilli in what appeared to be a large tumour in the region of the femero-tibiotarsal joint (Bucke and Mawdesley-Thomas, 1974). Such great numbers of *M.avium* are common in avian tuberculous lesions, as was also demonstrated by Mollhoff (1974) in the liver and gastrointestinal tract of a Saw-whet owl *Aegolius acadicus*. This bird

also had lesions present at the ventral base of the tongue and ventral surface of the larynx.

Tumour like masses up to 3 cm in diameter were also found in the ventral aspect of the wing of a Kestrel *Falco tinnuculus* (Blackmore and Keymer, 1969). These too showed typical granulomatous histology with many acid fast bacilli. Another unusual case of tuberculosis in a paralysed Kestrel, which had granular lesions pressing on the sciatic nerves, was recorded by Cooper (1968). The same author describes a localised infection of *M.avium* in the tibia region of an adult Buzzard. The lack of infection in the rest of the body would suggest that the infection originated from an external source possibly through entry of the bacilli following a wound. The Dutch Central Veterinary Institute have reported many such foot abcesses in Buzzards as well as common primary lesions on the breast, throat and limbs of raptors (Lumeiji *et al*, 1980), indicating generalised infection following contamination of a wound.

However, probably the most common cause of infection, leading to generalised tuberculosis, is the ingestion of infected prey. Such intestinal lesions were found in a Red-tailed Hawk *Buteo jamaicensis* in Wisconsin. These acid fast bacilli were identified as *M.avium* serotype 2 (Emerson *et al*, 1970).

There have been several cases of tuberculosis infection in Golden Eagles *Aquila chrysaetos* (MacDonald, 1965, Wilson and MacDonald, 1965). An attempt to identify the source of infection of those dying in the same Braemar district of Aberdeenshire, found that seven flocks of domestic poultry within a 20 mile range had been diagnosed with avian tuberculosis. Whether or not these hens had fallen prey to the eagles is however questionable. MacDonald (1965) reports the personal

communication of Dr. A. McDiarmid confirming avian tuberculosis infection in a Red Deer *Cervus elaphus* in the area of the eagles. It is likely that eagles would scavenge on any such carrion or the discarded viscera from shot deer. Other potential sources of infection: Mountain Hares *Lepus timidus scoticus*, Rabbits *Oryctolagus cuniculus* or Red Grouse *Lagopus scoticus*, which form the usual diet of the birds, were found to be free of tuberculosis. As eagles tend to spend considerable periods on the eyrie or on favourite perches, infected droppings may build up and heavily contaminate these areas providing a likely site of infection. Fortunately the habit of maintaining two eyries alternately (Bannerman, 1956) allows decontamination of the unused site by sun and rain.

The apparent first recorded case of avian tuberculosis in a wild Kenyan bird was reported by Kaliner and Cooper (1973) in an African fish eagle *Haliaeetus vocifer*. The source of infection is of interest, although its name would suggest otherwise, the fish eagle feeds largely on flamingoes on Lake Nakuru. Cooper *et al* (1975) report 14 Lesser Flamingoes *Phoeniconaias minor* with avian tuberculosis from this lake. *M.avium* serotype 1 was isolated from two of these birds and it is tentatively suggested that this originated from the migratory ducks and waders with which they share the lake.

The role of migration in the epizootiological importance of transmission of infection, was stressed by Thoen *et al* (1977), when a Sandhill Crane *Grus canadensis*, was found to be infected with *M.avium* serotype 1. The most common isolate from swine along the migratory flyways used by these birds was serotype 1, unusual in that serotype 2 is more commonly isolated from pigs.

Harrison and Hay (1959) recorded a tuberculous infection in a Moorhen *Gallinula chloropus* which resulted in the bird losing its toes. Other reports from water birds include two cases of tuberculosis in Cormorants *Phalacrocorax carbo* (Schaefer *et al*, 1973; Garden, 1961).

Serotyped *M.avium* strains from birds, isolated at the Tuberculosis Reference Laboratory, Cardiff, and Central Veterinary Laboratory, Weybridge, showed type 2 to be most prevalent (69%; 75/108), with 18% type 3 (19/108) and 13% type 1 (14/108) (Schaefer *et al*, 1973).

The disease in Woodpigeons *Columba palumbus* is somewhat unusual. Reports of avian tuberculosis in these birds are fairly frequent (Christiansen *et al*, 1946; McDiarmid, 1948; 1962; Soltys and Wise, 1967; Matthews and McDiarmid, 1979). The first point of interest is the unusual darkening of the plumage noted by McDiarmid (1948) and Harrison and Harrison (1956). Pathologically, the infection appears fairly typical (Francis, 1958). However, isolation of the tubercle bacilli proved difficult. This difficulty was overcome when the isolates were grown on the medium used for *M.paratuberculosis*, which contains mycobactin. These mycobactin-dependent strains of mycobacteria have been shown to have the pathogenic capability of *M.avium* in chickens and of *M.paratuberculosis* in calves (Collins *et al*, 1985b). Within general Woodpigeon populations there would appear to be quite high levels of incidence, up to 4% and not below 2% (Francis, 1958). Half of these infected birds have intestinal lesions and spread of infection may be through parent birds feeding the squab by regurgitation of food and production of 'milk' from the crop. Amongst birds being prepared for the table, tuberculous ones can usually be detected by the combination of dark plumage and yellow spots on the liver, and rejected.

There have been numerous records of the disease in free living wildfowl. Beer and Ogilvie (1972) cite cases in the swans, the disease being found in Mute Swans *Cygnus olor*, Whistling Swans *C. columbianus columbianus*, Trumpeter Swans *C.buccinator* and Bewick's Swans *C.columbianus bewickii*. Mr. E. Boughton of the Central Veterinary Laboratory, Weybridge, isolated *M.avium* serotype 2 from a Mute Swan, found in the Bristol area (personal communication). As part of this study the disease has also been identified in a Whooper Swan *C. cygnus cygnus* from which *M.avium* serotype 1 was isolated (Chapter ten). Francis (1958) quotes French (1904), who found that tundra swans *C. columbianus* captured on their return to Carolina, had very little resistance to *M.avium*. The disease in wild geese is less well documented, although there is a report of a tuberculous, moribund Barnacle Goose *Branta leucopsis* being picked up on the Solway Firth in 1973 (Dr. Myrfyn Owen, personal communication).

There have been many reports of the disease in free flying ducks. A study of 3,000 wild bird PM's in the Western Lakes area of USA, found avian tuberculosis in a Mallard *Anas platyrhynchos*, two Green-winged Teal *A. crecca carolinensis*, a Shoveler *A.clypeata*, two Pintail *A.acuta* and two Redheads *Aythya americana* (Quartrup and Shillinger, 1941). In Plum's extensive survey (1942) the disease was found in four Common Scoters *Melanitta nigra* and Sinkovic (1954) reported the disease in a Grey Teal *Querquedula gibberifrons*.

The disease has been reported in an adult drake Wigeon *Anas penelope*, found moribund in the Orkneys (Randall and Harrison, 1956); a bird of the same species found at Abberton, Essex (Wainwright, 1959); and a female Shelduck *Tadorna tadorna* in Kent (Harrison, 1957). The latter bird had a heavy infestation of four species of trematodes and two

species of cestodes possibly weakening the bird, and rendering it more susceptible to *M.avium* infection. This bird and a tuberculous Pochard *Aythya fuligula* found at Abberton (Harrison and Harrison, 1960), had both been partially predated, the Pochard having been lacerated about the neck possibly by a fox or otter. The authors suggest that these two cases and the case of the Short-eared Owl (Harrison, 1943) imply that birds weakened by *M.avium* infections may fall easy prey, yet predators seem to detect something unpleasant about the victim and discard it. Martin Brown of The Wildfowl and Wetlands Trust, reports many tuberculous birds as having an unusual 'sweet' smell (personal communication). Histology carried out on the liver of the Pochard revealed multiple caseous areas, with infiltration of "small round cells" and giant cells surrounding the caseation.

Garden (1961) carried out PM examinations of 173 Eiders *Somateria mollissima* from the same area on the Ythan Estuary, Aberdeenshire. Four birds were found to have tuberculous lesions in either the liver, spleen, intestines and also the pericardial sac.

The importance of thorough quarentine measures was emphasized by Thoen *et al* (1976) who isolated *M.avium* serotype 3 from one of ten White-faced Whistling Ducks *Dendrocygna viduata*, which were being imported from Nigeria to USA. The ducks were held in a pen with Garganey *Anas querquedula*, two of which also died.

Of strains of *M.avium* isolated from free flying wildfowl at the Tuberculosis Reference Laboratory, Cardiff and Central Veterinary Laboratory, Weybridge, 5/9 were serotype 2, 3/9 were serotype 1 and 1/9 was serotype 3 (Schaefer *et al*, 1973).

Avian Tuberculosis in Captive Wild Birds.

The situation of avian tuberculosis in poultry has already been mentioned earlier in this chapter. Flocks of turkeys, and domestic ducks and geese are susceptible but outbreaks are relatively rare, and the problem has been overcome in the same way as the poultry industry by the introduction of an early kill policy. This account deals with the disease in captive wild birds.

As might be expected, more is known about the disease within captive birds than those in the wild. It is however, of less importance in those birds kept in small numbers or closed communities, with the exception of birds of prey.

Avian tuberculosis is the most common bacterial infection of captive birds. There are occurrences in almost every variety of kept bird and frequent reports of the disease from many zoological and private collections (Fox, 1923; Ratcliffe, 1946; Appleby, 1952; Wilson, 1960; Graham-Jones, 1961; Dosza, 1964; Pattyn *et al*, 1967; Schaefer *et al*, 1973; Montali *et al*, 1976; Cromie *et al*, 1991a; 1991b). At the beginning of the century there were reports of high incidences of the disease at both London and Berlin Zoos. In 1923, 6.2% of 3,505 birds necropsied were found to be tuberculous at Philadelphia Zoo, (Fox). In this study the disease was found in 14 orders with the highest mortalities being found in the Galliformes and Columbiformes. In 1967 an investigation into the avian tuberculosis at Antwerp Zoo identified a number of strains of *M.avium* (Pattyn *et al*).

A study carried out at the National Zoological Park, Washington DC, over a seven year period found that 12/22 orders of exhibited birds were affected by avian tuberculosis (Montali *et al*, 1976). Their

highest annual mortality was 4% in 1975. Associated with the tuberculosis infection they found amyloidosis of the liver and spleen in approximately 20% of the cases. The avian tubercle bacilli were cultured from 30 birds and these were found to be serotype 1. The observation that some of the parenchymatous organs were all but replaced by coalescing granulomas is one that is often seen in the wildfowl studied in this thesis. The orders of birds to be most affected were the Charadriiformes, Galliformes, Passeriformes, Anseriformes and Gruiformes. The disease was found in adult birds (1-10 years old) and there was no sex predilection. Spread of infection from the wild birds that came in contact with collection birds, was thought to be unlikely as any wild birds found dead within the grounds were found to be non-tuberculous.

The highest incidences of avian tuberculosis in birds of prey are from zoological collections accounting for 1-30% of cases examined at PM. Cooper (1978) stressed the importance of clinicians to bear mycobacterial infections in mind when dealing with an unusual case, as often these are only diagnosed on pathological investigation. Lumeij *et al* (1980) report two such cases involving a female Goshawk *Accipiter gentilis*, and a Lanner Falcon *Falco biarmicus*. Fluid from the swollen knee joint of the second bird contained acid fast serotype 2 organisms. At PM the bird was found to have typical lesions in the liver and spleen suggesting an alimentary route of infection, although contamination of a knee wound directly is a possibility.

Cooper (1968) also described the disease in a female Goshawk and a female Saker Falcon *Falco cherrug*. He points out that if birds kept for falconary are to be fed sparrows or other dead birds, then this prey must be examined for any signs of tuberculosis. If a trained

falcon is flown at wild free living prey it may well pick out ailing or sick birds which are then a possible source of infection.

Dozsa (1964) reported mortality as high as 29.1% in birds of prey at Budapest Zoo over a five year period. Between 1938 and 1951, 16% of the Falconiformes at Edinburgh Zoo were found to be infected (Appleby, 1952). In London Zoo between 1958 and 1965, the disease was diagnosed in 3.1% of Falconiforme mortalities.

There have been many reports of avian tuberculosis in semi-domesticated or game birds. The disease in pheasants is often associated with foster-mothering by an infected hen. Boughton (1969) reports 63/120 pheasants dying of avian tuberculosis with 36 infected and moribund.

Incidences within tame pigeons have been quoted as high as 50% (McDiarmid, 1948). Wilson (1960) describes unusual tuberculous eye infections in pigeons as well as nodular skin lesions and arthritis.

Tuberculosis in parrots deserves special consideration, these birds as well as canaries, are more often infected with *M.tuberculosis*. This is due to their exposure to human tubercle bacilli rather than *M.avium* being less pathogenic in these species. Indeed parrots are also susceptible to bovine tubercle bacilli (Francis, 1958). The same author reports diagnosis of 25% (170/700) of pet parrots as tuberculous in a Berlin Veterinary School. Whereas incidences from zoological collections tend to be lower (5.4% of PM's), and these latter cases are caused by *M.avium* with a typical pathology of avian tuberculosis. It is likely that the incidence in pet parrots is now lower, due to the decline in the incidence of human tuberculosis in The Developed Countries.

The *M.tuberculosis* infection in parrots manifests itself as superficial cutaneous lesions commonly on the head, affecting the eyelids, nasal and oral cavities. When internal organs are affected it is usually the lungs rather than the abdominal organs. This unusual pattern of infection can be explained by the inability of many mycobacteria to grow at avian body temperature (41-43°C)(Boughton, 1969) hence the human tubercle bacilli grow at the skin where temperatures are lower.

This inability to grow at avian body temperatures means that other mycobacterial infections are rare, however there have been reports of *M.fortuitum* and "*M.aquae*" being isolated from Rooks *Corvus frugilagus* (Kubin and Matejka, 1967). *M.xenopi* is capable of growth at avian body temperatures but there are no definite reports of natural infections occurring. Kazda (1967) isolated a mycobacterium from chickens, that he named *M.brunense*. This was later determined by immunodiffusion analysis to be a variant of *M.avium* (Stanford and Grange, 1974) of which it was recognised as a subspecies (McIntyre and Stanford, 1986).

Table 1.2

Summary of Serotypes of *M.avium* isolated from Birds.

Species of Host	Serotype of Isolate.
(i) Wild Birds.	
Lesser Flamingo *Phoeniconaias minor*	1
Sandhill Crane *Grus canadensis*	1
Whooper Swan *Cygnus cygnus cygnus*	1
Red-tailed Hawk *Buteo jamaicensis*	2
Mute Swan *Cygnus olor*	2
White-faced Whistling Duck *Dendrocygna viduata*	3

Isolates made by the Tuberculosis Reference Laboratory, Cardiff and Central Veterinary Laboratory, Weybridge (Schaefer *et al*, 1973).

	Unselected isolates from birds	Wild British Anatidae
Serotype 1 :	14 cases	3 cases
Serotype 2 :	75 cases	5 cases
Serotype 3 :	19 cases	1 case

(ii) Captive Wild Birds.	
30 species of birds held at The National Zoological Park, Washington DC.	1
Lanner Falcon *Falco biarmicus*	2

It is important to realise that a well nourished and active bird, if infected, may wall off a tuberculous lesion with scar tissue so containing the organisms. If then the bird is stressed or develops another infection then the tuberculosis may reactivate. Such stress factors include: being recently imported which is then associated with a change of diet, possible different humidity and climatic conditions. These together with a lack of sunlight, overcrowding and generally poor standards of hygiene may all predispose the bird to infection. Ainsworth and Rewell (1949) suggest that aspergillosis is characteristic of birds newly arrived in captivity and that avian tuberculosis is characteristic of birds that have been in captivity more than six months. The remarks made by Crisp (1872) in his original descriptions of the disease in birds "I may observe that hens in confinement, where they do not get grass and insects, are very liable to take tubercle" still hold true today.

Avian tuberculosis in the captive collections of The Wildfowl and Wetlands Trust is discussed within this thesis.

CHAPTER TWO.

THE ANATIDAE, THE WILDFOWL AND WETLANDS TRUST AND AVIAN TUBERCULOSIS AT SLIMBRIDGE.

CHAPTER TWO.

THE ANATIDAE, THE WILDFOWL AND WETLANDS TRUST AND AVIAN TUBERCULOSIS AT SLIMBRIDGE.

The Anatidae.

Wildfowl, that is ducks, geese and swans, have been the subject of various taxonomic exercises, but one of the best classifications which reflects actual evolutionary relationships, was first proposed by Jean Delacour and Ernst Mayr in 1945. This classification emphasised similarities within tribes rather than division of species into an enormous number of subfamilies as earlier taxonomists had done. Johnsgard (1978) has since modified this slightly and it is this classification that will be described here.

Wildfowl are classified as the family Anatidae. This is one of two families in the order Anseriformes. This other family is the Anhimidae which contains only three species of birds; these are the Screamers of South America. The Anatidae on the other hand is an enormous family with 148 species (although some species may now be extinct), grouped into 43 genera and 13 tribes. 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, although highly varied depending on different adaptations for feeding, is characteristic with 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 layer of down lying beneath. 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, they show

an enormous amount of variation in size, shape and plumage colouration. The family Anatidae is divided into three subfamilies. The Anserinae and Anseranatinae contain the swans and geese; and the Anatinae includes the sheldgeese, shelducks and the typical ducks.

Subfamily Anseranatinae.

Tribe Anseranatini: the Magpie Goose *Anseranas semipalmata.*

This tribe is composed of only one species, that is the Magpie Goose of Australia and New Guinea. This has several peculiarities including only semi-webbed feet and it may be some sort of link between the Anhimidae and the Anatidae.

Subfamily Anserinae.

This subfamily includes the whistling ducks, swans, true geese and two other unusual species.

Tribe Dendrocygnini: the Whistling Ducks.

This tribe consists of nine species, all of which are so called whistling ducks due to their very distinctive multisyllabic whistle. The species are distributed around the world but are mostly from tropical areas.

Tribe Anserini: the Swans and True Geese.

This tribe consists of twenty species, all but three of which are found in cooler areas of the Northern Hemisphere. The other three are the Black Swan of Australia and the Black-necked and Coscoroba Swans of South America.

All of the Anserini are very large with relatively little sexual dimorphism. Most of them are strongly migratory with the exception of the Hawaiian Goose or Nene *Branta sandvicensis* which has become both

nonmigratory and semiterrestrial.

Tribe Cereopsini: the Cape Barren Goose or Cereopsis *Cereopsis novae-hollandiae.*

This unusual goose, from the islands off South Australia, is not particularly closely related to either the true geese or the sheldgeese but is possibly a survivor of a transitional group between the two.

Tribe Stictonettini: the Freckled Duck *Stictonetta naevosa.*

Despite its typical duck-like appearance this species shares more anatomical traits with those of the geese and swans. It lives in Southern Australia and Tasmania and like the Cereopsis is a unique contemporary form.

Subfamily Anatinae.

This subfamily is larger than the other two put together and contains the sheldgeese, shelducks and all of the typical ducks. There are generally two moults of the body feathers a year and there are therefore usually two plumages; breeding and eclipse, which may be very distinctive. There is often marked sexual dimorphism with frequent iridescent colouration particularly in the males. In the females this iridescence may be confined to the speculum. Where there is sexual dimorphism, the male tends to be larger and more aggressive.

Tribe Tadornini: the Sheldgeese and Shelducks.

This tribe consists of 14 species distributed around the world with the exception of North America. The sheldgeese are fairly goose-like and occupy a similar niche feeding by grazing whilst the shelducks are more dabbling feeders, eating aquatic invertebrates. The males are larger than the females and tend to be aggressive during the

competition for mates.

Tribe Tachyerini: the Steamer Ducks.

There are three species of Steamer Duck, two of which are flightless, and they are found off the coasts of South America where they feed on marine invertebrates. They are closely related to the shelducks and share the tendency to be highly aggressive during social display activities.

Tribe Cairinini: the Perching Ducks.

There are 13 species of perching duck distributed all over the world, in this rather heterogenous group. They have long claws to enable them to perch effectively and they tend to nest in cavities. They share many similarities with the dabbling ducks.

Tribe Merganettini: the Torrent Duck *Merganette armata*.

This highly adapted species of duck lives in the fast running streams of the Andean mountains. Its specialisation to this lifestyle has obscured many of its basic relationships to both the perching and dabbling ducks to which it appears to be most closely related. Several subspecies have been identified within South America.

Tribe Anatini: the Dabbling Ducks.

This tribe consists of 39 species with a worldwide distribution and constitutes the most successful of all wildfowl groups. All but three species are classified in the genus *Anas*. All the species surface-feed, dabble or tip up for food. They tend to be temperate breeding species that are adapted to marshy habitats. Some of the species occur on several continents and may have transcontinental migration patterns.

Tribe Aythyini: the Pochards.

This tribe consists of 16 species (although the Pink-headed Duck is probably extinct) of primarily fresh-water diving ducks, with a worldwide distribution. As an aid to diving all the pochards have large feet on widely placed legs that are situated quite far back, making the birds somewhat ungainly on land but enhancing their diving abilities. Their smallish wing surface area means that they must run over the surface of the water before becoming airborne. Nests tend to be just above the water, possibly on emergent vegetation.

Tribe Mergini: The Sea Ducks.

This classification includes the eiders, although others may put them in their own tribe: the Somateriini (Scott, 1961; Delacour, 1974). The twenty species of sea ducks are found in both fresh water and marine environments, generally in the Northern Hemisphere. All species are excellent divers. Plumage of the males tends to be elaborately patterned, often with black and white which may serve to enhance their visibility in marine habitats. The females are generally less patterned and those that nest on the ground tend to be cryptically coloured like the female dabbling ducks. Other species nest in cavities or under brush.

Tribe Oxyurini: The Stiff-Tailed Ducks.

This tribe consists of nine species of duck from all over the world. They 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. Their plumage is made up of small numerous feathers and the wings are particularly short so becoming airborne and flying are not easily achieved.

The exception to some of the stiff-tail traits is the Black-headed Duck *Heteronetta atricapilla* of South America. It bears instead, a close anatomical resemblance to the dabbling ducks and is possibly an evolutionary link between the two groups. It also has the peculiar tendency to parasitise other nests, dropping its eggs with a variety of hosts.

The Wildfowl and Wetlands Trust.

In 1946, Sir Peter Scott founded The Severn Wildfowl Trust at Slimbridge, Gloucestershire. This name was changed to The Wildfowl Trust in 1954 as worldwide interest in conservation and research grew. In 1989 the name was changed to The Wildfowl and Wetlands Trust to reflect the great amount of work done in preserving and creating the wetland habitats upon which wildfowl, and so many other species, depend.

The Aims of The Wildfowl and Wetlands Trust.

The aims of The Wildfowl and Wetlands Trust can be classified into four categories:-

(i) Research - into all aspects of wildfowl biology and how to conserve them and add to human knowledge.

(ii) Conservation - to preserve and protect wildfowl and their disappearing and threatened wetland habitats for now and the future.

(iii) Education - to increase people's appreciation of wildfowl and wetlands, and the species they support.

(iv) Recreation - to allow people to enjoy wildfowl and their habitats at close quarters.

To aid these aims they maintain nine centres in Great Britain at:- Slimbridge, Gloucestershire; Peakirk, Cambridgeshire; Welney, Cambridgeshire; Martin Mere, Lancashire; Washington, Tyne and Wear; Arundel, Sussex; Caerlaverock, Dumfriesshire; Llanelli, Dyfed; Castle Espie, County Down.

All of the centres provide refuges for our native wildfowl and people are brought close to these wild birds in their natural environment by means of hides.

Captive collections of wildfowl from around the world are held at all of these centres except Caerlaverock and Welney which are refuges for wild birds only.

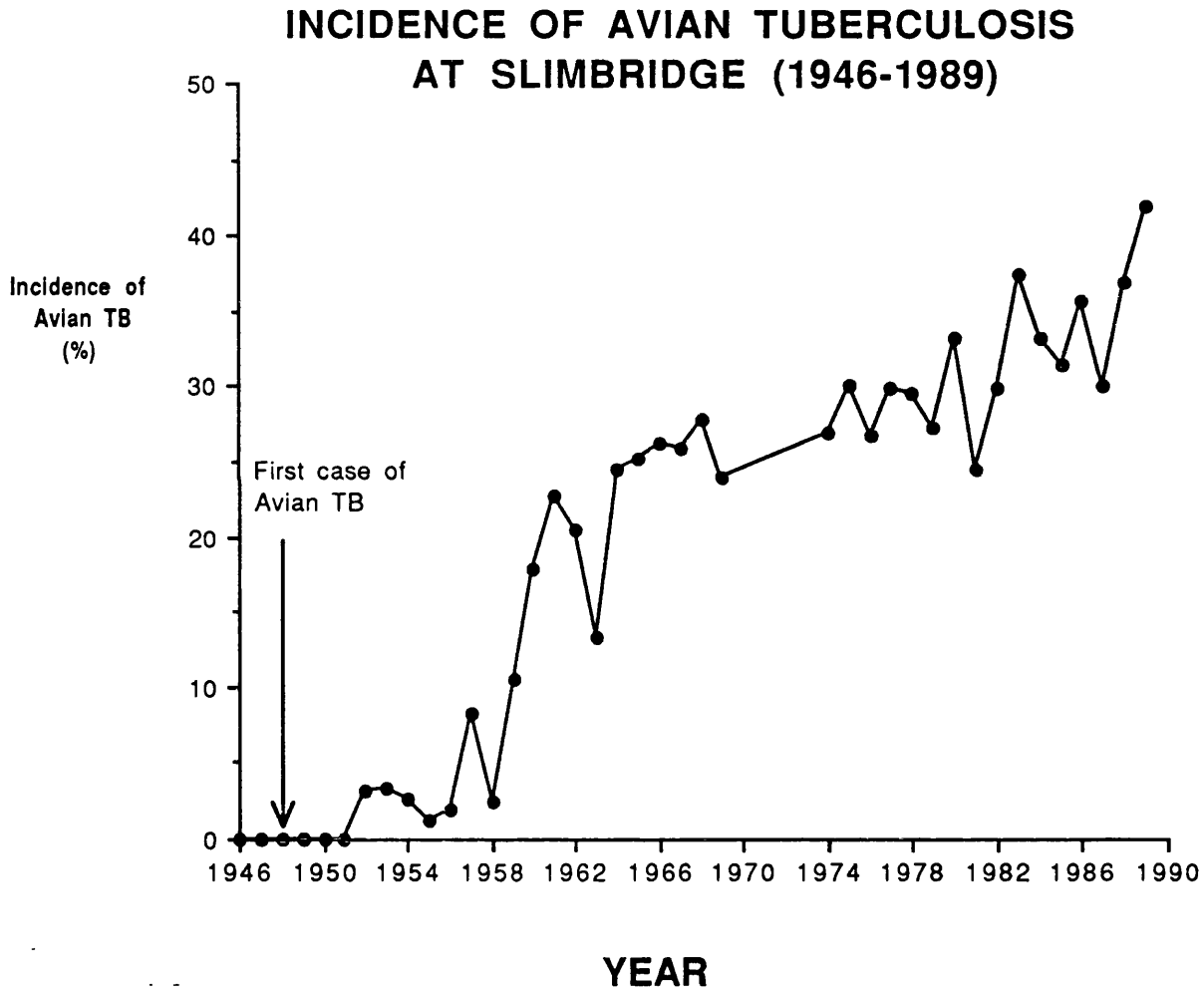
The Slimbridge Wildfowl and Wetlands Centre.

Slimbridge is the head quarters of The Wildfowl and Wetlands Trust and holds the largest and most comprehensive collection of wildfowl in the world, constituting a living museum.

Within the collection efforts are made to encourage the birds to breed successfully. This in turn enables the collection to be self sustaining and also raises stocks of rare or endangered species, some for possible reintroduction programmes. These collections form the basis for the four main aims of The Wildfowl and Wetlands Trust.

As well as the captive collection at Slimbridge, wild birds are attracted to the large refuge in very great numbers. In the winter well over 10,000 wildfowl use this refuge including thousands of White-fronted Geese *Anser albifrons*, the occasional Lesser White-fronted Goose *Anser erythropus* and hundreds of Bewick's Swans *Cygnus columbianus bewickii*.

Figure 2.1


INCIDENCE OF AVIAN TUBERCULOSIS
AT SLIMBRIDGE (1946-1989)
Incidence of Avian TB (%)
50
40
30
20
10
0
First case of Avian TB
1946 1950 1954 1958 1962 1966 1970 1974 1978 1982 1986 1990
YEAR

Avian Tuberculosis at Slimbridge.

The incidence of enzootic avian tuberculosis, caused by *M.avium*, has been rising gradually since 1948 (figure 2.1) and is now at epizootic proportions accounting for at least a third of adult mortalities. It is the main single cause of death in the captive collection. An analysis of the epizootiology of the disease from 1980-1989 is given in the next chapter.

The loss of birds at Slimbridge from avian tuberculosis is of great concern but there is also the problem of spreading the disease when translocating infected birds to other national and international collections. The risk of spreading disease to the wild populations must also be considered as infection could then be carried back to the summer breeding grounds of the many wild species that use the Slimbridge reserve.

Clinical Aspects.

Avian tuberculosis in wildfowl is a chronic disease with a slow insidious stage of development allowing the birds to remain in good condition for a long period with relatively few signs during the early stages of infection. Once the infection has progressed sufficiently, physical conditions are affected. The signs of the disease are not highly specific but eventually infected birds generally lose their appetite and become greatly emaciated, with particular wasting of the pectoral muscles so that the sternum becomes very prominent. There are enteric disturbances leading to diarrhoea inducing extreme weakness in the bird, so the birds tend to assume a sitting position. The plumage becomes dull and ruffled, and birds often become lame if there is involvement of the bone marrow. One of the most specific signs is the

distension of the abdomen due to hypertrophy of the liver and the massive build up of ascitic fluid. Unfortunately the disease signs may not be noticed until the bird is moribund.

Although infected birds may live for very many months in an advanced tuberculous state, death may be sudden as a consequence of a haemorrhage of an infected organ.

Pathology.

Pathologically the disease is characterised by numerous white caseous miliary tubercles. Lesions are found most commonly in the intestines and then probable intermittent bacillemia allows dissemination to the spleen and liver (figure 2.2). Tubercles vary in size from microscopic up to about 4 cm. Upon bacterial examination, they are found to be full of enormous numbers of acid fast organisms.

The disease may be widely disseminated and generalised infections are not uncommon with occasional involvement of kidneys, muscles, heart, lymph nodes, lungs, eyes and so on. This apparent ease with which systemic infections occurs may be due to the lack of well developed regional lymph nodes in birds.

It is a fairly common to find other infestations in tuberculous birds, such as *Amidostomum*, *Acuaria* and other parasitic worms. Amyloidosis is sometimes associated with chronic tuberculous infections, although this may be attributed to stress rather than to the causative disease.

Figure 2.2 : Advanced Avian Tuberculosis in an African White-backed Duck *Thalassornis leuconotus* showing numerous lesions in the liver.

Pathogenesis.

M.avium produces no toxins and, as in most mycobacterial infections, it is the protracted interactions between host and invading virulent organisms that cause disease. Disease is often the result of the replacement of functional tissue in organs such as the liver and spleen, by caseous tuberculous masses.

Transmission.

The disease is most likely spread by infected birds voiding tubercle bacilli with the faeces. This tends to contaminate the birds' feeding and loafing areas. The enormous number of bacilli exuded from ulcerated tuberculous intestinal lesions creates a constant source of virulent organisms. It is this infected faecal material, building up bacilli laden soil, that is the main vector in the dissemination of avian tuberculosis. The longer premises have been occupied by infected birds and the more concentrated the endemic populations, the more prevalent the infection is likely to be. It is therefore obvious that the problem at Slimbridge is exacerbated by the fact that there have been captive birds on the premises since 1946 and at huge and unnatural population densities.

M.avium may persist in the soil for great periods of time. Viable and virulent bacilli have been isolated four years on from an infected environment (Schalk *et al*, 1935; quoted by Thoen and Karlson, 1978). This ability to survive outside the host obviously increases risk of infection greatly. Infection appears to be most commonly acquired by the repeated ingestion of the organisms, hence the intestinal lesions.

Role of the Egg in Transmission.

Tuberculous lesions are rarely found in the ovary and oviduct and so it is unlikely that the egg constitutes a serious factor in the transmission of the disease. Cloacal contamination is also thought to be small. If eggs were infected, tuberculous hatchlings would be more prevalent and this is certainly not the case. Although it is possible to artificially infect hens' eggs with *M.avium* and therefore give rise to infected chicks, naturally infected birds generally lay uninfected eggs. Shchepilov (1956) did however, report isolating viable tubercle bacilli from duck eggs, from birds that had reacted positively to tuberculin testing. In artificially infected eggs, workers found that the bacilli would not survive after six minutes of boiling or two minutes of scrambling (Fritzsche and Allam, 1965; quoted by Thoen and Karlson, 1978).

The White-winged Wood Duck and The Worldwide Fund for Nature Project 406.

There have been cases of avian tuberculosis in practically all species of wildfowl at The Wildfowl and Wetlands Trust centres. However, some species have proved to be particularly susceptible and possibly the most susceptible is the rarest duck in the world, the White-winged Wood Duck *Cairina scutulata*. The reasons for its rarity, and the effects of avian tuberculosis on its captive breeding programme are discussed here.

Mackenzie and Kear (1976) and Green (1990) provide concise reports of the ecology and the conservation aspects of the White-winged Wood Duck. It is one of the largest of the perching ducks and one of the

largest ducks in the world at 30" long. It is related to the Muscovy Duck *Cairina moschata*, Hartlaub's Duck *Cairina hartlaubi* and the Comb Duck *Sarkidionis melanotos*. Its English name is derived from the white fore-wings which are very conspicuous in flight. Its local names in the areas of South East Asia that it inhabits, range from its Assamese name 'Raj-deohans' (Spirit Duck) which is derived from its ghostly wailing voice, to its Indonesion name of 'Itik hutan' (Forest Duck).

White-winged Wood Ducks were once widespread in lowland tropical evergreen forests and freshwater swamp forests of South East Asia. They are now almost certainly extinct in Malaysia and Java, but small populations still exist in pockets of tropical forest in North East India, Bangladesh, Sumatra, Vietnam and Thailand, and there may still be other populations in Laos, Burma and Cambodia.

The habitat requirements are forested areas interspersed with slow running streams and sheltered weed-infested pools. The ducks are most active at dawn and dusk, roosting in the shade of the forest during the day and flying to larger open areas of swamp just before dusk where they sometimes remain until just after dawn. Their omnivorous diet includes duckweed *Lemna*, annelids, molluscs, frogs, small reptiles and fish.

The birds are thought to form strong monogamous pairs which occupy on average 100 ha of optimal habitat, thus they are found at very low densities for a duck. They are most usually seen in pairs or singly and tend not to form flocks, although there have been reports of up to eleven birds in one flock. They nest in hollows or holes in larger trees, in tree stumps or on branches near the main stem, as high as 25 m off the ground. Green (1990) suggests the limited number of suitable nest sites may account for their low density.

During this century, and especially since World War II, the White-winged Wood Duck populations have been dwindling. It would appear that the main cause of this is the destruction of tropical forest habitat. This is all due to Man's disturbance with the exception of the heavy flooding of the Upper Assam plains following the 1950 earthquake. The disturbance has taken the form of clearing areas for timber extraction or agriculture, which tended to isolate birds, and the pressures of hunting and logging further reduced the remaining small populations. These lowland areas of forest allow easy logging and although the higher altitudes may be safer from the loggers, the water flow is usually too great to provide the sluggish streams and pools the ducks require. The increase in demand for timber has compounded the problem and now areas are being clear felled. In India some of these areas and being replanted with fast growing tree species providing unsuitable habitat with little cover and shrub layer.

Mackenzie and Kear (1976) summarise the threats leading to this recent rapid decline as a reduction in areas of suitable habitat; isolation of remaining ducks; an increase in disturbance; and an increase in hunting and collection of young.

Their present status is critical with a known population of only 200 birds left in the wild. King (1979) classified them as "vulnerable" in The Red Data Book. They are listed in the International Council for Bird Preservation (ICBP) "World Checklist of Threatened Birds" (1988) where it is again emphasised that decline in numbers is due to forest clearance, hunting, and disturbance. Although protected in India since 1937, and having been on the "Special Protected List" since 1952, the census figures of the International Waterfowl Research Bureau (IWRB) in 1966 suggested that immediate conservation actions had to be taken

in Assam if the species were to survive. It was for this reason that The World Wide Fund for Nature (WWF) Project 406 was initiated in 1968. The project, coordinated by The Wildfowl and Wetlands Trust, Slimbridge, was set up in order to conserve the remaining populations of White-winged Wood Ducks. Its three main objectives, or phases, being:-

i) to collect young White-winged Wood Ducks from the forest in order to create a number of captive breeding stocks in Assam, India; Slimbridge, England and Washington DC, USA.

ii) to breed and study the species in captivity.

iii) to establish as a sanctuary, a suitable area of unspoiled Assam primary plains tropical forest and to reintroduce captive bred birds into the area, or alternatively, into suitable sanctuaries in countries where they were thought to be extinct.

Phase 1.

During the years 1967-1970 12 White-winged Wood Ducks (seven males and five females) were collected as ducklings from Upper Assam from an area now largely destroyed and grazed by cattle. They were sent to Slimbridge where they were hand reared. Birds were also sent to Gauhati Zoo, Assam and USA via France. Captive breeding stocks are now held at The Wildfowl and Wetland Trust centres in Great Britain; various other British collections including Jersey Wildlife Preservation Trust; Washington Zoo, USA; Gauhati Zoo, Assam; Bordubi Tea Estate, Assam; Zoological and Botanical Gardens, Hong Kong.

Phase 2.

The birds were studied in captivity and were found to begin breeding mid to late March until June, moulting in July. Occasionally the birds breed at two years old but generally they breed at three years old. In

captivity they nest in boxes constructed somewhat like a dog kennel.

The most basic avicultural requirement for successful captive breeding would appear to be the provision of a shady pen. When kept on open ponds or with little cover, breeding does not occur. This is often the case with other forest ducks where bright light actually depresses activity and sexual display.

In the wild the birds would rarely experience temperatures below 5°C and therefore cold weather experienced in captivity may be stressful.

Phase 3.

Phase 3 of Project 406 has been drawn up by Sam Mackenzie and Mike Ounsted of The Wildfowl and Wetlands Trust with the following aims:-

i) to seek and encourage the establishment of 'suitable sanctuaries' within the present and former range of the species.

ii) to maintain healthy genetic stocks of White-winged Wood Ducks within the successful captive breeding units.

iii) where appropriate to reintroduce captive raised ducks safely into adequately protected 'suitable sanctuaries' within the present or former range.

This last point has now largely been superceded, due to the many intrinsic problems of reintroduction programmes. Instead, the main thrust of the project is the conservation of areas in which populations of ducks remain.

However, the captive breeding programme of the White-winged Wood Ducks within the Wildfowl and Wetlands Trust collections continues, to enable the Trust to aid Asian programmes, both by training overseas staff, and through the supply and exchange of birds. Additionally, research on the captive birds will provide information directly useful

to understanding wild populations and how to monitor them. The captive birds also contribute to the education and public awareness programmes in this country.

In the event of a further decline in wild populations, the birds held in Trust and other collections, may be necessary to preserve the species in captivity. The studbook for British birds was re-established in 1990 and is hoped to be extended to form an international studbook. Ideally, there will be genetic input from some of the unrelated birds bred in Asia, as these are more likely to be used for any reintroduction. To preserve a high proportion of the current genome, interchange of eggs/birds must be improved both nationally and internationally. Herein lies a problem.

The White-winged Wood Duck and Avian Tuberculosis.

White-winged Wood Ducks are susceptible to a range of diseases such as *Acuaria* infestations and aspergillosis, however the most devastating and important disease in captivity is avian tuberculosis. There are six PM reports from birds sent from Thailand to Slimbridge in 1955. One died of aspergillosis and five of avian tuberculosis. Of the birds sent as part of WWF Project 406 between 1967 and 1970, 23 original birds and their progeny died aged six months or over, all except three, of avian tuberculosis. Juvenile mortality was due to *Acuaria*, tapeworms, nephritis, aspergillosis and pneumonia.

A problem, only recently given the attention it warrants, in reintroduction programmes, is the risk of introducing diseases to wild populations. Unpublished reports of the health of captive White-winged Wood Ducks in the Mahao Wildlife Sanctuary in Arunachal Pradesh,

India, indicate one positive case of avian tuberculosis and several other reports of an infectious disease, which is almost certainly avian tuberculosis.

One of the points outlined in the third phase of the original WWF Project 406, is continued effort to be made on research into the control of disease, such as avian tuberculosis, amongst captive White-winged Wood Ducks and for this information to be passed on to those involved in the project.

The Epizootiology of Avian Tuberculosis in White-winged Wood Ducks at Slimbridge (1980-1989).

This short study of PM data from 1980 to 1989 analyses deaths caused by avian tuberculosis. Avian tuberculosis was the primary cause of death in most cases but those with sub-clinical infections have also been included. Diagnosis was made by the presence of lesions at PM examination backed up by the presence of acid-fast bacilli in Ziehl-Neelsen stained smears. All PM's were carried out by the same pathologist, namely Martin Brown of The Wildfowl and Wetlands Trust.

Statistical analyses have been carried out using Fisher's exact test.

Table 2.1.

Yearly Incidence of Avian tuberculosis in White-winged Wood Ducks.

Year	Estimate No. in colln.	No. PM's	TB cases	% min. mort.	% TB in colln.	% TB of PM's
1980	-	8	8			(100)
1981	-	5	5			(100)
1982	11	7	7	63.6	63.6	(100)
1983	5	2	2	40.0	40.0	(100)
1984	6	4	2	66.7	33.3	(50)
1985	12	1	1	8.3	8.3	(100)
1986	-	6	5			(83)
1987	44	5	2	11.4	4.5	(40)
1988	51	15	12	29.4	23.5	(80)
1989	33	19	19	57.6	57.6	(100)
Total		**71**	**63**			**(89)**

Estimate no. in colln. = Estimated number of White-winged Wood Ducks in collection.

% min. mort. = % minimum mortality

% TB in colln. = % avian tuberculosis in collection

- = data not available

Table 2.1 shows yearly incidence of avian tuberculosis in White-winged Wood Ducks over the last ten years. The most obvious point is the consistently high incidence. There appears to be no increase in incidence during this study period, a reflection of how long the disease has been prevalent within these birds at Slimbridge. A worrying point for the captive breeding programme is the high percentages of total numbers of birds in the collection dying from the

disease each year. Such losses will significantly reduce the size of the available gene pool.

Table 2.2

Incidence of Avian Tuberculosis according to Sex of White-winged Wood Ducks.

	TB	Total PM's	% TB
Males	31	36	**86.1**
Females	32	35	**91.4**
Total	**63**	**71**	**88.7**

As can be seen from table 2.2 there is no difference in the total numbers of males or females dying of avian tuberculosis, i.e. there is no sex predilection.

Table 2.3.

Seasonal Incidence of Avian Tuberculosis in White-winged Wood Ducks.

Season	TB	% TB	Other	% Other	Total
Winter	18	**(94.7)**	1	**(5.3)**	19
Spring	13	**(81.2)**	3	**(18.8)**	16
Summer	16	**(94.1)**	1	**(5.9)**	17
Autumn	15	**(78.9)**	4	**(21.1)**	19

Table 2.3 shows the incidence of avian tuberculosis deaths according to season. There would appear to be little difference in total mortality rates according to season. It can be seen that the greatest percentage of avian tuberculosis deaths occur in winter and summer.

Although numbers of deaths are too small to reach statistical significance the trend of deaths may be due to stress/energy demands associated with cold weather and breeding respectively, which reduce body condition.

Table 2.4.

Sex and Seasonal Incidence of Avian Tuberculosis in White-winged Wood Ducks.

Season	Males		Females	
	No. TB	%	No. TB	%
Winter	9	(50.0)	9	(50.0)
Spring	7	(53.8)	6	(46.2)
Summer	6	(37.5)	10	(62.5)
Autumn	9	(60.0)	6	(40.0)

As was seen from table 2.2 there is no overall difference in the numbers of males and females dying of the disease. However table 2.4 shows seasonal incidence divided into sex. The most notable point is the high female mortality in the summer, which is significantly greater than male mortality ($p < 0.04$). This is almost certainly due the stress associated with producing eggs and the subsequent incubation and brooding. Conversely, male mortality is highest in the autumn although this is not a statistically significant difference.

It is obvious from this study that avian tuberculosis is the main single cause of adult White-winged Wood Duck mortality at Slimbridge. As the disease is one of adult birds, and they do not breed until their second or third year, then hatching rates have more or less equated death rates and so captive numbers have remained relatively static.

The perching ducks as a whole are a particularly susceptible group with adult avian tuberculosis mortality rates as high as 49% (Hillgarth and Kear, 1981) although an even higher figure is recorded in the epizootiological survey carried out in the next chapter.

The problem of eradicating the virulent bacilli is great as the demand for plenty of shade to enhance breeding success does not allow for the sterilising effects of ultra-violet radiation from sunlight. Captivity also means that birds are kept in close contact with their own and pen mates' droppings. Stress may also play a part in susceptibility to infection. It may be brought on by captivity i.e. pinioning, being kept in unnatural high densities, disturbance from the visitors at The Wildfowl and Wetlands Trust centres and cold weather. Some reasons for the high incidence in the perching ducks as a whole are given in the following chapter.

Deaths from avian tuberculosis are very seriously affecting the White-winged Wood Duck captive breeding programme at Slimbridge and if it is to succeed, a means of rearing healthy disease free stock either by vaccination or different avicultural techniques must be developed.

If a vaccine could be developed it could at least increase the longevity of the birds to allow a few more breeding seasons to increase captive stocks. Vaccination as a possible control measure for avian tuberculosis is discussed in Chapter Four.

CHAPTER THREE.

THE EPIZOOTIOLOGY OF ENZOOTIC AVIAN TUBERCULOSIS AT SLIMBRIDGE.

CHAPTER THREE.

THE EPIZOOTIOLOGY OF ENZOOTIC AVIAN TUBERCULOSIS AT SLIMBRIDGE.

INTRODUCTION.

In an attempt to detect trends in the incidence and prevalence of avian tuberculosis within the captive birds at Slimbridge, this epizootiologic study has been carried out (Cromie *et al*, 1991a; 1991b). Differences in the occurrence of the disease in the different tribes, feeding groups, seasonal variation, and so on, are investigated. It is hoped that this will identify those groups most at risk of developing disease, give some information on how to control the disease, and ultimately how to assess if such measures have been of benefit.

This current study of the adult PM records of 1980-1989 is compared to a similar unpublished study carried out from 1948-1968 by J.V. Beer (The Wildfowl Trust, internal report). The first ten years of this study (1948-1957) were limited by the small numbers of PM's carried out, so most comparisons are made to the period 1958-1968.

MATERIALS AND METHODS.

Only adult PM data were analysed as avian tuberculosis is typically a disease of adult birds: an adult bird is defined as a bird that has survived to its first January or the equivalent for those southern hemisphere birds that breed earlier. In the last ten years there have been only 3/531 (0.6%) deaths of juveniles caused by avian tuberculosis: a juvenile is classified as a bird that is fully feathered but died in its first autumn or winter (before January 1st).

These three cases involved a Carolina *Aix sponsa*, a Maned Goose *Chenonetta jubata* and a Ruddy-headed Goose *Chloephaga rubidiceps*.

Diagnosis of the disease was made on macroscopic appearance of tuberculous lesions in the dissected body supported by the presence of acid fast bacilli in Ziehl-Neelsen stained smears. Although avian tuberculosis was the primary cause of death in most cases, PM data where there were dual infections/disease are also included i.e. some birds may not have died of avian tuberculosis, but had sub-clinical infection.

All PM's in the current study were carried out by the same pathologist, namely Martin Brown of The Wildfowl and Wetlands Trust. As a consequence there is a high degree of consistency in PM findings. During both this and the previous study period there have been a similar number of PM's carried out each year.

A total of 2384 PM's have been analysed in this study of which 787 PM's were diagnosed as avian tuberculosis. However, in the tables these figures may appear smaller due to occasionally incomplete data.

Incidences shown indicate the number of cases of avian tuberculosis found at PM as a percentage of the total number of PM's for that group.

The taxonomy used to divide the 148 species of wildfowl into 13 tribes was that described in Chapter two, i.e. that of Johnsgard (1978) which is based on both evolutionary and behavioural relationships.

Other classifications have also been used which divide the birds into feeding groups. Firstly, the birds have been split into three, by what

they ate: those whose diet consisted predominantly of vegetable matter, animal matter and those which fed on a mixture of the two. Secondly, how the birds obtained this food also allows the birds to be split into three groups according to feeding habits:-

(i) Grazers: those that fed on vegetable matter by grazing on land and took wheat from the feed provided.

(ii) Divers: those that fed on either wheat, sandeels or animal protein pellets by diving and taking food from the pelagic zone or benthos.

(iii) Dabblers: those that fed on grain, and/or mixed foodstuffs by sifting mud and silt on the water surface or just beneath it, generally in shallow water. They may also have upended to reach food below the water surface.

Statistical analyses have been done by Fisher's exact test.

RESULTS.

Annual Incidence of Avian Tuberculosis (1980-1989).

Table 3.1: Annual Incidence of Avian Tuberculosis (1980-1989).

Year	Collection size	PM's	TB	% Min. mort.	%TB in collection	%TB of PM's
1980	-	198	66			(33.3)
1981	-	266	65			(24.4)
1982	3253	221	66	6.8	2.0	(29.9)
1983	2622	264	99	10.1	3.8	(37.5)
1984	2737	273	91	10.0	3.3	(33.3)
Total 1980-84		1222	382			x=31.7% +4.9
1985	2516	241	76	9.6	3.0	(31.5)
1986	2904	235	84	8.9	2.9	(35.7)
1987	3018	282	85	9.3	2.8	(30.1)
1988	2928	187	69	6.4	2.4	(36.9)
1989	3076	217	91	7.1	3.0	(41.9)
Total 1985-89		1162	405			x=35.2% +4.7
TOTAL 1980-89		**2384**	**787**			**x=33.2% +5.1**
Data from the study of Beer 1958-68		2624	528			x=19.4% +8.1

% Min. mort. = % minimum mortality in the collection

- = data not available

The collection size is the annual census at the end of the preceeding year.

Table 3.1 shows the annual incidence of avian tuberculosis over the last ten years. The data are divided into five year periods (1980-1984 and 1985-1989).

Beer reported a mean annual incidence of the disease of 19.4% (± 8.1%) for the years 1958-1968 with 528/2642 PM's considered tuberculous. In the current study, this figure has risen to a mean of 33.2% (± 5.1%) with 787/2384 PM's considered tuberculous, a statistically significant rise ($p < 0.0000$). It should also be noted that the incidence of avian tuberculosis in the last five years (1985-89) has risen by a net 3.5%, which is an overall rise of 10.3%, compared to the first five year period (1980-84), a statistically significant rise ($p < 0.04$).

Table 3.2 : Incidence of Avian Tuberculosis according to Taxonomic Tribe.

Tribe	1980-84 PM's	TB	%TB	1985-89 PM's	TB	%TB	1980-89 PM's	TB	%TB
Cairinini	126	65	(51.6)	130	68	(52.3)	256	133	(51.9)
Mergini	136	60	(44.1)	96	53	(55.2)	232	113	(48.7)
Tadornini	53	16	(30.2)	35	15	(42.9)	88	31	(35.2)
Aythyini	94	28	(29.8)	64	22	(34.4)	158	50	(31.6)
Anatini	319	99	(31.0)	284	91	(32.0)	603	190	(31.5)
Dendrocygnini	73	24	(32.9)	96	29	(30.2)	169	53	(31.4)
Anserini	312	76	(24.4)	335	108	(32.2)	647	184	(28.4)
Oxyurini	47	13	(27.7)	62	14	(22.6)	109	27	(24.8)
Anseranatini	7	1	(14.3)	4	1	(25.0)	11	2	(18.2)
Cereopsini	2	0	(0)	4	1	(25.0)	6	1	(16.7)
Phoenicopteridae	44	0	(0)	37	2	(5.4)	81	2	(2.4)
Tachyerini	6	0	(0)	7	0	(0)	13	0	(0)
Anhimidae	4	0	(0)	4	0	(0)	8	0	(0)
Totals	1222	382	(31.3)	1162	405	(34.9)	2384	787	(33.0)

Table 3.2 shows the incidence of avian tuberculosis in the last ten years, divided into taxonomic tribes. Again the data are broken down into the same five year periods.

During the last ten years there have been cases of avian tuberculosis in all tribes with the exception of the Anhimidae (screamers) although there have been few PM's carried out in this group. Levels of incidence in both five year periods were generally similar, although incidences in the Tadornini, Mergini and Anserini showed upward trends, the increase in the latter tribe being statistically significant ($p < 0.01$).

Beer found the Cairinini (perching ducks) to have the greatest incidence, which was still true for this study period. However, there was a significant rise in the level of incidence (from 30.0% to 51.9%; $p < 0.0000$). Avian tuberculosis was by far the commonest single cause of death within this group.

In the Mergini, avian tuberculosis was also the most common single cause of death. The very high incidence is particularly interesting if the tribe is broken down into Mergini (sawbills, goldeneyes, and scoters) and the Somateriini (eiders) as some authors have done (Scott, 1961; Delacour, 1974).

From Table 3.3 it can be seen that the incidence in the eiders was significantly lower than the other seaducks ($p < 0.0000$), although there was a significant increase between the two five year periods ($p < 0.003$).

Table 3.3 : Incidence of Avian Tuberculosis in the Mergini and Somateriini.

Tribe	1980-84 PM's	TB	%TB	1885-90 PM's	TB	%TB	1980-89 PM's	TB	%TB
Mergini	71	51	**(71.8)**	62	39	**(62.9)**	**133**	**90**	**(67.7)**
Somateriini	65	9	**(13.8)**	34	14	**(41.2)**	**99**	**23**	**(23.2)**

If comparisons are made between the incidences reported previously and this study, the most notable point is the much higher figures in this study, particularly in the Mergini excluding the eiders, whose incidence has doubled (from 34.5% to 67.7%; $p < 0.0000$).

In this study, similar levels of incidence were found in the Aythyini (pochard, white-eye and scaup); Anatini (dabbling ducks); and Dendrocygnini (whistling ducks). Beer also found similar levels of incidence between these groups, although these previous levels were significantly lower (16.5%, $p < 0.003$; 17.5%, $p < 0.0002$; and 23.0%, $p < 0.01$ respectively). Avian tuberculosis was the most common single cause of death in all three of these tribes during this study.

Beer reported a figure of 12.5% of Tadornini mortalities due to avian tuberculosis, this figure now stands at 35.2%, a statistically significant rise ($p < 0.0000$). Some taxonomists group the Tadornini with the steamer ducks (Tachyerini) and Cereopsis Goose (Cereopsini) in the same tribe (Scott, 1961; Delacour, 1974). The Tadornini excluding the steamer ducks and Cereopsis Goose, had a very high incidence, with avian tuberculosis accounting for most of the deaths.

Even though the sample sizes are small, this is not the case in the latter two groups in which the incidences were very low.

The incidence of avian tuberculosis in the Anserini reported by Beer (10.0%) was found to be considerably higher at 28.4%, another statistically significant rise ($p < 0.0000$).

The Oxyurini was the only tribe in which incidence has fallen since Beer's previous study (from 28.5% to 24.8%) although this was not a statistically significant fall.

In Beer's previous study none of the 35 PM's of Anseranatini (Magpie Goose) were tuberculous, whereas 2/11 (18.2%) PM's were found to be so in this study.

The Phoenicopteridae (flamingoes) also had a very low incidence with only 2.4% (2/81) PM's considered tuberculous.

Incidence of Avian Tuberculosis according to Sex.

Table 3.4 : Incidence of Avian Tuberculosis according to Sex.

	1980-84			1985-89			1980-89		
	TB	(%)	Total	TB	(%)	Total	TB	(%)	Total
Male	200	**(32.9)**	608	188	**(32.8)**	573	**388**	**(32.9)**	**1181**
Female	178	**(29.2)**	610	217	**(37.0)**	586	**395**	**(33.0)**	**1196**
			($p < 0.003$)						
Total	378	**(31.0)**	1218	405	**(34.9)**	1159	**783**	**(32.9)**	**2377**

(The p value in table 3.4 indicates the level of significance of the difference in female tuberculosis mortalities).

Table 3.4 shows the incidence of avian tuberculosis according to sex. The sex ratio within the collection is considered to be very nearly 1:1. Although male mortality is slightly higher than female mortality in the first five year period, and female mortality is slightly higher than male mortality in the second five year period, the overall picture shows no sex predilection.

Seasonal Incidence of Avian Tuberculosis.

Table 3.5 : Seasonal Incidence of Avian Tuberculosis.

Season	TB deaths	(%)	Other	(%)	Total
Winter	225	**(34.7)**	423	**(65.3)**	648
Spring	176	**(24.9)**	531	**(75.1)**	707
Summer	207	**(40.7)**	302	**(59.3)**	509
Autumn	170	**(36.2)**	300	**(63.8)**	470
Total	778		1556		2334

The seasons are classified as spring (March-May), summer (June-August), autumn (September-November) and winter (December-February).

From the total numbers of deaths in table 3.5, it can be seen that the highest total mortality rates occurred in winter and spring. As percentage avian tuberculosis mortalities are therefore somewhat misleading, the actual number of avian tuberculosis cases are considered here.

The highest numbers of avian tuberculosis mortalities are in the winter and summer, a finding also made by Beer in his previous study.

Incidence of Avian Tuberculosis according to Sex and Season.

Table 3.6 : Incidence of Avian Tuberculosis according to Sex and Season.

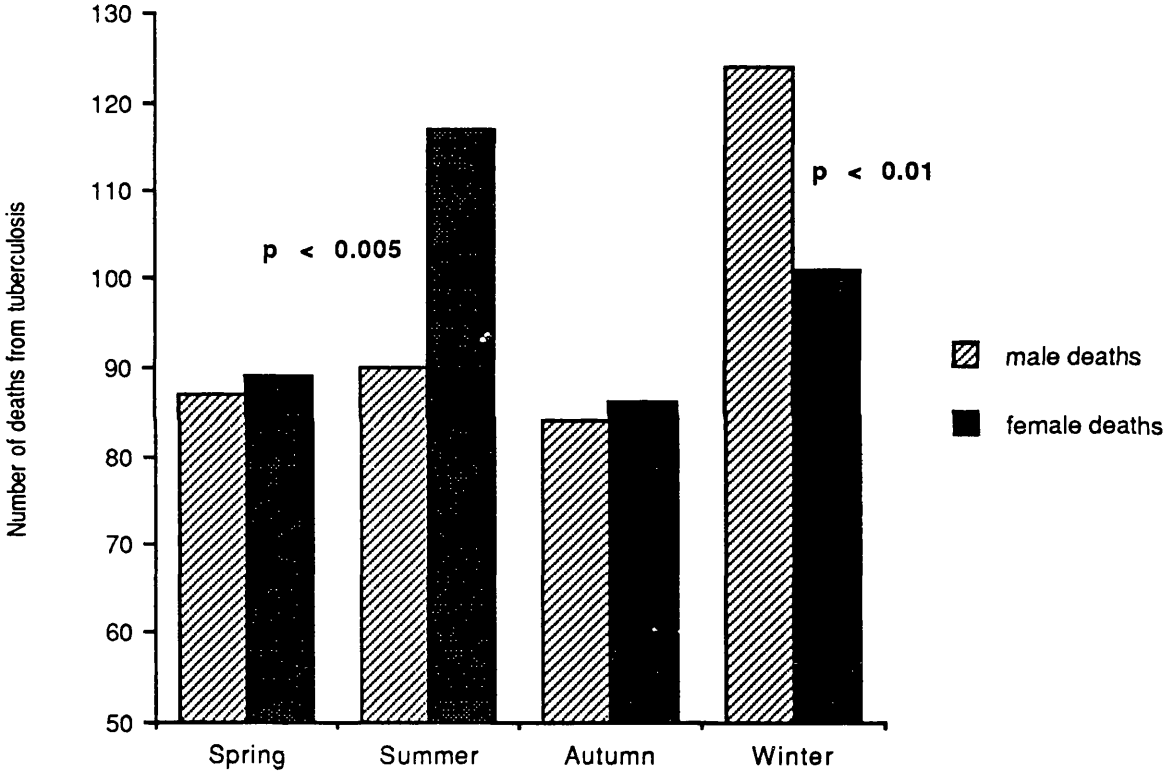
Season	TB Deaths				TB Deaths	Other Deaths	TOTALS DEATHS
	Male	(%)	Female	(%)			
Winter	124	(55.1)	101	(44.9)	225	423	648
		($p<0.01$)					
Spring	87	(49.4)	89	(50.6)	176	531	707
Summer	90	(43.5)	117	(56.5)	207	302	509
		($p<0.005$)					
Autumn	84	(49.4)	86	(50.6)	170	300	470
Total	385		393		778	1556	2334

(p values indicate significant differences in both male and female incidences in winter and summer, respectively).

Table 3.6 shows the incidence of death due to avian tuberculosis divided into male and female mortality as well as seasonal mortality. As has been seen earlier, there would appear to be no overall difference in susceptibility according to sex, but there are differences in when the sexes die, as can be seen from figure 3.1. Male mortality is significantly higher in the winter ($p < 0.01$) and female mortality is significantly higher in the summer ($p < 0.005$).

Figure 3.1

Incidence According to Sex and Season



Incidence of Avian Tuberculosis according to Climate of Country of Origin.

Table 3.7 : Incidence of Avian Tuberculosis according to Climate of Country of Origin.

	Climatic Zone.		
	Cold	Temperate	Hot
Total PM's	860	412	1108
TB Cases	292	122	372
% TB	**34.0**	**29.6**	**33.6**

Table 3.7 shows the incidence according to the climate of the bird's country of origin. Climatic zones are classified as hot (mean temperatures of 21°C and over), temperate (mean temperatures between 10°C and 21°C) or cold (mean temperatures of below 10°C). The lowest percentage of avian tuberculosis mortality was in those birds from temperate climates. This contrasts with the equally high mortalities in those birds that came from both hot and cold climates. In his previous study, Beer found the lowest mortality in those from cold climates, with higher similar figures for those from the other two climatic zones.

Table 3.8 : Incidence of Avian Tuberculosis according to Food and Feeding Habit.

	Food			Feeding Habit		
	Vegetable	Animal	Mixed	Diver	Grazer	Dabbler
Total PM's	1682	377	321	499	803	1079
TB cases	539	132	114	191	223	372
% TB	**32.0**	**35.0**	**35.5**	**38.3**	**27.8**	**34.4**
				$p < 0.002$		$p < 0.001$
Data from the study of Beer 1958-1968						
(%TB)	(18)	(28)	(31)	(18)	(22)	(30)

Table 3.8 and figure 3.2 show the incidence of avian tuberculosis in relation to food and feeding habits of the birds. The data were divided into (i) what the bird ate, and (ii) how the bird obtained its food, by either diving, grazing or dabbling. (p values show the significance levels of the differences between the diving and dabbling feeding groups related to the grazers).

There were no statistically significant differences from the results of actual foodstuffs taken. If this did affect the incidence of infection, then the mixed feeders should be intermediate between the other two groups. Beer found a lower incidence in the vegetable feeders, but again the mixed feeders were not the intermediate group.

However, when the feeding habits of the birds were considered there were statistically significant differences between the grazers and the other two groups (grazers v. divers $p < 0.002$; grazers v. dabblers $p <$

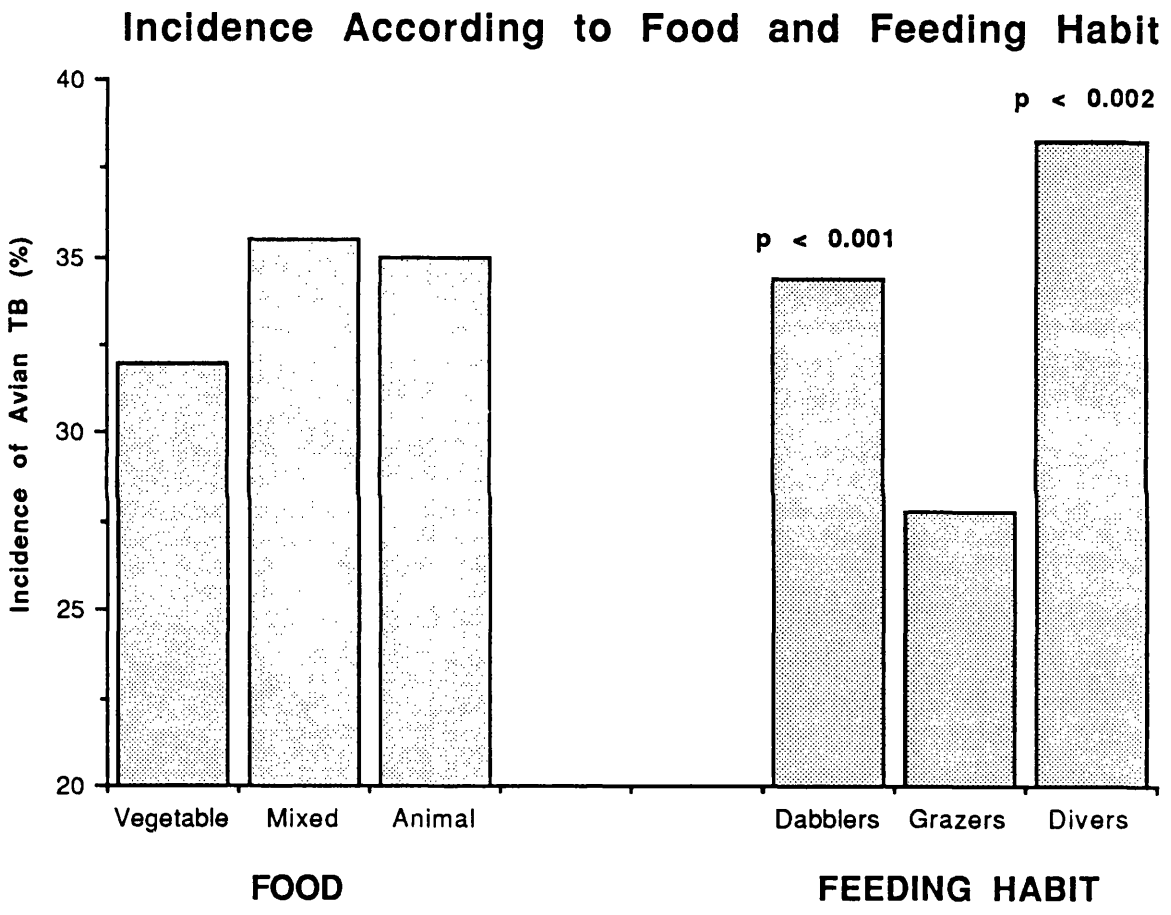
0.001). Although the incidence within the grazers was low, when some species were analysed separately a different picture was seen, for example in the Barnacle Geese *Branta leucopsis*. These birds had a relatively high incidence of avian tuberculosis with 42.1% (59/140) of PM's in this study period being due to the disease.

The only details of primary pulmonary tuberculosis were found in eight dabbling ducks (four of which were White-winged Wood Ducks).

The results from this recent study show that the divers had a particularly high incidence. If the divers were divided into freshwater divers (Oxyurini and Aythini) and seaduck divers (Mergini including the eiders) then there was a significantly higher incidence in the seaduck divers ($p < 0.001$).

Again the percentage of avian tuberculosis deaths in all groups was far higher in this recent study, with the most marked increase in the divers (from 22% to 38.3%).

Figure 3.2


Incidence According to Food and Feeding Habit
Incidence of Avian TB (%)
40
35
30
25
20
p < 0.001
p < 0.002
Vegetable
Mixed
Animal
Dabblers
Grazers
Divers
FOOD
FEEDING HABIT

Incidence of Avian Tuberculosis within the Grounds.

The grounds at Slimbridge cover about 100 acres and run from North East to South West (figure 3.3). The water supply is somewhat complicated with a mixture of sources from the canal, a rhine and mains water. The general flow of water is from the North East, through the grounds, discharging into the rhine at the South West end. At various points, water is actively pumped up from wells.

As there are very many individual pens, they have been grouped according to their proximity and shared water course. Data for the ten years study period have been pooled as individual years have too small a sample size. The results are summarised in table 3.9.

Figure 3.3

Map Of The Grounds

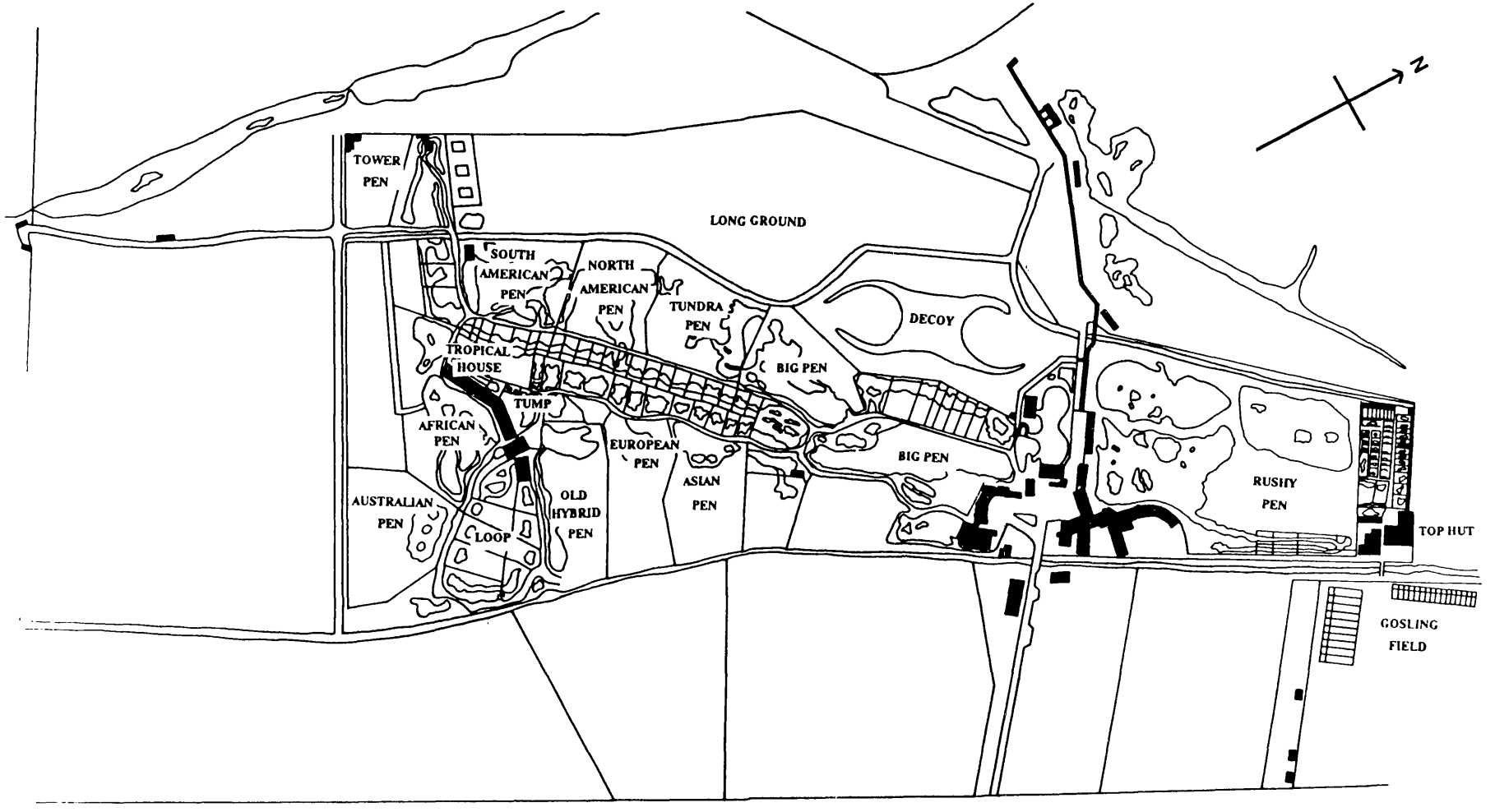


Table 3.9 : Incidence of Avian Tuberculosis within the Grounds.

1980-1989

	TB	TOTAL	% TB
Tropical House	22	40	(55.0)
Big Pen	183	394	(46.4)
Rushy Pen	32	79	(40.5)
African and Australian Pens	40	100	(40.0)
Pen 65, Asian and European Side Pens and Sea-duck Pen	27	73	(37.0)
Asian, European and Old Hybrid Pens	80	216	(37.0)
Pen 65, Side Pens of Big Pen, North and South American, and Tundra.	51	144	(35.4)
Tump	3	9	(33.3)
Mrs. Noah's, Tropical House Side Pen, Waterfall, Guiness and Vaughan Aviaries.	36	118	(30.5)
Top Hut, Top Pond, Top Aviary, Gosling Fields, Old Duckery	103	342	(30.1)
North and South American and Tundra Pens	52	183	(28.4)
Restaurant and Orchard Pens	13	51	(25.4)
Loop, and Loop Field, Tump and Side Pens, Australian Side Pens	50	205	(24.4)
Greater Flamingo House	3	15	(20.0)
Tower and Tower Side Pens	12	70	(17.1)
Patch/Youth Hostel	7	47	(14.9)
Lesser Flamingo House	5	36	(13.9)
Andean Flamingo House	4	29	(13.8)
Isolation, Sick Bay	1	8	(12.5)
Long Ground	3	37	(8.1)
Chilean Flamingo House	0	6	(0)

DISCUSSION.

The most obvious point to be made from this analysis is the very significant increase in incidence of deaths due to the disease in almost all groups of birds compared to the previous study carried out by Beer. The significant increase even over this ten year study period would point to a continuation in the increase of prevalence of the disease and that a plateau of mortality has not yet been reached (figure 2.1).

Schaefer *et al* (1973) reported isolating *M.avium* from nearly 50% (10/21) of Anatidae considered non-tuberculous at PM, indicating that birds infected with *M.avium* may not go on to develop pathological disease. From this the changes in mortality can be explained in two ways. Firstly, if the percentage of infected birds that develop disease remains constant, then increased infection will lead to increased mortality. This might be expected as the grounds at Slimbridge become increasingly contaminated with pathogenic strains of *M.avium*, concentrated and excreted by successive generations of tuberculous birds. On the other hand, if the level of infection has remained the same, the increase in mortality must be due to an increase in susceptibility. The data are considered in both these ways.

Incidence according to Taxonomic Tribe.

The high incidence of avian tuberculosis in the Cairinini and other causes of mortality are discussed by Hillgarth and Kear (1981). Why there should be such a high incidence is of interest. The group as a whole are somewhat heterogenous, feeding in different ways and originating from different climates (although the majority are from

hot climates). Some species are commonly and easily kept in captivity, whilst others are not so.

If the exceptionally high incidence of avian tuberculosis is a reflection of increased susceptibility in this group, it may be due to the fact that as perching ducks they would normally have less contact with the ground, and therefore mycobacteria, than other wildfowl groups. In evolutionary terms, mycobacterial immunity in arboreal species may have been of less importance than in other groups. Also, as the birds in captivity are pinioned and so spend all of their time at ground level in contact with their own and other birds' droppings, their risk of infection is increased. As has been mentioned in Chapter two, the White-winged Wood Duck is a particularly susceptible perching duck. Its requirements for a shady pen, where *M.avium* thrives in the lack of the sterilising effects of ultra-violet radiation, may be a factor also affecting the other perching ducks.

The high incidence in the Mergini may indicate high susceptibility. This could be explained by the fact that as birds evolved for sea life, they would rarely come into contact with *M.avium*, and so like the arboreal species there would not have been an evolutionary pressure towards mycobacterial immunity. Similarly, many of these birds live and breed in very cold climates in the far north thus encountering fewer pathogenic organisms, including mycobacteria. As discussed in Chapter one, there have been cases of avian tuberculosis in wild European Eiders *Somateria mollissima* (Garden, 1961) and wild Common Scoters *Melanitta nigra* (Plum, 1942).

The anomaly in incidences between the Mergini and Somateriini (Scott, 1961; Delacour, 1974) must indicate greater susceptibility in the former group, rather than environmental factors, as both these groups

share a similar ecology in the wild i.e. spending part of their lives at sea and feeding in the same way, namely diving for animal foodstuffs. In the captive collection they often share the same pens. Such an anomaly between these two groups was also found by Beer.

The Mergini excluding the eiders are a relatively difficult group to maintain in captivity, with infrequent captive breeding, and their susceptibility to diseases like avian tuberculosis, compounds the problem. A review of other diseases, as well as avian tuberculosis, in captive seaducks is given by Hillgarth and Kear (1979a).

Avian tuberculosis was the main single cause of death within the Tadornini. If this is a reflection of increased susceptibility in this group compared to the Tachyerini and Cereopsini, then it seems appropriate that the latter groups are separated by the taxonomy of Johnsgard (1978). Interestingly, the Tachyerini are not as easily kept or bred in captivity as the other sheldgeese and shelducks. A report of diseases within these birds is given by Hillgarth and Kear (1979b).

The Aythyini feed by diving, whilst the Anatini and Dendrocygnini feed mainly by dabbling; the effects of these feeding habits on incidence of the disease will be discussed later. The latter group are a particularly primitive group of birds, all from hot climates, the effect of which will also be discussed later.

The Anserini is kept and bred fairly easily in captivity, with the exception of some of the arctic species. Studies of the causes of mortality in northern geese in captivity showed that kidney failure was the main cause of death (Hillgarth and Kear, 1983), however avian tuberculosis in the Anserini as a whole is still a problem. They are a particularly long lived group of birds and Hillgarth and Kear (1983)

point out that this should raise their incidence of avian tuberculosis. This is not the case and may instead point to the group being less susceptible. However, the same authors found great differences in incidence between species of geese varying from 3% in Emperor Geese *Anser canagicus* to 47% in Lesser White-fronted Geese *Anser erythropus*. They suggest that as geese generally spend less time on potentially contaminated water than other groups, this may account for an overall lower incidence, which may also be related to their grazing feeding habits which will be discussed later.

Although there was a relatively low incidence in the Oxyurini as a whole, this is not general within the group. Hillgarth and Kear (1982b) found a high incidence in both the White-headed Duck *Oxyura leucocephala* and the White-backed Duck *Thalassornis leuconotus*.

There have been reports of the disease in wild Lesser Flamingoes *Phoeniconaias minor* from Kenya (Cooper *et al*, 1975), but the incidence in the flamingoes in captivity is very low. The effect of their feeding habit on their incidence is discussed later.

The low incidence found in the Anhimidae may be a reflection of immunity within this group, possibly due to their fairly distant evolutionary relationship to the Anatidae. However, there were few PM's carried out on birds from this group.

The pattern of tribal susceptibility reported by Beer has also been found in this study. The Cairinini remain a very susceptible group, and the Mergini excluding the eiders appear to be exceptionally susceptible.

Incidence according to Sex.

Beer also found that there was no overall sex predilection to avian tuberculosis. A similar situation was reported from other captive collections (Montali *et al*, 1976). Kear and Berger (1980) however, found a difference in the incidence between the sexes of Nenes *Branta sandvicensis*. They studied PM data of Nenes of Slimbridge origin, over 25 years, and found 10.0% (12/120) of males and 15.4% (23/149) of females dying of the disease. Although avian tuberculosis was a significant cause of death in these birds it was of less importance than both *Amidostomum* infestations and atherosclerosis.

In the study by Hillgarth and Kear (1983) into causes of mortality in northern geese, they found a 6.4% greater incidence in females than in males. In a similar study of whistling ducks, no sex predilection was found in 148 deaths caused by avian tuberculosis (Hillgarth and Kear, 1982a).

Incidence according to Season.

The high levels of total mortality in winter and spring are not unexpected as the stress associated with cold weather will predispose the birds to various infections and diseases. The effects of cold weather on mortalities of wild birds has been well documented by numerous authors in The Wildfowl Trust, Fifteenth Annual Report 1962-63. Beer (1964) described the effects of cold weather on collection birds at Slimbridge and found that smaller and medium sized birds (according to Scott, 1961) were more affected by cold weather than larger birds.

The high levels of mortality due to avian tuberculosis in both summer and winter could be due to the stress associated with both breeding and cold weather respectively. In the summer the birds are out of condition, due to the energy expended in breeding and the massive metabolic demand required for the summer moult. Seasonal weight changes in Lesser Snow Geese *Anser cærulescens cærulescens* show the lowest weights during winter and summer (Owen, 1980). Condition indices of Mallard *Anas platyrhynchos platyrhynchos* (Owen and Cook, 1977) also show this seasonal variation. Conversely the birds may be less susceptible to avian tuberculosis in the spring because this is a time when both sexes are in good condition in preparation for breeding.

Many viral, bacterial and parasitic epizootics in both captive and wild birds are shown to vary seasonally, for example: duck virus enteritis (Gough, 1984); avian influenza (Halvorson *et al*, 1985); avian cholera (*Pasteurella multocida*)(Price and Brand, 1984); *Campylobacter jejuni* (Hill and Grimes, 1984); necrotic enteritis (*Clostridium perfringens*)(Wobeser and Rainnie, 1987); botulism (*Clostridium botulinum*)(Shayegani *et al*, 1984) and acanthocephala (*Prolificollis botulus*) (Thompson, 1985). Whilst some of these have very sporadic outbreaks, usually in the spring and summer, deaths from tuberculosis can occur throughout the year reflecting the long incubation of the infection.

Incidence according to Sex and Season.

The high summer female mortality is probably due to birds becoming out of condition as a result of producing eggs and as most, and often all, of the incubating and brooding is carried out by the females. Weights of Lesser Snow Geese show a markedly lower female summer

weight than the males' (Owen, 1980). A similar pattern is also shown in Mallard condition indices (Owen and Cook, 1977). During this summer breeding period, female Mallard lose about 25% of their body weight (Owen and Black, 1990) and female Eiders *Somateria mollissima* may lose 50% of their prelaying body weight (Milne, 1976). In contrast to this, male Lesser Snow Geese experience their lowest weights in the winter (Owen, 1980) and male Mallard go through a progressive decline in body condition throughout the winter (Owen and Cook, 1977) which may increase their susceptibility to tuberculosis during this season.

The increase in female mortality in the latter five year period suggests that summertime stress has increased more than wintertime stress. There are two possible explanations for this; firstly there has been a 28% increase in the number of visitors to Slimbridge in the second five year period, particularly 1988 and 1989, compared to the first (from 0.91 million to 1.17 million per five years). The increase in summer visitors may prevent the females from leaving the nest as frequently to feed and drink. The long hot and dry summers of 1988 and 1989 would have increased the dehydration of incubating birds and exacerbated the problem. Secondly, there has been a slight shift in avicultural practices in the second five year period. To ensure maximum survival of offspring, eggs from many of the females are removed, hatched, and the young reared, in the duckery unit where there is less risk of predation and infection. In the latter years of this study, clutches were removed at a later stage of incubation. This may be more stressful in terms of anxiety to the bird, and as she has begun incubating, her body condition will be reduced from the longer period spent sitting on eggs.

Incidence according to Climate of Country of Origin.

Although few of the individuals held at Slimbridge are wild caught, they originate from all over the world, and so are adapted to a specific climate. This means that many of the birds must acclimatise to unusual conditions. Those birds evolved for life in either hot or cold climates may be stressed by the conditions experienced in Great Britain. As a consequence, birds from these hot or cold climatic zones appear to have a greater susceptibility to the disease. Beer made a similar finding although he found that those from cold climates had a similar incidence to those of temperate climates. This increase in incidence in the birds from cold climates (from 22% to 34%) may be due to stress associated with the unusually warm years experienced in the 1980's. Similarly the high mortality in birds from hot climates could be explained by the stress associated with cold weather.

Beer (1964) reported high mortalities in birds from tropical climes after the severe winters of 1961-62 and 1962-63, whereas the Anserini were hardly affected. In the studies by Hillgarth and Kear (1981; 1982a) mortality rates in the 'primitive' perching ducks (which originate mostly from hot climates) and the whistling ducks, were highest in the winter and spring. In contrast, the perching ducks from temperature climates and northern geese (Hillgarth and Kear, 1983) had lower mortality in the winter. This suggests that cold weather is the most critical stress factor in many of the birds from hot climates.

The susceptibility of many of the tropical species to cold is recognised and it is avicultural practice to provide extra heating for valuable birds such as Orinoco Geese *Neochen jubatus* and some of the whistling ducks in the winter. Cain (1973) reported that Red-billed Whistling Ducks *Dendrocygna autumnalis* were unable to tolerate a

temperature of -20°C for more than 72 hours, after which they died. The same author then suggested that temperature was the limiting factor in their distribution. Conversely, Beer (1964) found that overall mortalities in birds from hotter climates were lower than other birds in the summer.

Some groups of birds like the Oxyurini are less stressed by cold weather due to a substantial subcutaneous layer of body fat, an adaptation for prolonged spells on the water, a point which may partially explain this particular group's low incidence of avian tuberculosis.

Incidence according to Food and Feeding Habit.

Actual foodstuffs eaten did not appear to affect the incidence of avian tuberculosis. However, how the food was taken did affect the incidence. The usual pathology of avian tuberculosis suggests an oral route of infection and this could explain why feeding habit may be an important factor if the risk of infection is related to the bacterial load encountered by the birds. This seems to be the case if we assume that infection and mortality have a constant relationship.

The low incidence in the grazers may be due to the ability of these birds to selectively avoid contaminated vegetation, i.e. that vegetation which is covered in potentially contaminated faeces. Mycobacteria are generally found at air/water interfaces and grazers feed mainly on dry foodstuffs, so effectively reducing their risk of infection. In addition to this, much of their food is exposed to the sterilising effect of ultra-violet radiation. Beer also found the lowest incidence in the grazers and he suggests that if inhalation of contaminated dust were important in the transmission of the disease,

this group would be expected to have a relatively high incidence, which is not the case. Transmission by inhalation should also lead to typical pulmonary pathology, with frequent lung lesions. Although this is occasionally true, (in some dabbling ducks, see later) intestinal, liver and spleen lesions are far more frequent.

Some of the species that feed by grazing have been shown to have a high incidence, for example the Barnacle Geese. These birds are short-billed grazers and feed on shorter vegetation, cropping closer to the ground and possibly taking in a greater quantity of earth, and therefore mycobacteria, perhaps increasing their risk of infection.

The high incidence found in the dabblers may be explained by the birds being at an increased risk of infection, as the shallow water in which they sift out food is frequently defaecated in, and may potentially contain high numbers of *M.avium*. As "ducks of the microbial world" (Grange, 1987) mycobacteria are to be found at air/water interfaces and dabbling must be a most efficient way of encountering both pathogenic and saprophytic bacilli.

Dabbling may create aerosols which under the right conditions, lead to pulmonary infection. *M.avium* is certainly capable of inclusion in aerosols (George and Falkingham, 1989) and in this form can cause infections in man (Wenst *et al*, 1980). Stearns *et al* (1987) showed that inhaled aerosols in ducks could be found in the atria, infundibula and occasionally the air capillaries. The infrequency of cases of primary pulmonary tuberculosis is an indication of the lesser importance of inhalation of *M.avium* as a route of transmission of infection in the epizootiology of the disease.

The seaduck divers feed almost exclusively on animal foodstuffs, whereas the freshwater divers also take vegetable material. As has been seen, the actual content of the food does not appear to affect incidence, therefore the susceptibility of the seaduck divers is almost certainly not related to their feeding habit. Instead, the seaducks appear to be far more genetically susceptible to the disease.

The low incidence found in the flamingoes may be related to their food and feeding habits. They feed by filtering a special diet from troughs. These troughs are cleaned daily which may help reduce the risk of infection. Secondly, the high quality enriched food may even help to reduce susceptibility (Ratcliffe, 1946).

Incidence within the Grounds.

From table 3.9 it can be seen that by far the highest incidence was found within the Tropical House. This is an isolated unit served by its own water supply with no continuous movement of birds in or out. The temperature is regulated ensuring no fluctuations, so although it contains birds from tropical climates, cold stress is eliminated. The stocking density is also markedly lower than the rest of the grounds. However, this warm moist environment, with no sterilising ultra-violet radiation, must provide ideal conditions for the avian tubercle bacilli.

Incidence within the Big Pen was surprisingly high. Although there are very many birds held within this pen, it is very large and open i.e. exposed to high levels of ultra-violet radiation. There is a fairly high proportion of Anserini within this pen and the water supply has only travelled through one other pen (Rushy Pen) or directly from the canal. However, the flock of about 200 feral

Barnacle Geese use this pen extensively and as already discussed, this group had a high incidence of the disease. The water flow from the Rushy Pen is probably contaminated as this pen also had a high incidence of the disease.

In Beer's previous study, he found that disease was most prevalent in the Rushy Pen, infection then spread to the Big Pen and on to the European Pen. In the mid 1970's the Rushy Pen was transformed from a pen which visitors could walk through, with many collection birds, to one with no visitors and more provision for wild birds. Indeed, with many thousands of birds flying in in the winter, the Rushy Pen has by far the greatest density of birds anywhere in the grounds at this time. The level of incidence in this pen must indicate that these wild birds are at great risk of infection. Although this pen was dug out fairly comprehensively several years ago, the soil removed was used to construct banks within the pen. Whether or not this will reduce incidence of the disease by removing some of the source of infection will be seen within the next few years.

An equally high incidence was found in the African and Australian Pens. These pens receive water mainly from that which has travelled through the whole grounds i.e. it is at the end of the water system. There is obviously a greater risk of the bacilli being carried to these pens. Another factor to be taken into consideration is that birds within these pens are from hot climates; a group found to be susceptible.

There was a particularly high incidence in some of the individual pens in the central area of the grounds, for example the Asian Side Pens, (56.3%, 9/16). These pens have housed White-winged Wood Ducks which have died of avian tuberculosis and must now be heavily

contaminated. Both sets of side pens in this central area, had high incidences, with the American and Tundra side pens having incidences higher than their respective main pens. This may be due to the side pens tending to be both more crowded and shaded.

The Top Hut group of pens are designated for the rearing of young birds, and maintaining them until they are old enough to be put out in the grounds. With such a low age group this should mean a low incidence of avian tuberculosis. However, this is not the case and the high incidence is mainly due to moribund birds being brought out of the grounds to these pens. Such cases reduce incidence within the grounds but introduce the disease to pens that should be kept clean. These pens are now almost certainly contaminated, and this emphasises the need for a separate sick bay area.

The low incidence in the Loop Pens may be due to the high prevalence of Anserini in these areas. When considered separately, the side pens had a higher incidence (32.1%, 18/56), again possibly due to greater stocking densities and shading.

All the flamingo houses had a low incidence, these birds appear to be relatively resistant to infection and cases have tended to be in the ducks and geese from hot climates, sharing these enclosures.

To summarize, Beer found a trend in the spread of disease in the 1960's. By the 1980's the disease had spread throughout the grounds with no places free from infection. Beer found that the side pens tended to have have lower incidences, but he suggested that they merely lagged behind the main pens. This was certainly the case and they now exceed the main pens in many cases.

A number of pens have been renovated during the 1980's. This involved draining the water and mechanically pumping out the mud, which was then sprayed onto the back of the pens. Ideally this mud should have been removed and replaced with new topsoil. Whether this renovation will affect the incidence is too difficult to tell at this stage, but should become clear within the next few years. A point to note however, is that the same potentially infected birds have been put back in the newly renovated pens.

Limitations of the Study.

Although a high proportion of collection birds dying within the grounds are found and PM's are carried out, it should be realised that many bodies will go undiscovered. This is less likely in the larger, less gregarious birds, but may be true for the smaller birds that spend a great deal of time in groups or out of sight of the wardens. The discovery of a body also depends upon the pen which the bird is in, some pens are deliberately left very open whilst others are constructed to provide plenty of cover. Birds infected with a debilitating disease like avian tuberculosis will tend to skulk in available undergrowth.

Other limitations of this study are that many factors are not taken into consideration, for example, the role of stress in susceptibility to infection. Cold weather and seasonal body condition have already been mentioned, but there are many other potential stress factors. Although the majority of the birds in the collection are captive bred, some individuals are wild caught. There must be stress associated with the capture, transport and acclimatising to conditions of captivity.

Some of the collection birds are full-winged, but most are pinioned. In those strongly migratory species, or those that would naturally take to the air when in danger, being pinioned must present problems. Other birds, such as the Oxyurini, would use their ability to fly far less and might therefore, be less stressed.

Being kept in unnatural population densities and the presence of visitors everyday must also affect the birds. From a management point of view, attention must be paid to the ecology of the birds; whether or not the birds are social, whether males are usually found with females and so on. Gross *et al* (1989) showed that the nature and number of tuberculous lesions in chickens was affected by, not only their genetic make-up, but also by environmental factors in the form of different levels of 'social stress' they experienced. Mazanowski (1987) discusses the effect of the provision of an improved environment and diet on the prevention of disease in wildfowl.

Another factor to potentially skew the incidence of avian tuberculosis is sudden outbreaks of other unusual infections/causes of mortality. Such an example of this latter factor was the loss of birds in 1988 as a result of septicaemia following routine anti-duck virus enteritis vaccination. Out of the ten birds that died, nine were in the Anatini, and thus may appear to lower the incidence of avian tuberculosis. Cases of a fox getting into the grounds and killing many birds of the same species (18 Nenes in one case) are also present in the PM records. On the other hand, in 1985 a whole blood agglutination test for the disease was used with some degree of success, identifying many tuberculous Barnacle Geese and White-winged Wood Ducks, these birds were destroyed and in effect may give an unusually high incidence for that year. (The test was subsequently abandoned due to

high numbers of false positives).

A number of possible control measures were proposed by Beer, some of which were implemented. One such measure was the move away from the use of broody hens for incubation, as these had been found to be infected. However, Schaefer *et al* (1973) found a separate enzootic of type 2 *M.avium* in these birds, unlike the type 1 in the Anatidae.

An extensive environmental bacteriological survey would be useful especially when considering the distribution of the disease in the grounds. Schaefer *et al* (1973) isolated *M.avium* from 5% of all the soil, mud and water samples analysed, although no indication of their origin in the grounds is given. A bacteriological study of *M.avium* isolated from birds at PM since 1986 is discussed in Chapter ten.

No mention of the disease in wild birds associated with the collection has been made. This is mainly due to the lack of available PM data, as most bodies remain undiscovered or are predated before PM can be carried out. However, Martin Brown has found avian tuberculosis infections in 40% of 'local' Mallard (personal communication) i.e. a figure as high as that found in the captive birds. There have been cases of Bewick's Swans *Cygnus columbianus bewickii* too weakened by avian tuberculosis infection to make the return migration to Siberia in the spring and dying of the disease in the grounds at Slimbridge. In his previous study, Beer reported the disease in Black-headed Gulls *Larus ridibundus* that visit the grounds regularly. Like the wild Anatidae, such birds have the potential for spreading the disease over great distances.

This study has shown that those birds infrequently challenged with *M.avium* in their natural habitat may be more at risk under the

conditions of captivity at Slimbridge both in terms of exposure to mycobacterial infection and susceptibility to disease. Despite being bred at Slimbridge, many exotic species would have the evolutionary experience of their forebears reflected in their genes. In contrast, species from temperate zones and of freshwater habitats may have been selected for resistance to *M.avium* disease, and continue to exhibit this at Slimbridge. Thus, some of the wildfowl of the arctic regions and of the sea, have the increased incidence that might be expected of them.

The groups of wildfowl most at risk of dying of avian tuberculosis, and the factors which increase this risk, have been pinpointed. Hopefully it gives some indication of how and where control measures should be implemented to improve matters in the future.

CHAPTER FOUR.

THE PREVENTION AND CONTROL OF AVIAN TUBERCULOSIS.

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Prevention and Control of Avian Tuberculosis in Captive Wild Birds.

The poultry industry has lowered the incidence of avian tuberculosis, by firstly identifying foci of infection by means of a tuberculin test. This test identifies birds before the disease reaches a severe or chronic state, however this is only of use in birds which possess a wattle. Secondly, the industry attempted to remove infection from the environment by moving birds to new clean premises and maintaining high standards of hygiene. Whilst both measures have helped, the major factor responsible for the lowering of incidence is the reduction of the age of flocks.

Within captive collections of wild birds the disease is notoriously difficult to eradicate due to the tenacity of *M.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. Losses from the disease have increased in economic importance as many birds near extinction and individuals become far more valuable in both monetary and conservation terms.

As yet there is no reliable diagnostic test for those birds that do not possess a wattle, and so identification of tuberculous birds relies heavily on noticing signs in the individual, signs which generally indicate already advanced infection. The presence of acid

fast bacilli in the faeces is fairly indicative of infection but the absence of the organisms certainly does not indicate a non tuberculous bird, as the infection may not be intestinal, or merely that the bird is not shedding bacilli into the gut at that time.

Treatment of avian tuberculosis is not considered feasable, as the drugs involved are neither very effective nor cheap and the administration of long term chemotherapy is impractical (Beer, 1988). It is for these reasons that it is so important to ensure that new additions to a collection are free from the disease.

Generally the disease must be prevented or controlled by high standards of hygiene. This ideally involves a move to clean ground and the old infected ground should be limed and left free of birds for at least one year. The carcasses and droppings of tuberculous birds provide a ready source of infection and must be incinerated. Removal and incineration of all build up of droppings, grass cuttings and general organic refuse is advisable. Attention should also be paid to areas of possible contamination, for example footwear of workers, feed barrows, sacks and crates.

There has been a report of a lowering of incidence following an improvement of diet at Philadelphia Zoo with the addition of Vitamins A, D, B complexes and iodized salts, however this apparently lowered infection rates in most groups of birds but not the Anseriformes (Ratcliffe, 1946).

Prevention and Control at The Slimbridge Wildfowl and Wetlands Centre.

In the past at Slimbridge, there have been found to be two separate avian tuberculosis epizootics, one in the Anatidae and the other in the broody hens used for incubation (Schaefer *et al*, 1973). The former generally was infected with serotype 1, and the latter was infected with serotype 2. At this time the level of infection in the hens was quite high, but this has been controlled by the move to mechanical incubators and rearing of young beneath heat lamps in a duckery. In this system the standard of hygiene in the incubators and the duckery can be maintained. However, the infection within the Anatidae has become more prevalent and the problem of control is of greatest importance.

As has been mentioned, leaving ground free from birds is very efficient at controlling levels of infection, however at Slimbridge this is a fairly impractical measure. The following measures could reduce levels of *M.avium* in the birds' environment and so reduce the birds' risk of infection:-

(i) A sick bay area must be introduced to prevent the housing of moribund tuberculous birds in pens in the Gosling Field which is primarily designated for young birds.

(ii) When pens are being renovated, topsoil should be removed and replaced with clean soil. Posokhin (1954) suggested that soil should be lifted to a depth of 20 cm, the exposed surface should be quicklimed and covered again with 20 cm of clean soil.

(iii) Old nesting materials and nesting boxes should also be cleaned out annually.

(iv) The movements of birds, whether within the grounds or between

centres, from heavily infected pens; or very susceptible species, should be limited.

(v) Ultra-violet radiation is an effective sterilizing agent, therefore shady pens probably have higher levels of *M.avium*. Hence, it may be wise to cut back vegetation to some extent.

(vi) The flow of water from one pen to another constitutes an infection risk, with bacilli being transported throughout the grounds. If it were possible, particularly susceptible birds such as the White-winged Wood Ducks, should be kept on their own clean water supply.

(vii) Diet could be improved, and if this were provided in food troughs (with the availability of sufficient grit) it would reduce the time the birds spend feeding from an infected environment. However, this measure like some of the others is fairly impractical when feeding such an enormous numbers of birds, and would also lead to the less aggressive species or individuals being pushed out.

(viii) An overall reduction in the stocking density would be desirable.

Why Vaccination as a Possible Control Measure.

To summarise, although high standards of hygiene should be maintained it is usually impossible to keep moving birds to clean ground and the elimination of *M.avium* from the environment in this way is a practical impossibility. Even if there were a reliable diagnostic test some birds may be far too rare and valuable to cull. Culling of birds after their second or third year also presents ethical problems.

It is for these reasons that a vaccine could present a possible method of control. If such a vaccine could be developed it could establish immune stocks of captive birds and so lower the level of

infection, reducing the spread of the disease to the wild populations. A vaccine could improve the health and longevity of all species of the Anatidae, and in doing so increase breeding potential, which is of particular importance in species like the White-winged Wood Duck. This would then reduce the need for wild caught specimens.

All the birds at Slimbridge must be regularly challenged with potential infection, yet many species remain fairly resistant, indicating the existence of some protective immunity, as has been mentioned in the previous chapter. It is therefore proposed that the number of birds developing the disease could be significantly reduced by artificially boosting immunity by the use of a vaccine.

Previous Vaccine Attempts.

Previous vaccine attempts have mainly involved poultry. While some of the trials have shown increased resistance, most have been short term desultory attempts.

BCG has been used in a number of trials using chickens, with varying degrees of success. Harnack (1928) reported a degree of protection in chickens which were given BCG by a variety of routes. Guerden (1934) used BCG and then exposed birds to an infective environment for nine months. However, after this time 34.5% of vaccinates and 26.5% of the non vaccinates died of the disease. The same author did suggest later that vaccination delayed the onset of the disease.

BCG has also been used in an attempt to protect pigs from *M.avium* infection (Jorgensen, 1978). Whilst it appeared to be efficacious, it only reduced the number of viable organisms in the tissues and therefore was still of no clinical use. This vaccine has also been

used in rabbits, guinea pigs and mice (Engbaek and Jespersen, 1966). Whilst mortality in both the guinea pigs and mice was reduced considerably, all the rabbits developed the disease, although the onset was delayed following vaccination.

In a study carried out by Guindi (1960) both BCG and heat-killed avian tubercle bacilli were given either subcutaneously or intravenously to chickens. The intravenous vaccines gave the best results, the subcutaneous BCG afforded only slight protection whilst none at all was afforded by the heat killed organisms.

Other vaccines used in chickens include a heat killed smooth strain of *M.avium* (Petroff *et al*, 1932). The birds were then challenged orally. 56% of vaccinated birds did not develop the disease, but neither did 20% of the unvaccinated. They also noted that pathologically, the vaccinated birds' lesions were less progressive and more healing.

Rossi (1974) used a variety of inactivated absorbed vaccines of serotypes of *M.avium* and *M.intracellulare* in chickens. These included serotypes VI and VII of the *M.avium brunense* subspecies and Darden (*M.intracellulare* serotype 19), as well as three live vaccines of the same serotypes. The vaccines were given either intramuscularly or orally. The birds were then challenged and assessed on pathological changes. The oral dose (25 mg) of living *M.avium* serotype VI gave the best protection. In pullets the intramuscular inactivated and living *M.avium* serotype VII and Darden gave good results, as did the inactivated and living serotype VI in cockerels.

Vizy *et al* (1964) tried a killed decapsulated Gallinaceus type of *M.avium* in different species of poultry. The vaccine was administered

subcutaneously and intraperitoneally. Monitoring the vaccine showed that only the vaccinated birds gave positive slide agglutination tests. After both natural and artificial challenge they found that the vaccinated group had been protected whilst control animals died of the infection.

Feldman (1938) quotes the work of Friedmann who used a strain of *M.chelonei*, isolated from a turtle, both as a immunizing vaccine and an immunotherapeutic agent in chickens. Although he reported some degree of resistance, when a large scale trial was carried out in the 1920's it was shown to have no effect.

Chepik (1976) reports some degree of success using *M.smegmatis* in chickens although the sample sizes used were rather small.

From the above account it can be seen that vaccination in chickens is a possibility, and it is therefore hoped that the situation will be similar in wildfowl. Vaccination of wildfowl has been carried out previously against a variety of infections with some degree of success, for example duck virus enteritis (Lin *et al*, 1984a; 1984b; Weingarten, 1989), infectious serositis (caused by *Pasteurella anatipestifer*)(Layton and Sandhu, 1984; Floren *et al*, 1988; Timms and Marshall, 1989) and duck virus hepatitis (Davis, 1987; Luff and Hopkins, 1986). To appreciate how vaccination of such birds could work we must first consider the wildfowl immune system.

The Wildfowl Immune System.

There is a tendency to assume that all avian immunology is represented by that of the chicken, which has been much studied. This is mainly due to the Bursa of Fabricius providing such an ideal model for studying early B cell development. It was in bursectomised chickens that the first indication of a separate population of lymphocytes involved in human immunity was discovered (Glick *et al*, 1956). However, duck or wildfowl immunology would appear to be somewhat different from that of chickens, resembling instead that of reptiles and even amphibians. Indeed the Anatidae are a particularly ancient evolutionary group evolving some 40 million years prior to the Galliformes. Any research into wildfowl immunology has mainly concentrated on structure of duck immunoglobulins, in an attempt to gain information concerning evolution of such molecules in the animal kingdom as a whole.

The duck most widely studied is the domestic duck which was derived from the Mallard *Anas platyrhynchos*. It is assumed that this is a true reflection of the immune system within the Anatidae.

Wildfowl Lymphoid Tissues.

The Thymus.

The thymus consists of several flattened, irregularly shaped lobes lying along each side of the neck, close to the jugular vein and is concerned with lymphocyte formation. As well as lymphoid tissue, there are also reticular and epitheliod cells. The thymus is at its relatively largest during the first few weeks of life, after which it undergoes a fairly rapid involution with the approach of sexual

maturity. The larger wildfowl e.g. Snow Goose *Anser cærulescens*, do not reach sexual maturity until they are two years old and maintain a large thymus up until then. In some birds, including the Mallard, the thymus re-enlarges and recovers the original juvenile status for some weeks following the first and even later sexual cycles (Höhn, 1947; 1956).

In mammals thymic atrophy may occur in response to stress due to hypersecretion of adrenal glucocorticoids. In birds such atrophy may occur as a result of stress, such as infectious diseases.

The Cloacal Bursa (Bursa of Fabricius).

The cloacal bursa is unique to birds and consists predominantly of lymphoid tissues. The structural and morphological features of the vascular component are described in detail by Scala *et al* (1989). The bursa, like the thymus, undergoes a rapid involution as sexual maturity approaches, again this process may be slower in geese where sexual maturity is not reached until two years old. Once involution has occurred there is no re-enlargement. This can be used at PM, or through cloacal probing of the larger wildfowl, to assess whether the bird is immature i.e. prior to first sexual cycle, or has reached sexual maturity. Once involution has occurred the remnant of the bursa persists as a tiny saccule.

The Spleen.

The spleen is a rounded organ similar in histology to that of mammals and is situated to the right of the glandular part of the stomach. The white and red pulp are less distinct than in mammals. The former is made up of typical lymphatic tissue and is concerned with lymphopoiesis. The latter consists of venous sinuses separated by

cords of cells including lymphocytes, macrophages and the elements of the circulating blood, and is concerned with phagocytosis of old erythrocytes. Antibody production occurs in both the red and white pulp.

Unlike mammals, the splenic circulation is thought to be 'open' i.e. with no direct vascular connections between veins and arteries. It also appears not to act as a significant reservoir of blood.

The importance of protein-linked oligosaccharides in many biological systems including the immune system, has now been recognised (Reading, 1984). Changes in their structure may be indicative of a number of features including infection. It was thought that oligosaccharides with terminal N-acetylglucosamine (GlcNAc) were rarely found in animal tissues. However, Chechik and Fernandez (1989) have described terminal GlcNAc cluster antigen patterns within the spleen, intestine and colon of both ducks and geese.

Duck Immunoglobulins.

Ducks possess three major immunoglobulins which have been characterised, one higher weight immunoglobulin similar to mammalian IgM, and two lower weight immunoglobulins, IgG's, which form the bulk of the duck's humoral responses to a variety of antigens. These antibodies bind well to antigens and are capable of efficient virus-neutralisation (Toth and Norcross, 1981a), however the secondary activities are often limited. Ducks rarely produce antibodies capable of complement fixation or precipitating antigen. Their agglutinating antibody responses are also weak, although the work of Sari and Thain (1983) showed that this response could be enhanced by using an antiglobulin reagent when agglutination testing ducks for antibodies

to *Salmonella spp.* This limited secondary activity must be due to structural differences in the duck immunoglobulins.

Duck IgG's.

Chickens possess a low molecular weight antibody characteristic of mammalian IgG. Similarities include:-

(a) a sedimentation coefficient of about 7S

(b) low ionic strength buffers allowing elution from DEAE-cellulose

(c) being synthesised after the synthesis of high molecular weight antibodies.

One major difference, however, is the increased molecular weight of the heavy chain, about 10 kDa greater than the mammalian γ-chain (Benedict and Yamaga, 1976).

Ducks also possess a very similar 7.8S immunoglobulin. Its characteristics include:-

(a) a molecular weight of 178 kDa

(b) the heavy chain has a molecular weight of 62-66 kDa

(c) the light chain has a molecular weight of 23 kDa

(d) the heavy chain differs from that of mammalian IgG in a number of ways (i) the amino acid composition differs significantly, (ii) the total carbohydrate content is higher in ducks, ranging between 5-6.4% (Zimmerman *et al*, 1971; Acton *et al*, 1972), (iii) there are a greater number of cysteines than in human γ-chains.

(e) there is a greater anodal migration in the papain produced Fc than Fab.

(f) the most striking evidence for homology is from the results of immunodiffusion analysis, in which rabbit anti-duck 7.8S immunoglobulin recognises antigenic determinants on chicken 7S

immunoglobulin (Zimmerman *et al*, 1971).

(g) structurally, like the chicken immunoglobulin, there are only three N-terminal amino acid residues on the light chain, these are Ala-Leu-Thr (Hood *et al*, 1970).

Biologically, this 7.8S antibody is capable of sensitising duck skin for passive cutaneous anaphylaxis reactions and fixing duck complement (Grey, 1967).

In addition to this 7.8S immunoglobulin, ducks also possess an antigenically similar 5.7S IgG molecule. Chickens lack this smaller immunoglobulin but a structurally similar immunoglobulin has been found in the sera of some turtles, the lungfish and groupers (Kubo *et al*, 1973).

This smaller immunoglobulin has a molecular weight of 118 kDa and is made up of two heavy chains (molecular weight 35 kDa) and two light chains (molecular weight 23 kDa)(Zimmerman *et al*, 1971). It differs from the 7.8S molecule by absence of a portion of the Fc terminal of the heavy chain (Zimmerman *et al*, 1971). The fact that only the larger 7.8S molecule is transmitted to the duckling *in ovo* suggests that recognition of this region is required during this process (Liu and Higgins, 1990).

The 5.7S immunoglobulin is neither a precursor nor a breakdown molecule of the larger IgG and is independently produced (Grey, 1967). In immunodiffusion analysis the antigenic determinants on chicken 7S immunoglobulins recognised by rabbit anti-duck 7.8S immunoglobulin were not present on the 5.7S molecule (Zimmerman *et al*, 1971), and some monoclonal antibodies raised to the 5.7S immunoglobulin react specifically with it and not the larger 7.8S molecule.

The amino acid composition of the heavy chain is like that of the chicken 7S heavy chain, but markedly different from that of the human γ-chain (Benedict and Yamaga, 1976). Like the duck 7.8S immunoglobulin, there are a greater number of cysteines per chain than found in mammalian IgG chains. The 5.7S molecule has a low carbohydrate content of 0.6%, which is associated with the Fd fragment and light chain. It can also form 'half molecules' with a molecular weight of 58 kDa as a result of mild reduction.

Biologically, the haemagglutinating capacity can be destroyed, and antigen binding capacity decreased considerably, following reduction with 0.1 M mercaptoethanol and alkylation. Such reduction has little effect on the 7.8S immunoglobulin (Grey, 1967). This smaller immunoglobulin also appears incapable of fixing duck complement and eliciting a passive cutaneous anaphylaxis reaction. This is almost certainly due to the lack of a portion of the Fc region. Grey (1967) also demonstrated the ability of the 7.8S IgG to agglutinate passively sensitised erythrocytes far more than the 5.7S IgG.

Toth and Norcross (1981c) showed that ducks actively immunised with chicken or sheep red blood cells produced very low levels of haemagglutinins in direct-haemagglutination tests. They suggest from this, and other work confirms (Toth and Norcross 1981a, 1981b), that duck immunoglobulins are somewhat deficient in those reactions that require functional bivalency. The structural studies of Zimmerman *et al* (1971) of duck IgG's show the γ-heavy chains are held together by a higher number of disulphide bonds than found in mammalian γ-heavy chains. This renders the duck IgG's less flexible in the Fab portions around the hinge region, a flexibility which is important in both precipitation and agglutination reactions.

Higgins (1989) has shown that ducks can produce considerable amounts of precipitating 5.7S IgG to human IgG. These antibodies were found to contain two populations of heavy chains with molecular weights of 37 kDa and 41 kDa, unlike the non-precipitating IgG which had heavy chains of 37 kDa. It is, therefore suggested that there is the possibility of structurally and functionally distinct subclasses of the 5.7S IgG.

The immune response to bovine serum albumin produces both 7.8S and 5.7S IgG's, and during early responses there are similar amounts of each. However, in hyperimmunised White Pekin ducks, three weeks post primary immunisation, $^{3}/_{4}$ of the total antibody was 5.7S IgG (Grey, 1967).

Duck IgM.

Although the bulk of duck humoral responses is made up of the lower weight immunoglobulins they also produce a high molecular weight immunoglobulin. This IgM molecule has a sedimentation coefficient of 17S and is produced in response to soluble antigens. It is homologous to that found in chickens, indeed in the vertebrates as a whole. Although functionally these molecules are similar there are some differences in structure, for example the number of monomers, comprising two heavy chains and two light chains, may vary. Ng and Higgins (1986) have proposed that duck IgM may be structurally like that found in other avian species but instead of a pentameric structure it may be tetrameric, with a molecular weight of 800 kDa, with heavy chains of 86 kDa. The total carbohydrate content of the molecule is 5.3%, similar to that of chickens.

Bile Immunoglobulin.

In the search for a duck secretory immunoglobulin, Ng and Higgins (1986) demonstrated a single class of IgM-like immunoglobulin in White Pekin duck bile. This was found to be antigenically similar to serum IgM but carried additional determinants not recognised by rabbit anti-duck serum IgM. Bile immunoglobulin had a molecular weight of 890 kDa with heavy chains of 75 kDa. They found that this was produced independently of serum IgM and proposed a nomenclature of "IgX". Hadge and Ambrosius (1988) used radioimmunoassays to elucidate the different antigenic properties between this secretory immunoglobulin and galliform birds' biliary immunoglobulins. Neither of these molecules showed cross-reactive properties with human or porcine IgA.

Duck Lymphocytes.

In order to investigate how ducks respond to infectious agents, something must be known of their lymphocytes and monocytes. Higgins and Chung (1986) purified duck lymphocytes, which in itself is difficult due to the similarity in size between lymphocytes and thrombocytes. Once purified, they were able to study their surface markers. E rosettes were not formed, similarly EA rosettes were not formed with sheep erythrocytes sensitised with rabbit antibodies. A high proportion expressed surface immunoglobulin but no receptors for *Helix pomatia* lectin were found. Some cells with receptors for peanut agglutinin were found, but these, and those with surface immunoglobulin, were distributed fairly randomly throughout the lymphoid system, i.e. without the expected distribution of T and B cells. Two bands of cells were produced in Percoll gradients. These showed no difference in surface immunoglobulin or peanut agglutinin receptors but only the upper band responded to a variety of mitogens.

Lymphocytes could also be divided into adherent and non-adherent cells when passed through a nylon wool column. The adherent population responded to mitogens but again there were no differences in surface immunoglobulin and peanut agglutinin receptors.

Once it was possible to obtain high yields of lymphocytes, then Higgins and Teoh (1988) optimised the culture conditions required for responses to phytohaemagglutinin. These conditions included a high cell concentration (8 x 10^5 cells/well of a 96 well micro-titre tray) and culture in 10% pooled adult duck serum at 41.6°C (duck body temperature). Culture of optimum cell concentrations in 20% foetal calf serum, and 5, 10, and 20% chicken serum also supported lymphocyte transformation. These workers and personal observations have noted the obvious clumping of stimulated cells around macrophages when viewed under phase contrast microscopy. It would appear that the macrophage plays an essential role in antigen/mitogen presentation, indeed the work of Vainio and Ratcliffe (1984) showed that chicken lymphocytes' response to pokeweed mitogen depends upon the presence of macrophages.

Higgins and Teoh (1988) and personal work have also noted that to increase the longevity of duck mononuclear cells there must be stimulant present. The fact that duck cells respond to phytohaemagglutinin at least shows that they express receptors (sugars within glycoproteins and glycolipids on the cell surface) to this mitogen, although this may be of little consequence to the immunological responses required during infection. In an attempt to identify and characterise sub-populations of duck lymphocytes Higgins (1990a) demonstrated three different patterns of transformation response to various common mitogens possibly indicating different functional sub-populations, (if not T and B cells), with different

surface receptors and with different response kinetics. The responses of duck cells to mycobacterial antigens will be reported within this thesis.

Communication between cells during immune responses is due to cytokines. Whether or not ducks produce such products is open to question, but has received much attention recently with the work of Dr. D.A. Higgins (Hong Kong University) and Prof. H.-D. Flad (Forschungsinstitut Borstel) and the author, at The First International Duck Cytokine Workshop (Hong Kong University). The investigations tried to determine whether ducks produced, or responded to, cytokines similar to those produced in mammals (Higgins *et al*, 1991). It was shown that mitogen stimulated duck lymphocyte cultures did not respond to either recombinant or Jurkat cell line human IL-2. Also, the addition of supernatants from other stimulated (by either mitogens or mixed lymphocyte reactions) cultures could not increase, and often markedly decreased, ^{3}H-thymidine uptake of lymphocyte and macrophage cultures. These supernatants were assayed for mammalian IL-1, IL-2, IL-6 and tumour necrosis factor (TNF), none of which were found. RNA preparations are currently being examined with probes for human IL-1, IL-2, IL-4, IL-6 and TNF. Although, so far most of the results have been negative it seems fairly unlikely that ducks do not produce cytokines, and it is possible that any cytokines produced are so different from those produced by mammals that there was no cross reactivity with those used in the assays. It is also possible that the assays were not appropriate and that any cytokines produced may be autocrinely used, bound either intracellularly, within the cell membrane or within the medium and therefore avoid detection. However, Nosik *et al* (1989) demonstrated interferon production after treating

duck peripheral blood lymphocytes and spleen cells with high levels of both viral and non-viral inducers.

CHAPTER FIVE.

THE DEVELOPMENT OF TECHNIQUES TO MONITOR THE EFFICACY OF THE STUDY VACCINES IN THE MALLARD.

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THE DEVELOPMENT OF TECHNIQUES TO MONITOR THE EFFICACY OF THE STUDY VACCINES IN THE MALLARD.

The first step in this project was to develop the techniques for the immunological tests to be used to assess vaccine efficacy. These tests are the lymphocyte transformation test LTT, skin test and the enzyme-linked immunosorbent assay, ELISA.

The recipes for the buffers and solutions are summarised in appendix 5.1.

1. LYMPHOCYTE TRANSFORMATION TEST.

This test is used to detect sensitisation to pathogens/vaccine by the stimulation of lymphocytes to increase protein synthesis and enlarge to become blast cells. Antigen driven lymphocyte transformation had not been carried out previously with duck or goose cells and consequently the test had to be developed and optimised. Details of how this was done and the precise experimental details of the optimisation of the test have been reported (Cromie *et al*, 1988; Cromie *et al*, 1989).

A major problem to overcome was maintaining living lymphocytes over the distance between the site of the experimental birds in Slimbridge, and the laboratory in London. This resulted in a five hour delay prior to cell separation. A number of different systems were tried in an attempt to overcome the problem and a method has been developed producing satisfactory results.

Materials and Methods.

Blood Collection and Transport.

Blood was collected from the medial metatarsal vein using a 5 ml syringe (Plastipak, 050025150) fitted with a 23 gauge needle (Monoject, 0.6 mm X 25 mm). This site is surrounded by supporting muscles and tendons that assist in haemostasis (Dein, 1982; Murdock and Lewis, 1964).

Transport of whole heparinised blood over the distance between Slimbridge and London resulted in lymphocyte death within 24 hours, therefore transport of whole blood in different media at different temperatures, and transport of purified lymphocytes, were tried.

Blood was taken into an equal volume of either 0.1 M phosphate buffered saline PBS pH 7.2 (Higgins and Chung, 1986), containing 10 U/ml of sodium heparin (Monoparin, Heparin Injection B.P., Weddel Pharmaceuticals Ltd.) or heparinised Roswell Park Memorial Institute 1640 medium (RPMI 1640 medium with 25 mM HEPES buffer with L-glutamine, Gibco Ltd.). Samples were then transported in siliconised Vacutainers (No additive, Silicon coated Vacutainer, Becton Dickinson).

Lymphocyte Separation.

2.5 mls of the blood/buffer mixture was layered over an equal volume of lymphocyte separation medium, specific gravity 1.077 (Flow Laboratories)(Higgins and Chung, 1986) in Vacutainers. These were centrifuged at 200 x *g* for 25 minutes (Higgins and Chung, 1986). Cells collected from both the lymphocyte separation medium and the interface were washed three times in RPMI.

Preparation of Antigens.

In addition to the mitogen phytohaemagglutinin, PHA (Sigma L-9132), the following species of mycobacteria were used for preparation of antigens: *M.avium*, *M.vaccae* and Glaxo BCG (Glaxo Laboratories Ltd., Greenford, Middlesex). Antigens were prepared from fresh cultures, which had been grown on Sauton's medium solidified with 1% agar. Bacilli were suspended in 0.15 M borate buffered saline pH 8.0, and treated for 15 minutes in an MSE 100 Watt ultrasonic disintegrator with an amplitude of 6-8 um (Stanford *et al* 1975a). The resultant mixture of residual whole organisms, broken cell walls and cytoplasm was then centrifuged at 3000 rpm for 20 mins. The supernatant was filtered serially through a 0.45 um and two 0.2 um membrane filters (Schleicher and Schuell). Protein concentration was measured spectrophotometrically by the method of Warburg and Christian (1941), based on absorption of ultra-violet light at 260 and 280 nm. These preparations were used in different final concentrations (1, 5, 10, 20, 50, 100 ug/ml).

Lymphocyte Transformation Test Technique.

20 ul of antigen in RPMI (or 20 ul of RPMI alone in control wells) was incubated at 41.6°C with 100 ul of 20% serum supplemented RPMI in 96 well flat bottomed microtitre trays (Microwell, Nunc)(Higgins and Teoh 1988). To this, 80 ul of cells suspended in RPMI at 2.5 x final concentration was added. All RPMI used contained streptomycin sulphate B.P. (Evans) at 100 ug/ml and sodium benzylpenicillin BP (Crystapen, Glaxo) at 100 U/ml. After culture for four days at 41.6°C (Higgins and Teoh, 1988) in a humid atmosphere with 5% carbon dioxide, to maintain pH, newly synthesised DNA in proliferating lymphocytes was radiolabelled for 17 hours with 0.6 uCi of ^{3}H-thymidine per well.

Cells were washed onto cell harvester paper (Skatron AS) with distilled water using a semi automatic cell harvester (Skatron AS). Discs were placed in 4 mls of scintillation fluid (Ecoscint A, National Diagnostics). Counts per minute (cpm) were measured using a liquid scintillation counter (1211 Rackbeta, LKB) for one minute per sample.

Stimulation indices (SI's) were calculated from the mean cpm of triplicate antigen stimulated cultures divided by the mean cpm of triplicate unstimulated control cultures (Timms, 1979).

Using this method the following experiments were carried out in an attempt to find the optimum conditions for both the transport and culture of the lymphocytes.

Blood samples were taken into equal volumes of either heparinised PBS or heparinised RPMI. Half of each sample had the lymphocytes separated at Slimbridge immediately after the blood was collected and the separated cells were transported to London in RPMI. The remaining half samples were transported to London prior to separation of the lymphocytes. In both cases the samples were transported on ice, at ambient temperature, or at 40°C. Lymphocytes were then cultured in 10% heat inactivated (for one hour at 56°C) Cherry Valley pooled duck sera CVPDS (from clinically healthy seven week old White Pekin ducklings *Anas platyrhynchos*, supplied by Cherry Valley Farms Ltd.) or 10% autologous sera at two cell concentrations, 4 and 8 x 10^5 cells/well.

Results.

The transport of purified lymphocytes at various temperatures suspended in RPMI resulted in cell death within 24-48 hours and reduced yields of lymphocytes and macrophages. There was also a higher percentage of contaminating cells (erythrocytes, thrombocytes and granulocytes) probably due to the imperfect speed control of the centrifuge used (Zinkl, 1986).

Lymphocytes purified in London from the blood transported on ice or at ambient temperature appeared unhealthy and died within 24 hours. Whole blood transported in heparinised RPMI at 40°C, gave the best transformation results (table 5.1). From the samples transported in this way, lymphocytes gave their highest SI's when cultured at 8×10^5 cells/well (table 5.2) and in autologous sera (table 5.3).

Discussion.

It can be seen that wildfowl lymphocytes transform in the presence of mycobacterial antigens, providing the transport and culture conditions are right. The culture conditions differ from those previously recommended for mitogen driven transformation (Higgins and Teoh, 1988). The most obvious critical observation is the use of autologous sera at high cell concentrations.

The probable explanation of RPMI increasing the longevity of the cells is that the sodium bicarbonate buffer (2 mg/ml) temporarily controls the carbon dioxide levels. Also, the slightly more acidic conditions in the RPMI would reduce loss of macrophages by adherence to the Vacutainer.

The effect of temperature on the longevity of lymphocytes was found to be considerable. Those transported on ice or at ambient temperature appeared dead after 24 hours with no thymidine uptake after two, three or four days' incubation. This would indicate dead or non metabolising cells rather than cooled cells simply being more sluggish in their responses. Washing solutions, stored at 4°C, are now prewarmed to avoid uneccessary temperature shock.

The superior results for the higher cell concentrations suggest that a closer proximity of cells improves survival and enables them to respond better to antigens. In addition, the close cell to cell contact probably allows a greater amount of antigen presentation. Higgins and Teoh (1988) suggested the use of U or V shaped culture wells to increase cell contact.

Higgins and Teoh (1988) also found that 20% foetal calf serum supported duck LTT to phytohaemagglutinin at lower cell concentrations, as did 5, 10 and 20% chicken sera. From the results of the cells cultured in CVPDS it can be seen that thymidine incorporation in antigen stimulated cells may be similar, but SI's tend to be lower due to higher values for unstimulated cells. Microscopic observations of lymphocyte morphology in control wells showed some degree of clumping and clustering of lymphocytes around macrophages suggesting a degree of antigen presentation. The CVPDS is heat inactivated to destroy complement activity, but this does not destroy circulating immunoglobulins and it is possible that these non-self molecules have alloantigenic properties so causing stimulation of the cells.

The lymphocytes cultured in autologous sera gave higher SI's than those cultured in CVPDS which is derived from White Pekin ducks *Anas platyrhynchos* and would therefore be considered allogeneic. Although these two breeds of duck share the same species name it is highly likely that they have genetically diverged since White Pekin ducks were domesticated from wild Mallard and appeared in Chinese literature as early as 1597 AD (Shaw, 1940). In evolutionary terms this is relatively recent. However the processes used in domestication are likely to induce genetic divergence in a relatively short time. As the physiology, anatomy and behaviour of the two breeds of duck are also distinct (Delacour, 1964; Sossinka, 1982), it is probable that Mallard lymphocytes recognise some aspects of the CVPDS as foreign.

In conclusion, wildfowl mononuclear cells are temperature sensitive and should be kept as near to the bird's body temperature as possible. Antigen driven lymphocyte transformation is better at higher cell concentrations; and is better in autologous sera than in pooled sera.

Table 5.1 : LTT responses of Mallard lymphocytes collected from blood travelling in RPMI or PBS prior to separation.

Travelled in:-	(i) RPMI			(ii) PBS		
Stock antigen Concentration ug/ml	Mean cpm (Mean SI)	SD	*n.*	Mean cpm (Mean SI)	SD	*n.*
PHA						
500	1186 (6.8)	174 (1.2)	3	704 (4.0)	378 (1.3)	3
100	1042 (5.8)	304 (0.9)	3	500 (3.1)	294 (1.8)	3
10	433 (2.5)	206 (1.2)	3	319 (1.6)	88 (0.6)	3
M.vaccae						
1000	450 (2.6)	261 (1.5)	3	281 (1.6)	149 (0.5)	3
100	448 (2.6)	298 (1.7)	3	230 (1.4)	118 (0.6)	3
10	193 (1.1)	39 (0.2)	4	226 (1.4)	132 (0.8)	3
M.avium						
1000	394 (2.2)	95 (0.6)	4	228 (1.4)	124 (0.7)	3
100	254 (1.4)	80 (0.4)	4	183 (1.1)	85 (0.3)	3
10	285 (1.6)	69 (0.5)	4	185 (1.1)	82 (0.2)	3
Control unstimulated wells	180	21	4	171	76	3

Transport and culture conditions include: blood travelled at 40°C, the cells were cultured in 10% CVPDS at $8x10^5$ cells/well for 4 days.

SI, stimulation index; SD, standard deviation; *n*, number of samples.

Footnote: mean cpm of control unstimulated wells shown represent cpm of the maximum number of birds in each group. Mean SI's are calculated from individual SI's not mean cpm divided by mean control unstimulated cpm.

Table 5.2 : LTT responses of Mallard lymphocytes at different cell concentrations.

Stock Antigen Concentration ug/ml	8 x 10^5 cells/well Mean cpm (Mean SI)	SD	n.	4 x 10^5 cells/well Mean SI (Mean SI)	SD	n.
PHA						
500	1726 (3.3)	1230 (1.4)	3	667 (1.1)	345 (0.7)	3
100	674 (1.5)	252 (0.1)	3	824 (1.2)	637 (0.5)	3
10	585 (1.4)	161 (0.3)	3	1099 (1.4)	770 (0)	2
BCG						
1000	1088 (1.6)	1084 (0.2)	4	240 (0.6)	0 (0)	1
500	935 (1.5)	554 (0.4)	4	679 (1.0)	542 (0.2)	3
200	794 (1.5)	378 (0.3)	4	747 (1.0)	666 (0.3)	3
100	914 (1.4)	550 (0.4)	4	642 (0.9)	537 (0.2)	3
50	892 (1.5)	456 (0.5)	4	605 (0.9)	394 (0.1)	3
10	1041 (1.3)	1056 (0.4)	4	614 (0.9)	443 (0.2)	3
Control unstimulated wells	701	505	4	651	435	3

Transport and culture conditions include: the blood travelled at 40°C in RPMI and cells were cultured in 10% CVPDS for 4 days.

See footnote from table 5.1.

Table 5.3 : LTT responses of Mallard lymphocytes in 10% CVPDS and autologous sera.

Stock Antigen Concentration ug/ml	10% Autologous sera Mean cpm (Mean SI)	SD	n.	10% CVPDS Mean cpm (Mean SI)	SD	n.
PHA						
500	908 (4.8)	453 (2.7)	5	793 (2.8)	663 (2.3)	4
100	2629 (13.3)	1574 (10.5)	4	1222 (4.2)	365 (2.7)	5
10	837 (4.1)	490 (2.4)	5	922 (4.1)	1039 (2.9)	4
M.vaccae						
1000	518 (2.6)	213 (1.2)	5	557 (1.4)	378 (0.6)	4
100	435 (2.1)	290 (0.9)	5	590 (1.5)	169 (1.0)	3
10	355 (1.8)	35 (0.5)	4	399 (1.1)	163 (0.1)	4
Control unstimulated wells	204	56	5	365	181	5

Transport and culture conditions include: the blood travelled at 40°C in RPMI and cells were cultured at $8x10^5$ cells/well for 4 days.

See footnote from table 5.1.

2. SKIN TEST.

In humans, skin testing is used widely to assess the effects of BCG vaccination or sensitisation following contact with mycobacteria. Tuberculin testing in poultry is a useful diagnostic test determining those individuals that are either infected, or have come into contact, with the avian tubercle bacillus.

The test in poultry involves injection of avian tuberculin into the dermis of the wattle. The birds are then examined after 48 hours and using the un-injected wattle as a negative control, the degree of swelling of the injected wattle is measured. This swelling is caused by oedema in the connective tissue between the layers of the dermis and also by an infiltration of mononuclear histiocytic cells, eosinophils and a variable number of lymphoid cells. This swelling usually subsides after 48 hours and has disappeared within five days (Thoen and Karlson, 1978). Occasionally false positives occur or more frequently false negatives, especially in advanced states of infection. However, for use in large flocks this test is approximately 80% accurate. False positives do not occur in birds that are retested after one month i.e. the dose of tuberculin in not sufficient to sensitize non-infected birds.

Tuberculin testing in turkeys is less effective, Hinshaw *et al* (1932) tried various sites for injection, including the wattle, the snood, the skin at the edge and the centre of the wing web, and the mucosa of the anus. These workers found that the wing web gave the most consistent results agreeing with 75.7% of PM findings.

The use of the test in exotic birds (Montali *et al*, 1976) and raptors (Lumeij *et al*, 1980) is considered to be unsatisfactory,

however Stehle (1965) demonstrated a positive skin test reaction after 48 hours in the skin near the ear of a tuberculous kestrel.

Shchepilov (1955) tuberculin tested both ducks and geese intradermally under the jaw, and found those positive reactors, to contain tuberculous foci at PM. Other than this report, there appears to have been little use of skin testing in ducks for diagnosis and apparently none for use when assessing response to potential vaccines. For this reason the following test was developed.

Materials and Methods.

Mallard from the vaccine study (Chapter six) were divided into three groups of seven birds (A, B and C) containing equal numbers of representatives from different vaccine groups.

Three sites were chosen for injection:

Site 1- the wing web

Site 2- a plucked area on the back

Site 3- the foot web (on the opposite foot to the vaccination site).

Skin Test Reagents.

The skin test reagents were prepared as for the LTT antigens (Stanford *et al*, 1975a) and were kindly supplied by Dr. J.L. Stanford. The following reagents were used with 0.1 ml being administered intradermally, using Monoject 1 ml syringes fitted with 26 guage needles (Microlance, 0.45 x 10 mm):

(i) BCG 2ug/ml

(ii) BCG 20ug/ml

(iii) Vaccin 20ug/ml

Birds were injected with each reagent, so as to allow each reagent to be tested separately at all three sites as follows:

Group A	Site 1	-	BCG 2ug/ml
	Site 2	-	BCG 20ug/ml
	Site 3	-	Vaccin 20ug/ml
Group B	Site 1	-	BCG 20ug/ml
	Site 2	-	Vaccin 20ug/ml
	Site 3	-	BCG 2ug/ml
Group C	Site 1	-	Vaccin 20ug/ml
	Site 2	-	BCG 2ug/ml
	Site 3	-	BCG 20ug/ml

Results.

The skin test results are summarised in table 5.4.

Unlike the reactions in poultry no obvious oedema could be observed, however in some of the sites there was a definite area of erythema and this was considered to be a positive skin test reaction. This area was measured in millimetres and scored accordingly. These measurements were made at 17, 24, 41, 48, 65 and 72 hours post administration of skin test reagent. From table 5.4 it can be seen that the foot web gave the greatest number of positive reactions for all three skin test reagents, with maximum reactions at 41 hours. Positive reactions were given by both vaccinated and control unvaccinated birds. The other sites gave scanty results with no responses to either BCG 20ug/ml or Vaccin 20ug/ml on the back.

Table 5.4 : Results of Skin Tests at 41 hours post administration of Skin Test Reaction.

Skin Test Reagent	Site of administration		
	Foot Web	Wing	Back
Bird response			
BCG 2ug/ml	++	+	+
	++		
	+		
	+		
BCG 20ug/ml	+++	+	
	++		
	++		
	+		
Vaccin 20ug/ml	++	+	
	++	+	
	+		
	+		
	+		

Discussion.

The birds used in the development of this test were 13 months of age and it would be expected that they had had contact with both saprophytic and possibly pathogenic environmental mycobacteria. A degree of skin test positive reactions would be expected to reflect this, as well as sensitisation following vaccination. The foot web was the only site to show this and as such would appear to be a suitable site for skin testing.

The foot web is also preferable in practical terms i.e. the site is already exposed and more accessable than either of the other sites. These latter sites provided practical problems in that the skin in the wing web is very thin and vasculated allowing easy injury to the area. The site on the back had to be plucked causing a small amount of

injury. Here the skin was thicker but very close to the backbone and it was therefore difficult to administer the reagent. There was also the problem of feathers beginning to grow back obscuring the skin test site after only 48 hours.

It is hoped that skin test positivity is a reflection of sensitisation to mycobacterial antigens either from the vaccine or environmental contact. Skin testing in the foot web will thus be used to monitor the effect of the vaccine.

Histology of Skin Test Sites.

Subsequently, six Mallard and seven Mandarin Ducks *Aix galericulata*, were skin tested with Vaccin 20 ug/ml in the foot web and biopsies (3 mm punch biopsy, Downs Surgical) were taken 41 hours later. The biopsies were taken under local anaesthetic using a 10 mg dose of lignocaine (Xylocaine aerosol spray, Astra).

Histology was carried out by Dr. Sebastian Lucas and staff of the Histopathology Department, University College and Middlesex School of Medicine. Biopsies were embedded and sections were stained by haematoxylin and eosin. They were then examined for the following and scored accordingly: oedema, tissue eosinophilia, eosinophil degranulation, perivascular chronic inflammation (PVCI), diffuse dermal chronic inflammation (DDCI) (i.e. excluding eosinophils) and collagen degeneration.

The results are summarised in table 5.5. It can be seen that clinical reaction related to the level of tissue eosinophilia, and eosinophil degranulation. Oedema was present in most of the clinically positive skin test reactions and absent in the clinically negative responses. A higher degree of diffuse dermal chronic inflammation was found in

those birds with the greatest clinical responses, again this was negative in those birds with negative clinical responses. There was collagen degeneration in only one biopsy, namely that of SB2940, which also had the most marked eosinophilia.

The areas of biopsy on the foot webs of all the birds healed over neatly within several weeks.

Table 5.5 : Results of Histology from skin test biopsies.

Bird	Clinical Reaction	Oedema	Eosino-philia	Eosinophil degranul-ation	PVCI	DDCI	Collagen Degeneration
Mallard.							
LBA5	+++	+	++	+	+	++	-
RBA3	+++	+	++	+	+	+	-
RBB1	++	+	++	+	+	+	-
LBA2	++	-	++	+	+	+	-
RBA9	+	+	++	+	-	+	-
LYC8	+	-	+	-	+	-	-
Mandarin.							
SB2934	+++	+	++	+	+	++	-
SB2940	++	+	+++	+	+	++	+
SB2974	++	-	++	+	+	-	-
SB2937	++	-	+	-	+	-	-
SB2961	++	-	+	-	-	-	-
SB2933	-	-	+	-	+	-	-
SB2949	-	-	+	-	+	-	-

PVCI = perivascular chronic inflammation.

DDCI = diffuse dermal chronic inflammation

3. ENZYME LINKED IMMUNOSORBANCE ASSAY (ELISA).

This test was used to measure directly, levels of anti-mycobacterial antibodies. This solid phase ELISA was originally described by Nassau *et al* (1976). Before this test could be carried out, a conjugated anti-duck antibody had to be made. An anti-duck antibody is commercially available (Nordic Immunological Laboratories Ltd.) however this is raised with complete Freund's adjuvant and is therefore of no use in anti-mycobacterial assays. Mouse anti-duck total immunoglobulin and mouse anti-duck IgG were therefore made using the following methods.

Precipitation of Total Duck Immunoglobulin.

Total duck immunoglobulins were precipitated from the sera of seven week old White Pekin Ducks. The sera was collected from clinically healthy birds at slaughter and was supplied by Cherry Valley Farms Ltd. 25 ml of sera was stirred and 3.6 g of sodium sulphate (Hopkin and Williams, 810600) was added. This was stirred for a further 30 minutes. It was centrifuged at 20,000 rpm for 30 minutes. The supernatent was discarded and the precipitated immunoglobulins were redissolved in 20 ml of PBS. This was stirred as 2.8 g of sodium sulphate was added and stirred for a further 30 minutes. This was centrifuged at 20,000 rpm for 30 minutes. The last steps of redissolving, stirring and centrifuging were then repeated.

This time the precipitate was redissolved in a minimum volume of PBS and dialysed against several changes of PBS at 4°C. The immunoglobulins were then purified using the following technique.

Affinity Purifying Antibodies with CNBr-activated Sepharose 4B.

This method is taken from that described by Pharmacia who supply the Sepharose beads.

The powder was weighed out (1 g of CNBr Sepharose powder swells to 3.5 ml of swollen gel) and suspended in 1 mM HCl (BDH 1789). The gel swells immediately and it was washed in 1 mM HCl for 15 minutes on a sintered glass filter, (200 ml of 1 mM HCl was used per gram of freeze dried powder).

The gel was washed in coupling buffer (sodium bicarbonate, BDH 10247, 0.1 M pH 8.3 containing sodium chloride, BDH 10241, 0.5 M). The ligand (immunoglobulins to be purified) was dialysed against coupling buffer overnight at 4°C. This ligand (5 ml per gram of powder) was mixed with the gel by rotating end over end overnight at 4°C.

This was then packed into a column in a 5 ml syringe (Plastipak, 050025150) with a loose bung of scrubbed nylon wool fibre (Fenwal Laboratories, 4C2960). The column outlet was attached to an Ultraviolet Absorbitometer (LKB Uvicord II, type 8303A) to detect eluted proteins which were recorded on a two-channel chart recorder (LKB Recorder 2210). Excess ligand from the column was washed away with coupling buffer. Any remaining active groups were blocked with Tris HCl buffer (0.1 M pH 8)(Trizma base, Sigma 5753) overnight at 4°C.

The immunoglobulins were eluted using three cycles of alternating pH:- (i) 0.1 M acetate buffer pH 4, containing 0.5 M sodium chloride.

(ii) Tris buffer (0.1 M pH 8 containing 0.5 M sodium chloride).

The immunoglobulins were dialysed against several changes of PBS at 4°C. Protein concentration was measured using the method of Warburg and Christian (1941).

Isolation and Purification of Duck Immunoglobulin G from Serum on Protein A-Sepharose.

This method (Hudson and Hay, 1980) has apparently not been used before for separating duck IgG from serum but it worked effectively.

IgG was purified from a pool of healthy duck sera (supplied by Cherry Valley Farms Ltd.). This was done using a 3 ml column of Protein A (Extracellular) Agarose Gel (Sigma, P-5906) packed in a 5 ml syringe constructed as in the previous method. The column consisted of approximately 0.25 g of protein A/ml of column, dissolved in PBS-azide (sodium azide, BDH 30111). Eluted proteins were recorded on a two-channel chart recorder (LKB Recorder 2210). The column was then washed through with: an excess of PBS-azide, the elution buffer, and PBS-azide again to remove any possible impurities.

3 ml of filtered duck serum was diluted in 3 ml of PBS-azide and this was run very slowly through the column overnight to allow binding of the IgG to the protein A. Unbound proteins were washed out with PBS-azide until the recorder returned to baseline.

The bound IgG was eluted with glycine-HCl buffer pH 2.9 (glycine, Sigma G-7126). The IgG fraction was collected and the column was washed through with PBS-azide.

The IgG was titrated to neutrality with sodium hydroxide solution (BDH 10252). The immunoglobulin was dialysed against several changes of PBS at 4°C and the protein concentration was measured as described

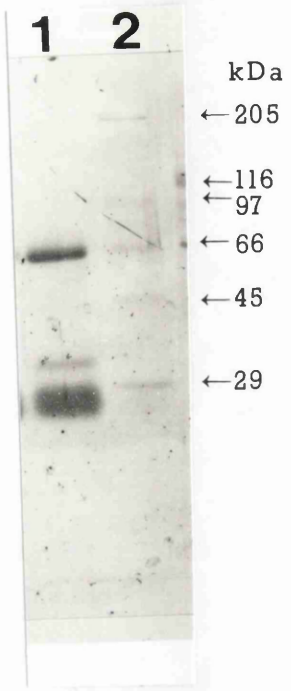
previously.

The column was washed thoroughly with PBS-azide and stored with this buffer to prevent fungal and bacterial growth.

To ensure that it was in fact IgG that had been eluted from the column it was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A 12.5% acrylamide resolving gel was used according to the discontinuous buffer system of Laemmli (1970) using the Hoefer Mighty Small II slab gel electrophoresis unit SE 250 and the method and materials indicated by Hoefer. The gel was stained with Coomassie blue as described by Hoefer.

When compared to molecular weight markers (Sigma MW-SDS 200) of 29, 45, 66, 97, 116 and 205 kDa, the sample showed the characteristic three bands of duck IgG (Ng and Higgins, 1986) indicating the two heavy chains of different size (approximately 66 and 35 kDa from the 7.8S IgG and the 5.7S IgG respectively) and the thicker band of the light chain (approximately 23 kDa)(figure 5.1).

Figure 5.1: Duck IgG on SDS-PAGE showing two sizes of heavy chain and one size of light chain.



Key:

1. Duck IgG
2. Protein marker (29, 45, 66, 97, 116 and 205 kDa)

Once the affinity purified total antibodies and IgG had been collected they were used to raise anti-duck antibodies in Balb/c mice using the following method.

Immunisation with Immunoglobulin (taken from Steele, 1985).

An equal volume of the prepared immunoglobulins at 250 mg/ml were mixed with incomplete Freund's adjuvant. These were homogenised into a meringue-like consistency by repeated mixing under pressure in 1 ml syringes that had been jammed into 25 ml universal containers.

For each immunoglobulin, 50 ul was injected into both hind legs of 10 mice. This was repeated twice at 10 day intervals using only half the dose of immunoglobulin. Two weeks after the last inoculation the mice were bled by cardiac puncture and the serum was collected.

It was confirmed that antibodies had been raised to the duck immunoglobulins by carrying out an ELISA using the method described in Hudson and Hay (1980) with the total immunoglobulins and the purified IgG as antigens.

The mouse immunoglobulins were then precipitated from the mouse sera using the same method as described for the duck's sera earlier in the chapter.

The protein concentration of the mouse anti-duck immunoglobulin and mouse anti-duck IgG were measured spectrophotometrically and they were stored at 4°C. These immunoglobulins were then conjugated to horseradish peroxidase using the following technique.

Method for Conjugation of Horseradish Peroxidase to Immunoglobulins.

This method is described by Hudson and Hay (1980) and is a modification of that described by Wilson and Nakane (1978).

4 mg of horseradish peroxidase (HRP)(Sigma P-8375), was dissolved in 1 ml of distilled water. To this, 0.2 ml of freshly prepared 0.1 M

sodium periodate (Sigma S-1878) solution was added. The resulting green/brown mixture was stirred for 20 minutes at room temperature. This was dialysed overnight against 1 mM sodium acetate buffer pH 4.4 at 4°C.

The pH was raised to 9-9.5 by adding 20 ul of 0.2 M sodium carbonate buffer pH 9.5. Immediately 8 mg of immunoglobulin in 1 ml of 0.01 M sodium bicarbonate buffer pH 9.5 was added. This was stirred for 2 hours at room temperature.

0.1 ml of fresh 4 mg/ml sodium borohydride solution (BDH 30114) was added to reduce any free enzyme. This was left to stand for two hours. It was then dialysed against 0.1 M borate buffer pH 7.4 overnight at 4°C. This was diluted 1:2 in 60% glycerol (Fisons G/0600) in borate buffer containing sodium azide (BDH 30111).

Conjugates made by this method appear to be stable for at least one year when stored at 4°C.

The ELISA technique.

Once the conjugates had been prepared the ELISA could be developed. The following procedure is essentially as described by Nassau *et al* (1976) and Bahr *et al* (1980).

Methods and Materials.

Antigens were prepared in the same way as for the LTT. These were diluted in coating buffer (0.05 M carbonate/bicarbonate buffer at pH 9.6) to a concentration of 10 ug/ml.

Wells of 96 well micotitre plates (Nunc, Denmark) were coated with 100 ul of the antigen and this was incubated in a damp box for 3 hours at room temperature.

The antigen was washed out using washing incubation buffer (1 M PBS pH 7.4 with 0.05% Tween 20). The wells were washed out by completely filling the wells and leaving for three minutes, this was repeated three times.

The plate was then blocked using 100 ul/well of 2% Bovine serum albumin BSA (Sigma A-9647 Fraction V) diluted in washing/incubation buffer. This was left overnight in a damp box at 4°C.

The wells were washed as before and 100 ul of the test sera was added using a 1:200 dilution of sera in washing/incubation buffer. Each serum/antigen combination was duplicated. This was left for 2 hours at room temperature in a damp box.

After this time the wells were washed out as before.

The conjugate was added at 1:1000 dilution in washing/incubation buffer using 100 ul/well. This was left on overnight in a damp box at 4°C.

Again the wells were washed out as before and 100 ul of substrate 2, 2 azino-di-(3 ethyl benthiazoline sulphonic acid) ABTS (Sigma A1888) diluted in citrate phosphate buffer (0.1 M pH 4.1) was added to each well. The colour was left to develop in the dark in a damp box for approximately 30 minutes.

The reaction was stopped by the addition of 50 ul of stopping solution to each well.

The optical density was measured on a micro ELISA auto-reader (Dynatech Labs., Ltd., Sussex) at 650 nm using a well in which test sera had been substituted by washing/incubation buffer alone, as a blank.

Control sera were included in each ELISA run to faciltate standardisation of runs carried out at different times.

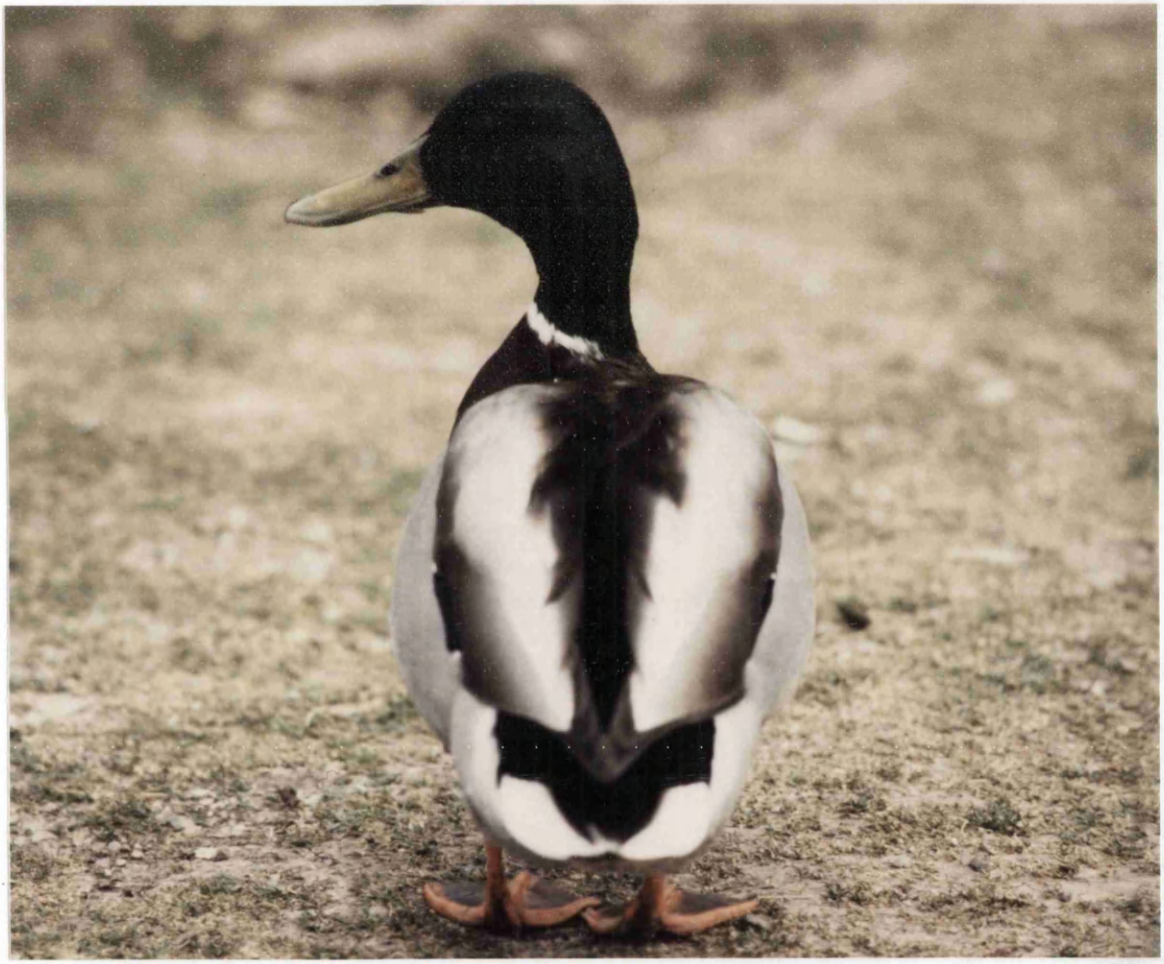
Dr. D.A. Higgins, of Hong Kong University, kindly made several affinity purified anti-duck antibodies. These were then conjugated by the author, using the method described previously. One of the antibodies was raised in a rabbit with incomplete Freund's adjuvant. The conjugate made from this, was selected as the most specific anti-duck and had the lowest anti-mycobacterial backgrounds (i.e. lowest amount of conjugate binding to the antigen). This conjugate was then used in preference to those made by the author.

Once these three immunological tests had been developed the monitoring of the immunological responses of the birds in the vaccine studies began.

CHAPTER SIX.

A SERIES OF POTENTIAL VACCINES:

THE MALLARD STUDY.



CHAPTER SIX.

A SERIES OF POTENTIAL VACCINES: THE MALLARD STUDY.

INTRODUCTION.

The pathogenicity of mycobacteria is determined by the host's immune responses to either all or some of their antigens. Similarly, protection from mycobacterial disease relies on immune recognition of various antigens. Which antigens evoke such a protective response is open to debate, but it would appear that recognition of the common mycobacterial antigens facilitates protective immune responses. These common antigens are referred to as the group i antigens and they are shared by all species of mycobacteria (Stanford and Grange, 1974). At least some of these antigens are also shared by *Nocardia*, *Listeria* and *Corynebacteria* (Stanford *et al*, 1978; Grange, 1985). Evidence to support the role of common antigens in immunogenicity is shown in that BCG can be protective against both leprosy and tuberculosis in man (Brown *et al*, 1968). However, as Rook (1987) points out, immunisation with common antigens may provoke protective immune responses by promoting a rapid recognition of the species specific ones and this latter response may be the target for bactericidal effector mechanisms.

BCG is the vaccine strain of *M.tuberculosis* used in man. It is named after the workers Albert Calmette and Camille Guérin. They produced the vaccine strain at Lille in France, where they subcultured the bovine tubercle bacilli repeatedly on medium containing ox bile every three weeks from 1906 until 1918. It has now been in use since 1921 and is widely used despite setbacks, like the one at Lübeck where a

batch of vaccine was accidently prepared from virulent *M.tuberculosis*, resulting in the deaths of 72 children.

In man BCG is administered as a live vaccine. Live vaccines have long been considered most immunogenic, suggesting that the ability to survive *in vivo* is required for appropriate protective immune responses (Collins, 1971). As most invading potentially infective living organisms are overcome, this would appear to be the case.

The work of Lefford *et al* (1980) showed BCG to protect mice from *M.avium* infection. As has been discussed in Chapter four, BCG has also been used with varying degrees of success to protect chickens from avian tuberculosis. Although duck immunology differs somewhat from that of chickens, BCG was chosen in this study as a potential vaccine to protect wildfowl from the disease.

It would appear that all species of mycobacteria are capable of inducing some level of mycobacterial immunity in mammals. Whereas the slow growers may produce more necrotic and less protective responses, some fast growers can suppress necrotic responses and induce bactericidal mechanisms (Stanford, 1983b). Certainly the role of environmental saprophytic mycobacteria in 'immunising' individuals prior to BCG vaccination has been described (Palmer and Long, 1966; Comstock and Webster, 1969).

The potential use of *M.vaccae* as a vaccine against both leprosy and tuberculosis has been recognised where immune recognition is elicited by its high concentration of group i common antigens (Stanford *et al*, 1978; Swinburne *et al*, 1985; Stanford *et al*, 1989; Ghazi-Saidi *et al*, 1989). *M.vaccae*, named in 1964 by Bönicke and Juhasz, is a rapidly growing variably chromogenic environmental saprophyte. It bears an

antigenic similarity with *M.leprae* in that it possesses the antigens common to all mycobacteria but lacks groups ii and iii antigens associated with either the slow growing or rapid growing mycobacteria respectively (Stanford and Grange, 1974; Stanford *et al*, 1975b).

The synergistic effect of *M.vaccae* on the outcome of BCG vaccination has also been documented (Stanley *et al*, 1981; Bahr *et al*, 1986; Stanford *et al*, 1989). Indeed the immunogenicity of *M.vaccae* has warranted its use as an immunotherapeutic agent for leprosy and tuberculosis (Stanford *et al*, 1988a; Stanford *et al*, 1990; Bahr *et al*, 1990a; Bahr *et al*, 1990b). There have been no reported cases of *M.vaccae* infections in birds so this non-pathogenic saprophytic mycobacterium was chosen as a potential vaccine for the wildfowl in this study.

Whilst many vaccines are attenuated live vaccines e.g. BCG, poliomyelitis and smallpox, killed vaccines have been shown to produce specific protection (Weiss, 1959; Rook, 1980). As previously mentioned, the ability of a vaccine to survive *in vivo* may be linked to immunogenicity. However, the work of Youmans and Youmans (1969) showed that protection could be afforded in CF 1 mice when they were immunised with *M.tuberculosis* $H_{37}Ra$; a strain that has limited ability to multiply in the host. Stanford *et al* (1990) used *M.vaccae*, killed by irradiation, as an immunotherapeutic agent in the treatment of pulmonary tuberculosis in humans and similarly this killed organism has been used in the prevention of bovine tubercle bacilli infection in badgers *Meles meles* (Stainsby, 1989). Sinha *et al* (1987) also found that killed *M.vaccae* was better at sensitising guinea pigs and mice for delayed-type hypersensitivity than the live bacilli. Killed *M.vaccae* was therefore tried as a potential vaccine in this study.

Mammalian immunity to mycobacteria is mediated by specific antigen responsive T-cells, which in turn release lymphokines which then activate non-specific killing mechanisms of macrophages. The inability as yet, to distinguish between T and B cells in wildfowl, and the difficulty in demonstrating the presence of cytokines, may suggest different protective mechanisms. However, it is plausible to assume that wildfowl immune responses are somewhat similar, if less sophisticated, to those found in mammals and chickens. Within wildfowl no work has as yet, been carried out to determine which epitopes are recognised by appropriate lymphocytes. It is hoped that if an optimal vaccine is to be produced, then all individuals will recognise the same, or a similar, set of antigenic determinants rather than different epitopes being recognised by different individuals.

METHODS AND MATERIALS.

The Choice of Bird for the Study.

Mallard *Anas platyrhynchos platyrhynchos* were chosen as the study bird. As members of the Anatini, which also feed by dabbling, they are relatively susceptible to avian tuberculosis. There have been many cases of the disease in captive Mallard at Slimbridge and also reports of 40% of 'local' wild Mallard being infected (Martin Brown, personal communication). Due to the abundance of these birds at Slimbridge, it was relatively easy to raise a sufficient number of young birds for the study.

Eggs were hatched and the ducklings raised in the hygienic duckery unit at Slimbridge. There have been no cases of avian tuberculosis in ducklings under the age of eight weeks, with only one isolated case of

a juvenile Carolina *Aix sponsa* dying of advanced infection at eight weeks of age when in the outdoor duckery (Martin Brown, personal communication). The possibility of infected eggs is considered very slight, as the ovaries and oviduct are infrequently infected and cloacal contamination is unlikely. Ducklings were therefore considered to be healthy and free from avian tuberculosis at vaccination.

The Potential Vaccines.

The strain of *M.vaccae* used, was a stable rough variant of that isolated by Stanford and Paul (1973) from the mud of Lake Kyoga in Uganda: R877R (NCTC 11659). The *M.vaccae* was grown on Sauton's medium solidified with 1% agar, which unlike Löwenstein-Jensen medium contains no egg protein. When the bacteria were at the end of their logarithmic phase they were harvested and suspended in 0.067 M sterile borate buffered saline (pH 8.0) at a concentration of 1 mg wet weight of bacteria per ml, this is approximately equivalent to 10^9 bacilli per ml. From this stock they were diluted to the following concentrations:- 10^7 and 10^8 bacilli per ml. It was administered either live, or killed, using γ-irradiation (2.5 M rads.) emitted from a ^{60}Cobalt source.

The BCG used was Glaxo BCG (Glaxo Laboratories Ltd. Greenford, Middlesex) which was reconstituted from its lyophilized form with sterile distilled water. This was diluted to the same concentrations as the *M.vaccae*.

Route for Administration of Vaccine.

Oral vaccination against tuberculosis has been shown to be largely protective in man (Weill-Halle and Turpin, 1925; Von Rosemberg, 1954), guinea pigs (Sadelkow, 1951; cited in Weiss, 1959) and badgers

(Stainsby, 1989). Such a route for administration is usually used when stimulation of the tonsil and gut lymphoid tissue is required. From Chapter four we have seen that a variety of routes of vaccination in birds have been tried, with degrees of success from the following routes: oral (Rossi, 1974), subcutaneous (Vizy, 1964; Guindi, 1960), intraperitoneal (Vizy, 1964) and intravenous (Guindi, 1960). However, in man BCG is administered intradermally or by scarification. Such a route where the antigen is not introduced to the venous circulation is considered important for mycobacterial immunity. If the vaccine antigen is carried to the spleen there is a great deal of antigen/antibody complexing and a possible 'trapping' of specifically responsive lymphocytes in organs such as the spleen (Lagrange *et al*, 1974; Rowley *et al*, 1972). For these reasons an intradermal route of vaccination was tried for this study.

Age for Vaccination.

BCG appears to protect humans by either inducing anti-mycobacterial responses in those who have yet to encounter such organisms, or increasing appropriate immune responses in those who have already been naturally challenged with mycobacteria, pathogenic or otherwise. The responses to the vaccine are therefore influenced strongly by age of the recipient at vaccination. For this reasons two ages for vaccination were chosen, namely one day old and six weeks of age.

Vaccination.

In the previously described doses, and live or killed forms, the vaccines were administered intradermally into the foot web of one day old or six week old Mallard ducklings. Each vaccine was administered to ten ducklings. Each bird received 0.1 ml of vaccine, i.e. a dose of

either 10^6 or 10^7 bacilli. Both doses were given to different groups of day old birds, but only the high dose was given to the six week old birds. The live *M.vaccae* was only given in the high dose to the six week old birds.

Vaccination was carried out by Martin Brown of The Wildfowl and Wetlands Trust and a previous student who left for personal reasons.

Table 6.1 : Summary of Vaccines Administered to Mallard.

Age	Live/ Killed	Vaccine	Dose	Darvic ring number
BCG Vaccinated Birds				
1 day	Live	BCG	10^6	LBA 1-10
"	"	"	10^7	LBB 1-10
6 weeks	"	"	10^7	RBB 1-10
***M.vaccae* vaccinated Birds**				
1 day	Killed	*M.vaccae*	10^6	LGA 1-10
"	"	"	10^7	LGB 1-10
6 weeks	"	"	10^7	RGB 1-10
"	Live	"	10^7	RWB 1-10
Controls				
30 birds were left unvaccinated as controls				

All the birds were ringed and pinioned, and when they were several months of age they were put into the Asian Side Pens in the grounds at Slimbridge. As seen from the epizootiological study (Chapter three) these particular pens had a high incidence of avian tuberculosis.

Although the route of infection of *M.avium* would appear to be primarily oral, as shown in the epizootiological study, some of the dabbling ducks develop pulmonary lesions. Such pathology may be indicative of primary infection or a result of dissemination of the disease. Whichever the case, the route for artificial challenge is unknown. The dose of the challenge is also unknown, as the birds at Slimbridge may be in frequent daily contact with the avian tubercle bacilli.

An artificial challenge would involve housing the birds within restricted isolator units which would present an unnaturally stressful environment, which may alter the nature and course of the disease (Gross *et al*, 1989). The ethics of The Wildfowl and Wetlands Trust being involved in essentially overt animal experimentation must also be taken into consideration. For these reasons the birds were exposed to a totally natural challenge since it is this, from which it was hoped to protect them.

Due to the early age of vaccination, base line bleeds or skin tests could not be taken as a reference pre-vaccination point.

Tests used to Assess Immune Responses.

The tests described in Chapter five, namely LTT, skin test and ELISA were all used to follow the immune responses of the birds during the $3^1/_2$ years following vaccination. At each time for testing, representatives from each vaccine group were caught up and bled for LTT and ELISA. In the following week the birds were skin tested. The tests were always carried out in this order to prevent any chance of skin testing affecting the LTT and ELISA results.

Antigens Used in the Tests.

All the antigens were prepared by the method shown in Chapter five (Stanford *et al*, 1975a).

(i) Lymphocyte Transformation Test.

The following antigens were used in LTT's at the following stock concentrations (i.e. final concentrations were $^{1}/_{10}$ of these):

M.vaccae R877R	100ug/ml
" "	50ug/ml
BCG	100ug/ml
"	50ug/ml
M.fortuitum	100ug/ml
"	50ug/ml
M.avium GWT*	100ug/ml
"	50ug/ml
" RBP*	100ug/ml

* these strains of *M.avium* were isolated from birds dying of avian tuberculosis within the Slimbridge collection. The GWT is an abbreviation of Green-winged Teal *Anas crecca crecca* (PM number 86/136) from which this strain was isolated. The RBP is an abbreviation of Red-billed Pintail *Anas erythrorhyncha* (PM number 87/65) from which it was isolated.

(ii) Skin Test.

Initially the skin test reagents were kindly supplied by Dr. J.L. Stanford and latterly they were prepared (Stanford *et al*, 1975a) by the author. The reagents included Vaccin 20 ug/ml, Avumin A 2 ug/ml and *M.avium* GWT 20 ug/ml.

Table 6.2 : Summary of Skin Test Reagents used for Mallard.

(a) *M.vaccae* Vaccinated Mallard.

Months of age	Skin Test Reagent
17 months	Vaccin 20ug/ml
20 "	Avumin A 2ug/ml
23 "	*M.avium* GWT 20ug/ml
26 "	Vaccin 20ug/ml
29 "	"
32 "	"
35 "	*M.avium* GWT 20ug/ml
38 "	"
43 "	"

(b) BCG Vaccinated Mallard.

Months of age	Skin Test Reagent
18 months	Vaccin 20ug/ml
21 "	Avumin A 2ug/ml
24 "	*M.avium* GWT 20ug/ml
27 "	Vaccin 20ug/ml
31 "	"
34 "	*M.avium* GWT 20ug/ml
37 "	"
44 "	"

(iii) ELISA.

Again the antigens were prepared by the method of Stanford *et al* (1975a) and the following were used at 10 ug/ml:

M.vaccae R877R, *M.avium* GWT, *M.avium* RBP, BCG, *M.fortuitum* and *M.gordonae*.

Although a previous student had vaccinated the birds, the techniques for monitoring vaccine efficacy had not been developed. This development started some five months after vaccination and took some time, and as a consequence there are no results from the birds for the first 15 months post-vaccination. Although this is disappointing, it is hoped that as the disease is one of older birds the critical time when infection could be expected has not been missed. After this time, representative birds from each vaccine group were tested every three months until they were three years of age and once more subsequently at $3^1/_2$ years of age.

Statistical Tests Used to Analyse the Data from the Vaccine Studies.

Lymphocyte Transformation Test.

A two way analysis of variance was used to analyse the LTT results. This test allows the estimation of the effects of two independent variables, namely time and vaccine group, on a dependent variable i.e. the response of the individual birds within a vaccine group. The test uses the raw data which was analysed using a Macintosh Statview 512+ computer package.

The two way analysis of variance gives three *p* values, the first of which indicates whether or not time has a significant effect on the

response. The second, whether or not the vaccine has an effect on the response and the third whether or not there is interaction between the two variables. This interaction indicates a different relationship and prevents us from drawing conclusions about the other two relationships (i.e. renders the other two values invalid).

Student's *t* tests were also used to compare mean SI's to those of the controls at given time points as an indication of exactly when a difference in immune response was taking place and the nature of this difference.

Skin Test.

The area of skin test reaction was measured and scored accordingly. This measurement was then expressed as a fraction of the maximum individual response seen at each time of testing. Group results were then pooled. Fisher's exact test was used to analyse the results at the two times found to give the maximum skin test reaction, namely 41 and 48 hours post administration of the skin test reagent.

ELISA.

Student's *t* tests were used to analyse the ELISA data and compare mean antibody levels of the vaccine groups against those of the controls. Paired Student's *t* tests were used to assess changes in antibody levels within the same birds over time.

RESULTS.

First, it should be noted that vaccination with any of the potential vaccines did not seem to affect the birds in any adverse way and there were no observed side effects. All of the birds went through normal moult phases. Fecundity was not affected, as many of the females produced normal clutches of eggs in spite of the disturbance caused by regular catching, bleeding and skin testing. These clutches hatched and produced healthy offspring.

The fact that the birds were released into pens within the grounds at Slimbridge exposed them to the dangers and stresses that the other collection birds face daily. This led to non-tuberculosis mortality, greater than would have been expected if the birds had been housed in laboratory conditions. Several of the young birds died of renal coccidiosis, a fairly common protozoal infection in ducklings, when they were first released into the grounds. Many of the female birds were lost due to drowning and trauma as a result of forced copulation by over zealous male Mallard in the spring and summer months. This is a common occurrence in Mallard and the problem was compounded by the females in this study being pinioned so preventing their escape. As a consequence, towards the end of the study there are few females in the vaccine or control groups. Other causes of mortality included: lead poisoning, egg peritonitis and haemorragic enteritis. However, table 6.3 details the seven birds that did die of avian tuberculosis.

Table 6.3 : Deaths of Mallard from Avian Tuberculosis.

Vaccine Group Bird	Sex	Age at death
***M.vaccae* vaccinated.**		
1 day old / low dose / killed		
LGA4	Female	22 months
6 weeks old / high dose / live		
RWB3 (Primary cause of death was trauma but *M.avium* infection present).	Male	26 months
	(2/40 = 5%)	
BCG vaccinated.		
1 day old / low dose		
LBA4	Male	27 months
1 day old / high dose		
LBB2	Female	35 months
6 weeks old / high dose		
RBB8	Female	46 months
	(3/30 = 10%)	
Controls		
RBA9	Female	20 months
LYC5	Male	28 months
	(2/30 = 7%)	

It can be seen from table 6.3 that there have been deaths from tuberculosis in all the vaccine groups with the exception of the 1 day old/ high dose/ killed *M.vaccae*. Similar numbers of males and females have died. The *M.vaccae* vaccinated birds and the controls died on average at 24 months of age, whilst the BCG vaccinated birds died on average at 36 months of age. However, too few birds have died to form conclusive evidence for vaccine efficacy.

Data from these birds when in the latter stages of infection (three months prior to death) show generally low SI's from LTT, due to either very high cpm for both stimulated and unstimulated background control cultures or very sick cells that either die or respond very weakly. Skin test responses were occasionally negative in the control birds but present in vaccinates. Antibody levels to all the antigens increased dramatically during late infection in both vaccinates and controls. The results from these birds are summarised in appendices 6.1 - 6.3 and have been omitted from the rest of the analyses as the ELISA data in particular, skews the data considerably.

***M.vaccae* vaccinated Mallard.**

Lymphocyte Transformation Test.

The results of two way analysis of variance show differences in the responses given by the vaccine groups. The *p* values show the level of significance of the difference, and the type of difference (i.e. whether it is greater or smaller) is shown from plots of the data and Student's *t* tests. The responses to all the antigens, with the exception of those of the group vaccinated with killed *M.vaccae* at six weeks of age to *M.avium* GWT, differed significantly over time as can also be seen from figures 6.1 - 6.6. In some cases there was

interaction between the two independent variables i.e. time and vaccine group, which then prevented the analysis being carried out.

The following results showed significant differences between the vaccine group and the control group:

(i) There was a significant difference in the response of the group receiving the low dose of killed vaccine at one day old to the *M.fortuitum* antigen ($p < 0.04$). (There was interaction in the responses to *M.vaccae* and *M.avium* RBP).

(ii) The group receiving the high dose of killed vaccine at one day old responded significantly differently to the *M.vaccae* antigen ($p < 0.0005$), the *M.avium* RBP antigen ($p < 0.001$) and the *M.fortuitum* antigen ($p < 0.0001$). (There was interaction in the response to *M.avium* GWT).

(iii) The group receiving the high dose of killed vaccine at six weeks of age responded significantly differently to the *M.vaccae* R877R antigen ($p < 0.02$) and the *M.fortuitum* antigen ($p < 0.02$). (There was interaction in the response to *M.avium* RBP).

(iv) The group receiving the live vaccine at six weeks of age showed no differences in response from the controls to any of the antigens.

The results of Student's *t* tests of vaccine groups' responses compared to the controls' responses are shown in table 6.4 and are summarised as follows:-

(i) the group receiving the low dose of killed vaccine at one day old gave significantly greater responses to *M.avium* RBP 100 ug/ml and *M.fortuitum* 50 ug/ml at 29 months of age.

(ii) the group receiving the high dose of the killed vaccine at one day old responded consistently better than control birds at many time points and to many antigens. At 26 months of age responses were

greater to BCG 50 ug/ml and both concentrations of *M.fortuitum*: 100 ug/ml and 50 ug/ml. Responses were greater to *M.avium* GWT 100 ug/ml at 29 months of age and again at 35 months of age to both concentrations. Also at this time there were greater responses to both concentrations of *M.fortuitum*. At 38 months of age there were greater responses to *M.vaccae* R877R 50 ug/ml, both concentrations of *M.fortuitum* and *M.avium* GWT 50 ug/ml. At 43 months, the greater responses were to both concentrations of *M.fortuitum*. However at 17 months of age the controls responded significantly better than this group to BCG 100 ug/ml.

(iii) the group receiving the high dose of killed vaccine at six weeks of age gave greater responses to both concentrations of *M.avium* GWT at 29 months of age and to BCG 100 ug/ml at 38 months of age.

(iv) the group receiving the live vaccine at six weeks of age responded significantly less than the control group to BCG 100 ug/ml at 17 months, but significantly more at 23 months of age to *M.avium* RBP 100 ug/ml and *M.avium* GWT 100 ug/ml at 29 months of age.

Results of *M.vaccae* vaccinated Mallard LTT responses are summarised in appendix 6.4 and LTT results to *M.vaccae*, *M.avium* and *M.fortuitum* are shown in figures 6.1 - 6.6.

Table 6.4 : Student's *t* tests of *M.vaccae* vaccinated Mallard LTT responses over time when compared to control birds' responses.

Vaccine group	Months of age						
	17	23	26	29	35	38	43
Day 1, killed, low dose.				*M.fort* 50 $p < 0.05$ *M.avium* RBP 100 $p < 0.01$			
Day 1, killed, high dose.	BCG 100 $p < 0.025$ LESS		BCG 50 $p < 0.01$ *M.fort* 100 $p < 0.05$ *M.fort* 50 $p < 0.025$	*M.avium* 100 $p < 0.005$	*M.fort* 100 $p < 0.025$ *M.fort* 50 $p < 0.01$ *M.avium* GWT 100 $p < 0.05$ *M.avium* GWT 50 $p < 0.05$	R877R 50 $p < 0.025$ BCG 50 $p < 0.05$ *M.fort* 100 $p < 0.05$ *M.fort* 50 $p < 0.025$ *M.avium* GWT 50 $p < 0.05$	*M.fort* 100 $p < 0.025$ *M.fort* 50 $p < 0.025$
6 week, killed, high dose.				*M.avium* GWT 100 $p < 0.025$ BCG 100 $p < 0.025$ *M.avium* GWT 50 $p < 0.05$			
6 week, live, high dose.	BCG 100 $p < 0.01$ LESS	*M.avium* RBP 100 $p < 0.05$		*M.avium* GWT 100 $p < 0.01$ *M.avium* GWT 50 $p < 0.05$			

(*M.fort* = *M.fortuitum*)

Key for figures 6.1 - 6.14.

Legend	Vaccine group
day1/k/low	= 1 day old, low dose of killed *M.vaccae*
day1/k/high	= " high dose " "
6week/k/high	= 6 weeks old, high dose " "
6week/l/high	= " " live *M.vaccae*

Figure 6.1

M.vaccae vaccinated Mallard LTT resposes to M.avium GWT: Day 1 vaccinates and controls

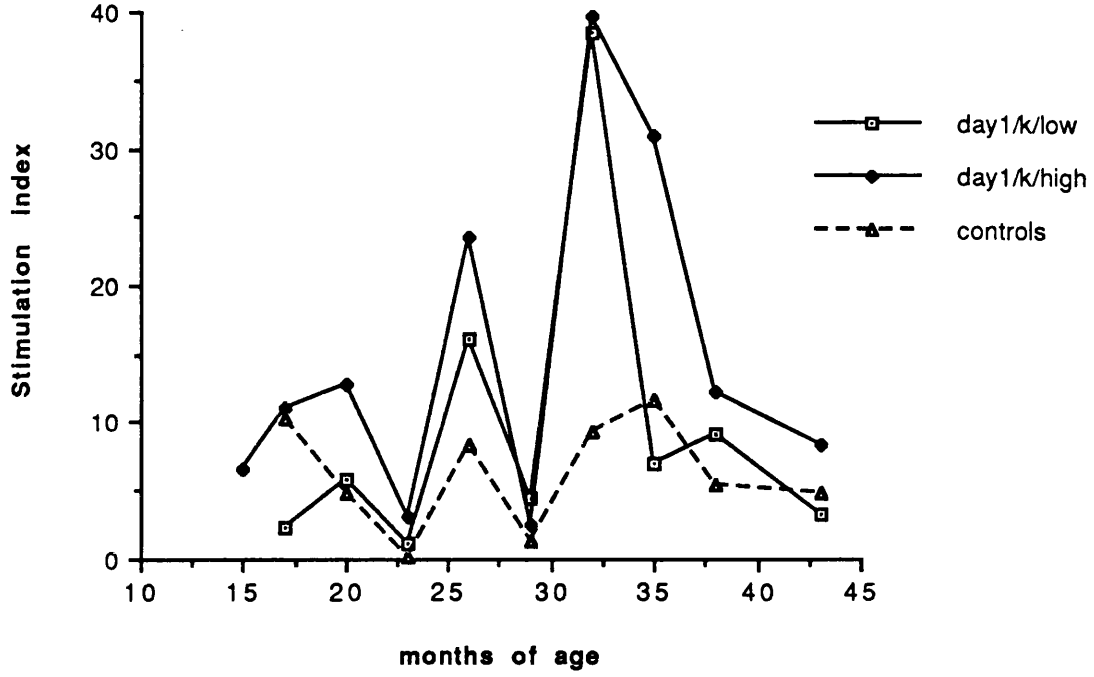


Figure 6.2

M.vaccae vaccinated Mallard LTT responses to M.avium GWT: 6 week vaccinates and controls

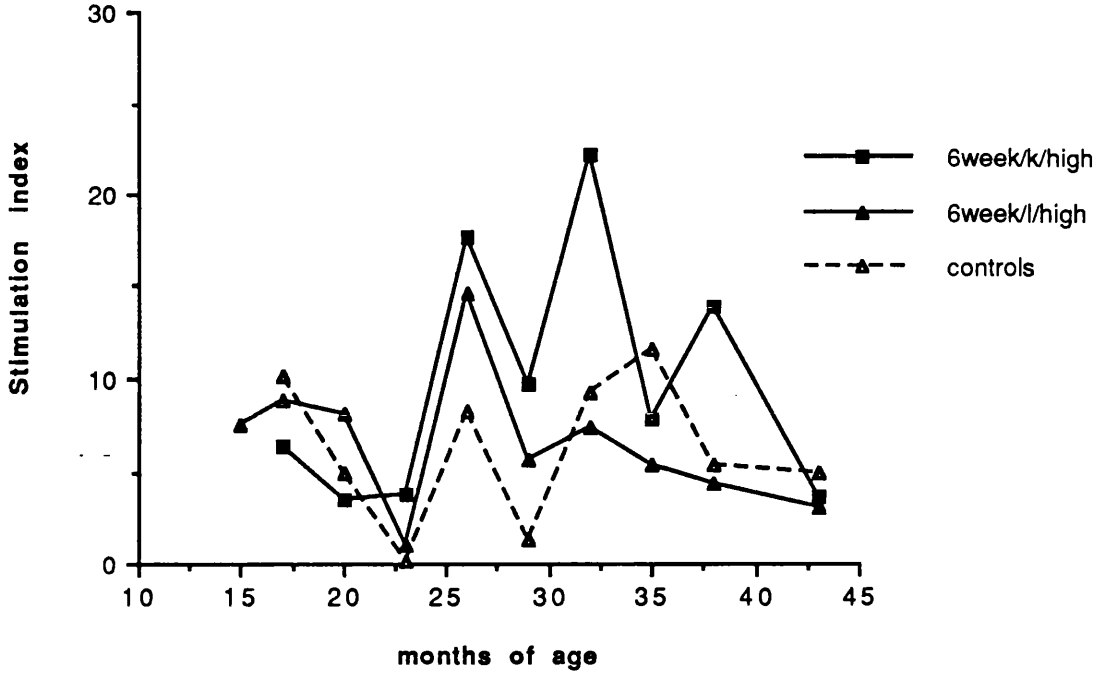


Figure 6.3

M.vaccae vaccinated Mallard LTT responses to M.vaccae: Day 1 vaccinates and controls

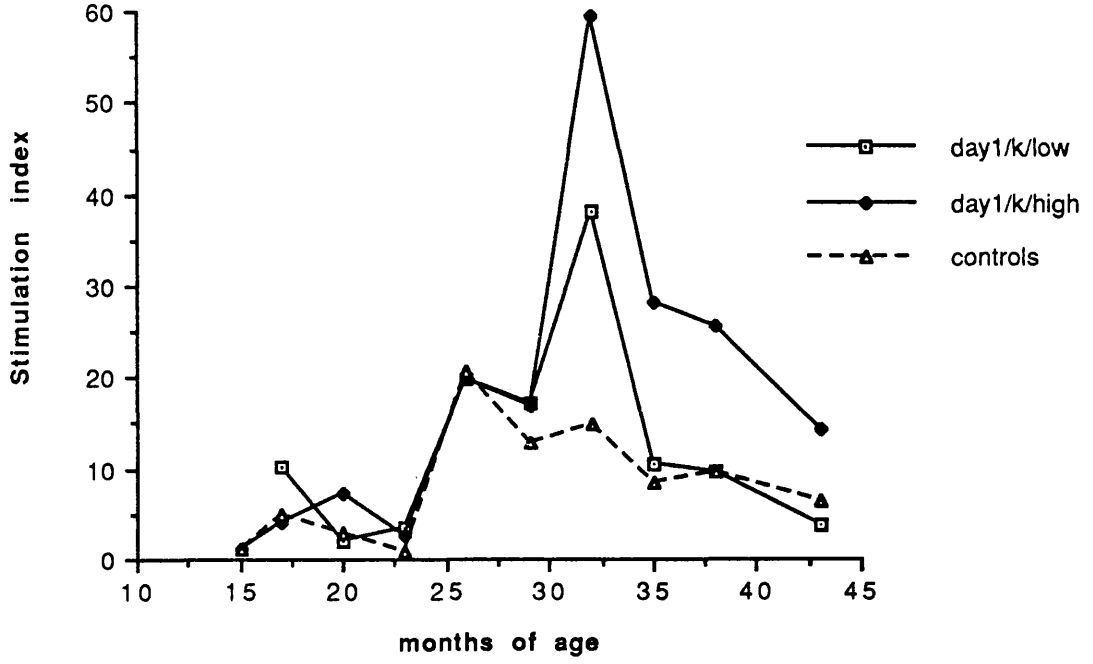


Figure 6.4

M.vaccae vaccinated Mallard LTT responses to M.vaccae: 6 week vaccinates and controls

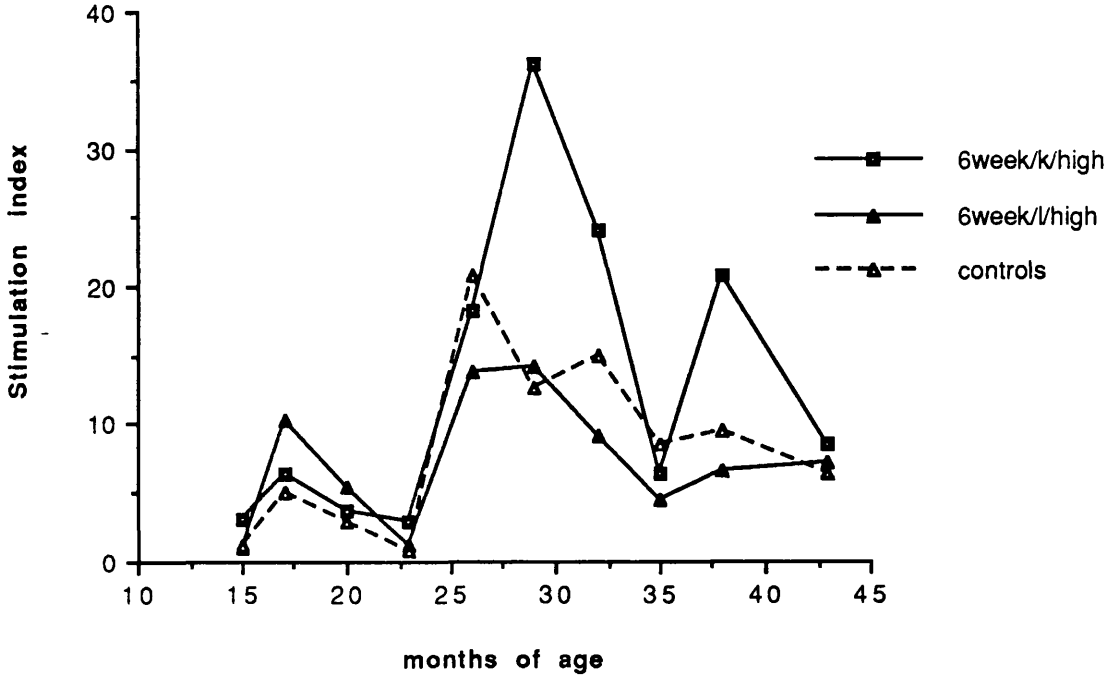


Figure 6.5

M.vaccae vaccinated Mallard LTT responses to M.fortuitum: Day 1 vaccinates and controls

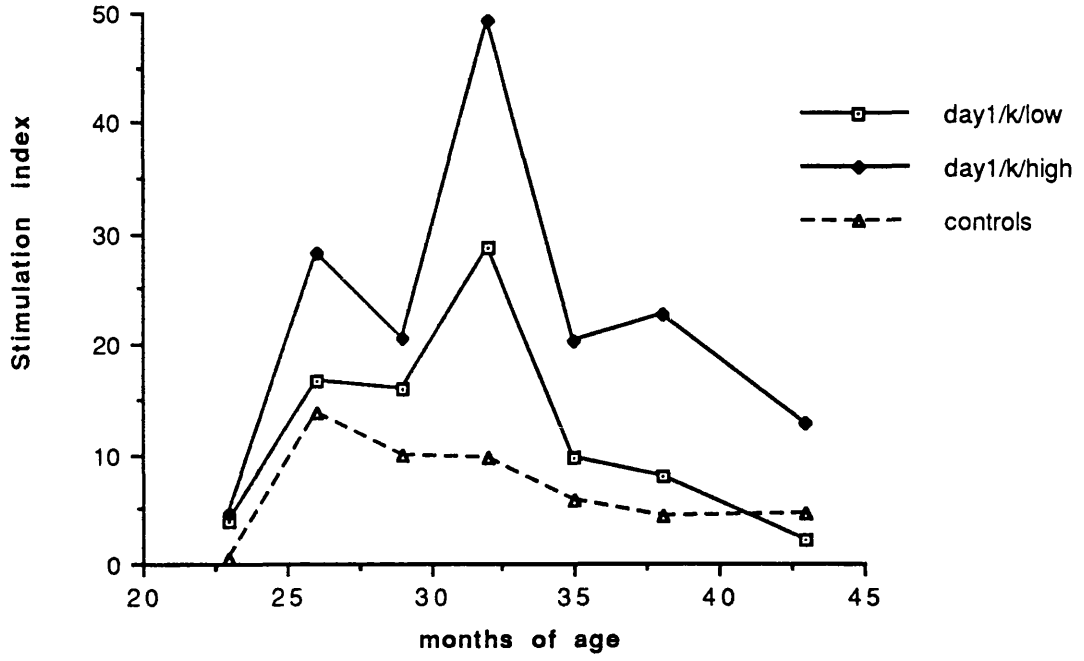
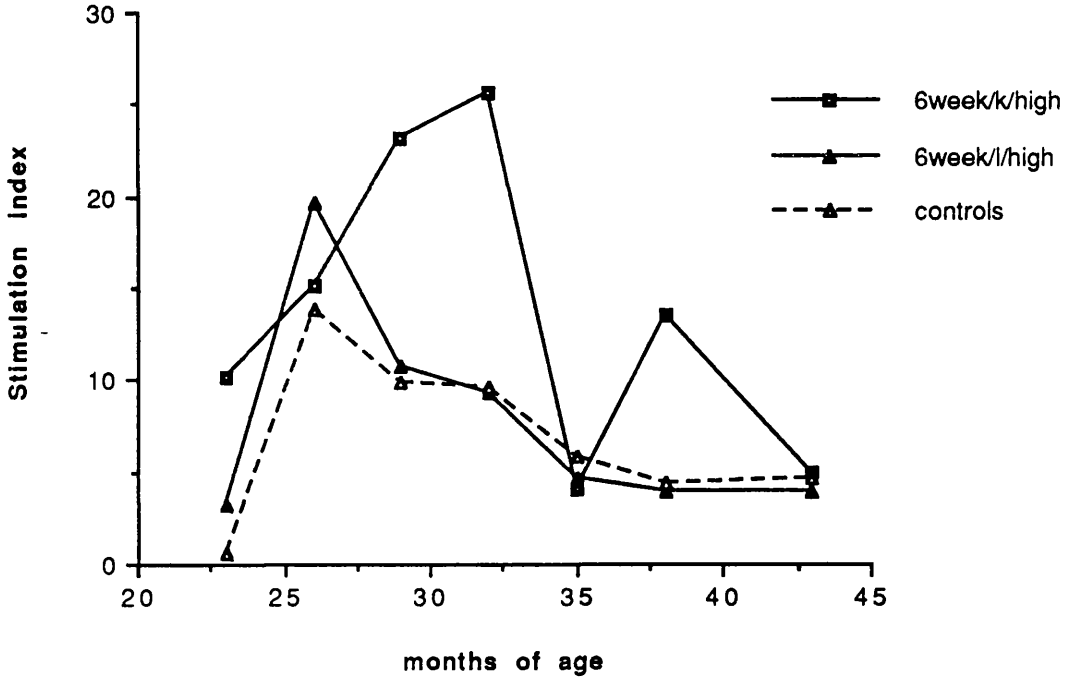


Figure 6.6

M.vaccae vaccinated Mallard LTT responses to M.fortuitum: 6 week vaccinates and controls



Skin Test.

Skin test results are summarised in appendix 6.5 and the responses at 41 hours post administration of skin test reagent are shown in figures 6.7 and 6.8.

The results of Fisher's exact tests used to analyse the skin test reactions of vaccine groups against those of the control group, at 41 and 48 hours post administration of skin test reagent, are shown in table 6.5.

The following statistically significant results were obtained from successive skin tests:-

(i) the group receiving the low dose of killed vaccine at one day old, produced significantly greater skin test responses than the control group at 17, 26 and 38 months of age, at either one or both 41 or 48 hour skin test measurements. At the 26 months of age skin test, this groups' response was also significantly greater than that elicited by the group vaccinated at six weeks of age with the killed vaccine (41 hours: $p < 0.02$). At 35 months skin testing this vaccine group produced significantly greater responses than the live vaccine group (41 hours: $p < 0.02$).

(ii) the group vaccinated with the high dose of killed vaccine at one day old gave significantly greater skin test responses than the control group at 17, 20, 38 and 43 months of age at either one or both 41 and 48 skin test measurements. This vaccine group also produced greater responses than other vaccine groups' at a number of skin tests, namely 20 months when responses were greater than those of the group vaccinated at six weeks of age with the killed vaccine (41 hours: $p < 0.002$; 48 hours: $p < 0.008$); 35 months, when responses were greater than those of the live vaccine group (41 hours: $p < 0.001$; 48

hours: $p < 0.05$). At the 38 month skin test, responses were greater than those of all the other vaccine groups at the 48 hour measurement (low dose, killed vaccine at one day old: $p < 0.03$; high dose, killed vaccine at six weeks of age: $p < 0.009$; high dose, live vaccine at six weeks of age: $p < 0.03$). At the 43 month skin test, this group's responses were significantly greater than those of the group vaccinated at six weeks of age with the killed vaccine (41 hours: $p < 0.04$).

(iii) the group vaccinated at six weeks of age with the high dose of killed vaccine gave significantly greater responses than the control group at the 17 and 35 months skin tests. In addition the responses at the 17 and 35 month skin tests were greater than those of the live vaccine group (41 hours: $p < 0.01$ and $p < 0.002$ respectively).

(iv) the group receiving the live vaccine at six weeks of age gave significantly greater responses than the control group at only the 48 hour measurement at the 17 and 38 month skin tests.

Table 6.5 : Fisher's exact tests of skin test responses of *M.vaccae* vaccinated Mallard over time when compared to control birds' responses.

Vaccine group	Months of age				
	17	20	35	38	43
Day 1, killed, low dose.	41 hours $p < 0.009$ 48 hours $p < 0.002$			41 hours $p < 0.0000$ 48 hours $p < 0.02$	
Day 1, killed, high dose.	41 hours $p < 0.02$ 48 hours $p < 0.02$	41 hours $p < 0.01$ 48 hours $p < 0.008$	41 hours $p < 0.02$	41 hours $p < 0.0000$ 48 hours $p < 0.0000$	41 hours $p < 0.01$ 48 hours $p < 0.03$
6 week, killed, high dose.	41 hours $p < 0.0007$ 48 hours $p < 0.003$		41 hours $p < 0.03$		
6 week, live, high dose.	48 hours $p < 0.03$			48 hours $p < 0.02$	

Figure 6.7

M.vaccae vaccinated Mallard skin test responses at 41 hours: Day 1 vaccinates and controls

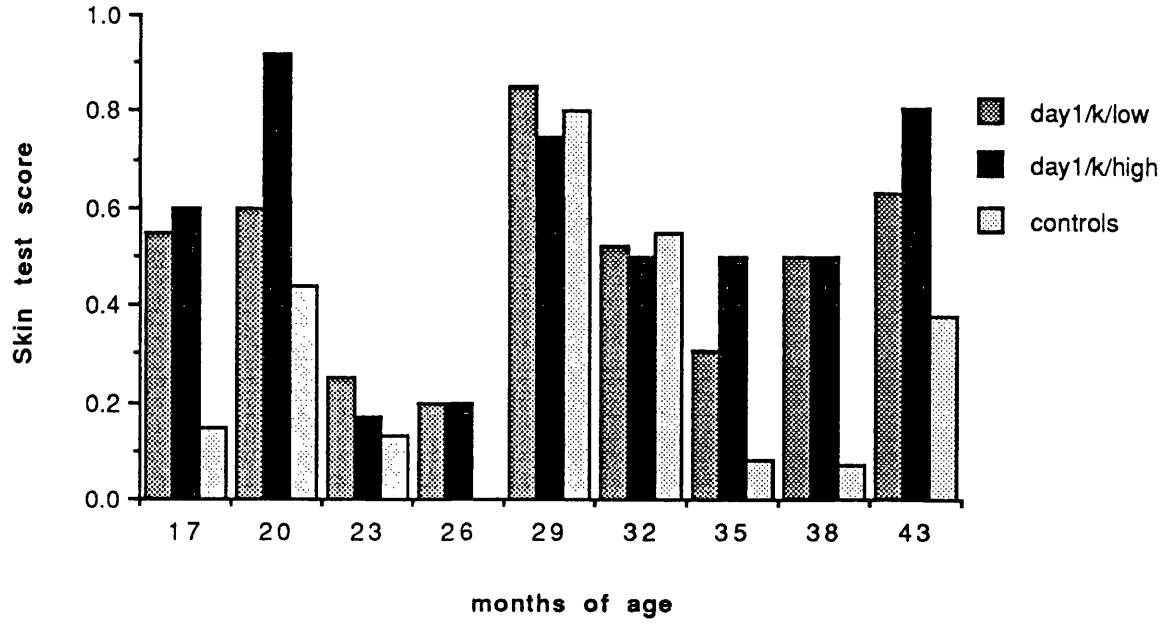
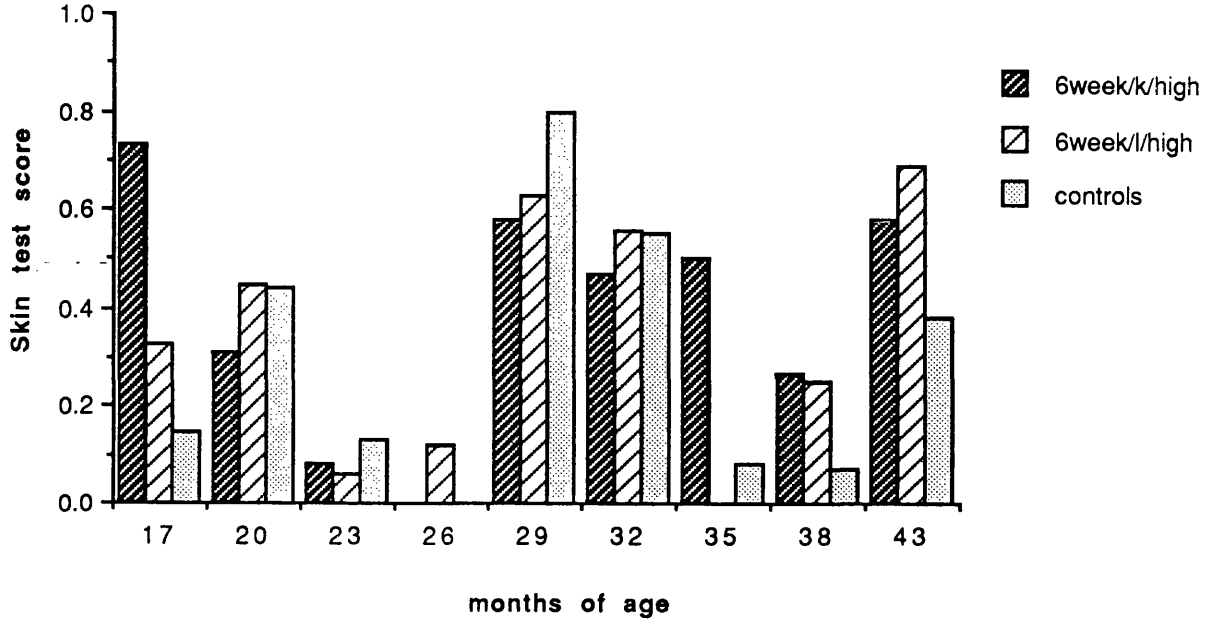


Figure 6.8

M.vaccae vaccinated Mallard skin test responses at 41 hours: 6 week vaccinates and controls



ELISA.

The results of antibody levels over time reveal a number of points, firstly a statistically significant rise in antibody levels from the vaccinated and control groups as a whole to all six antigens over time as can be seen from figures 6.9 - 6.14 ($p < 0.005$ for BCG, *M.avium* GWT and RBP, *M.gordonae* and *M.fortuitum*; and $p < 0.01$ for R877R in a paired Student's *t* test). The second notable point is the relatively few statistical differences in antibody levels between the different vaccine groups (table 6.6). Where statistical differences were observed these were mainly between the two groups vaccinated with the high dose of killed vaccine at either one day old or six weeks of age and the control group. These differences showed antibody levels of the vaccine groups to be consistently lower than those of the control group.

When figures 6.9 - 6.14 are compared it can be seen that there are few differences in antibody responses to all six antigens, i.e. the graphs are a similar shape although absolute levels may vary (e.g. levels to *M.fortuitum* are always higher than to BCG). Superimposed on top of all the graphs is a quite marked seasonal variation with highest levels to be found in the summer and lower levels in the winter. These results are summarised in appendix 6.6.

Table 6.6 : Student's *t* tests of *M.vaccae* vaccinated Mallard antibody levels over time, compared to control birds'.

All *p* values indicate a significantly LOWER antibody level than that of the control group

Vaccine group	Months of age						
	11	23	26	29	32	35	43
Day 1, killed, low dose.							
Day 1, killed, high dose.				*M.gordonae* $p < 0.05$	*M.gordonae* $p < 0.05$	*M.avium* RBP *M.gordonae*, BCG *M.fortuitum* $p < 0.025$	
6 week, killed, high dose.	All 6 antigens $p < 0.05$		*M.avium* RBP $p < 0.025$ BCG, *M.fortuitum* $p < 0.05$	*M.gordonae* $p < 0.01$	BCG $p < 0.05$ *M.fortuitum* $p < 0.005$		*M.gordonae* $p < 0.025$
6 week, live, high dose.						*M.gordonae* *M.fortuitum* $p < 0.05$	

M.vaccae vaccinated Mallard
antibody levels to BCG

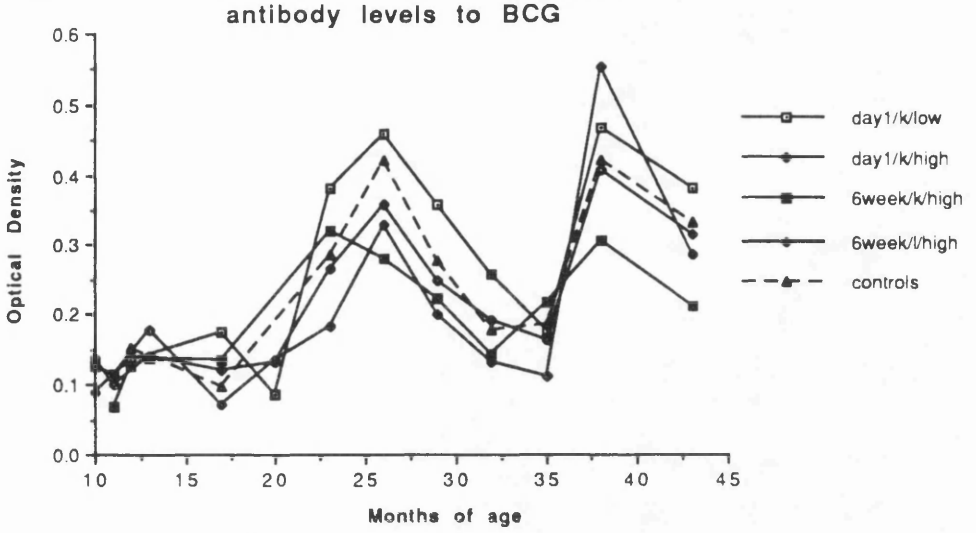


Figure 6.10

M.vaccae vaccinated Mallard
antibody levels to M.avium GWT

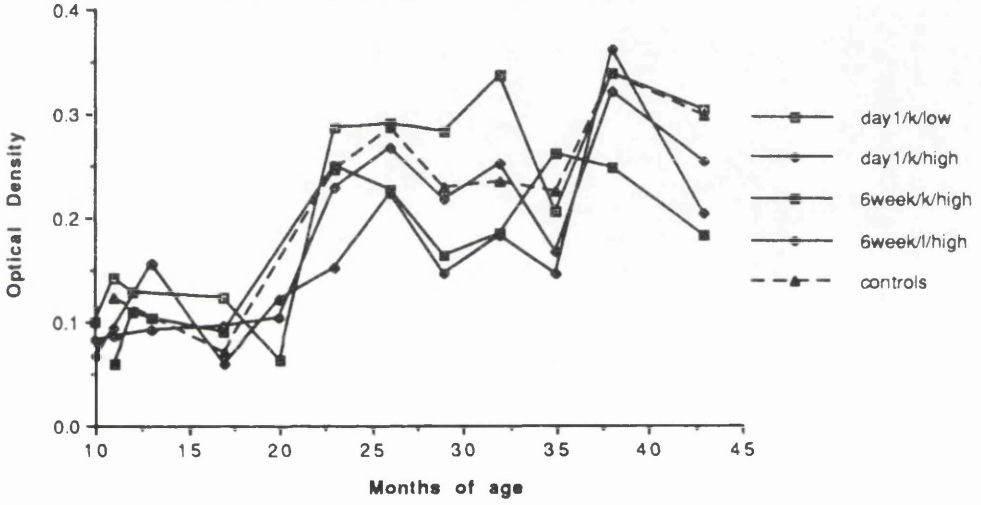


Figure 6.11

M.vaccae vaccinated Mallard
antibody levels to M.fortuitum

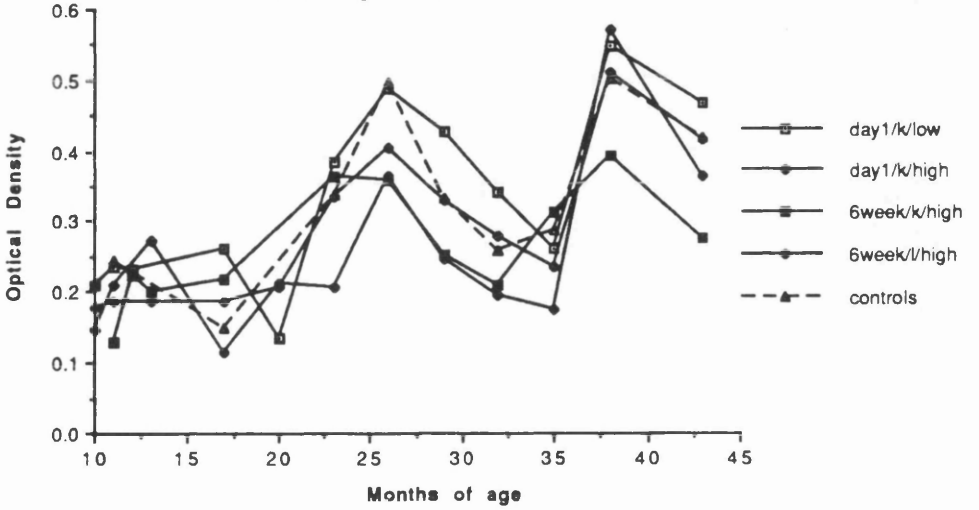


Figure 6.12

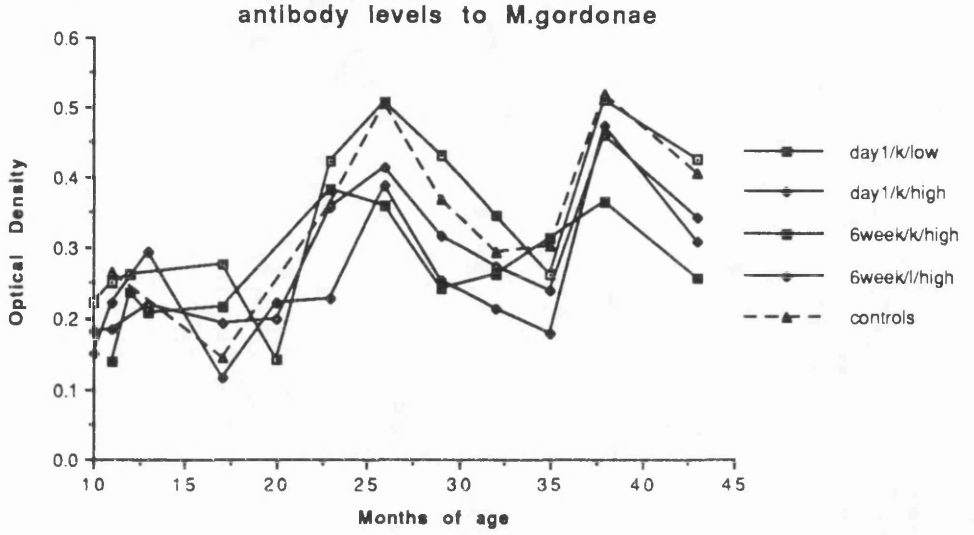


Figure 6.13

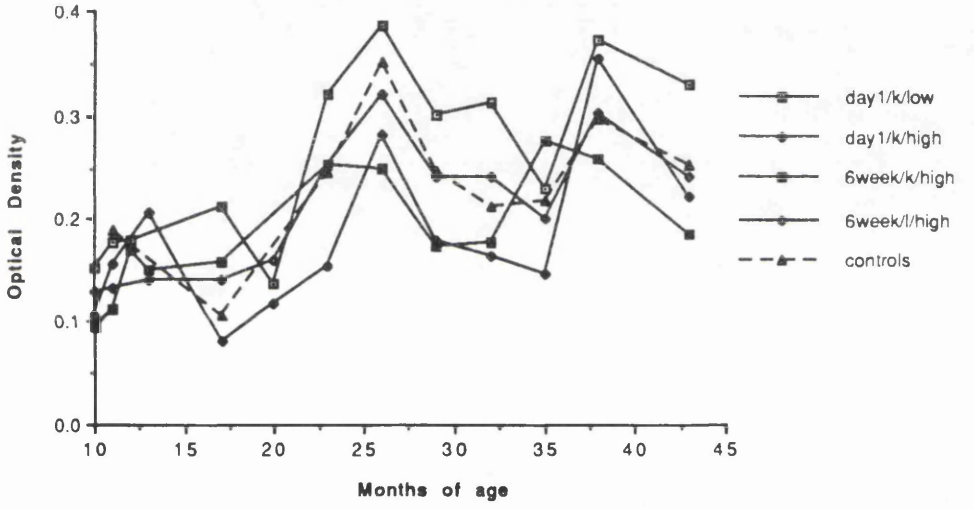
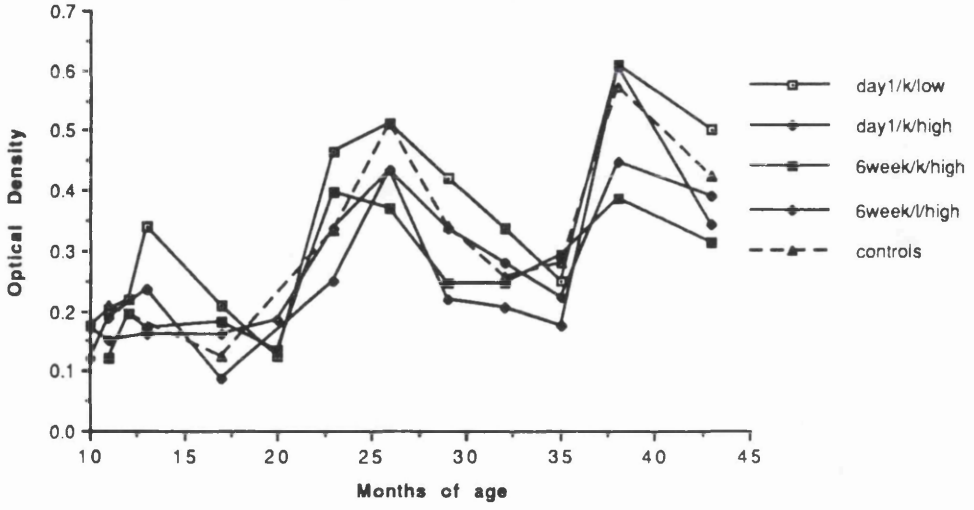


Figure 6.14



Lymphocyte Transformation Test.

The results of a two way analysis of variance show no overall statistically significant differences in responses between the BCG vaccine groups and the control group at any time to any of the antigens. However, there were significant differences in the responses to all the antigens over time ($p < 0.05$) which can be seen clearly from the responses to BCG and *M.vaccae* in figures 6.15 - 6.16.

Student's *t* tests were also used to analyse the data and compare the vaccine groups' responses to those of the control group. The only significant responses obtained, were at 18 months of age when the group vaccinated with the high dose at one day old gave greater responses to *M.vaccae* R877R and BCG (both $p < 0.05$). Also at this time the group vaccinated at six weeks of age gave significantly greater responses to BCG ($p < 0.05$).

LTT results are summarised in appendix 6.7.

Key for figures 6.15 - 6.23.

Legend	Vaccine Group
day1/low	= 1 day old, low dose of BCG
day1/high	= 1 day old, high dose "
6week/high	= 6 weeks old, high dose "

Figure 6.15

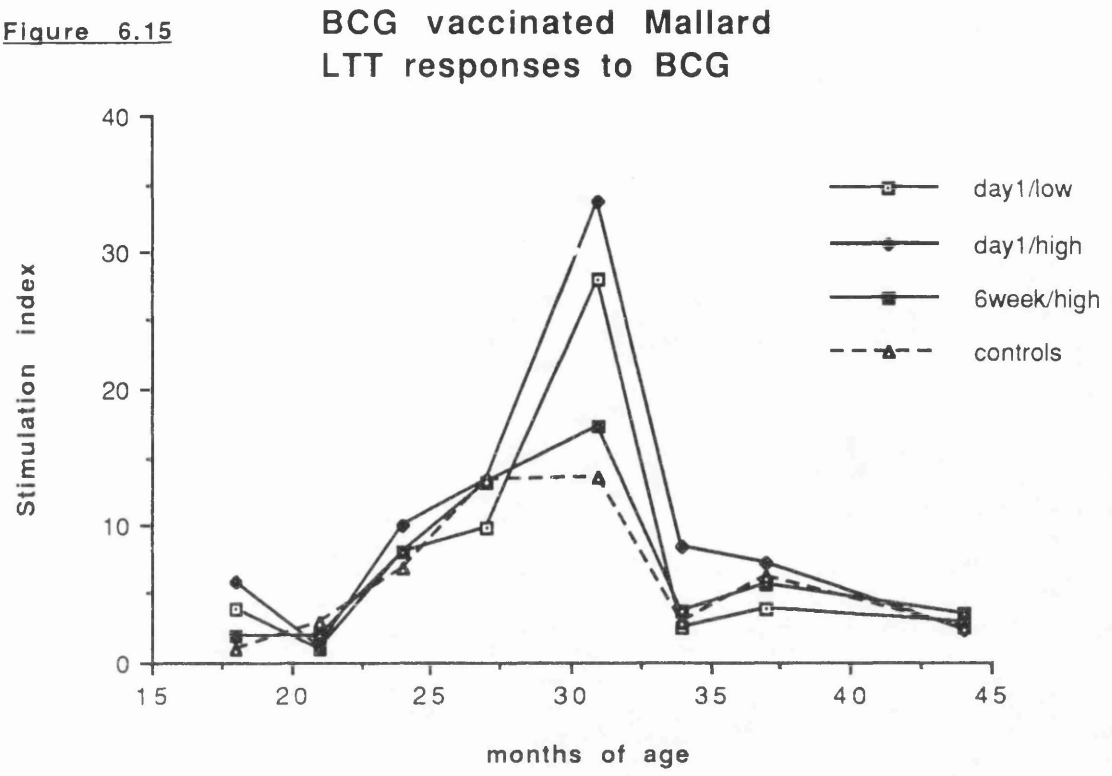
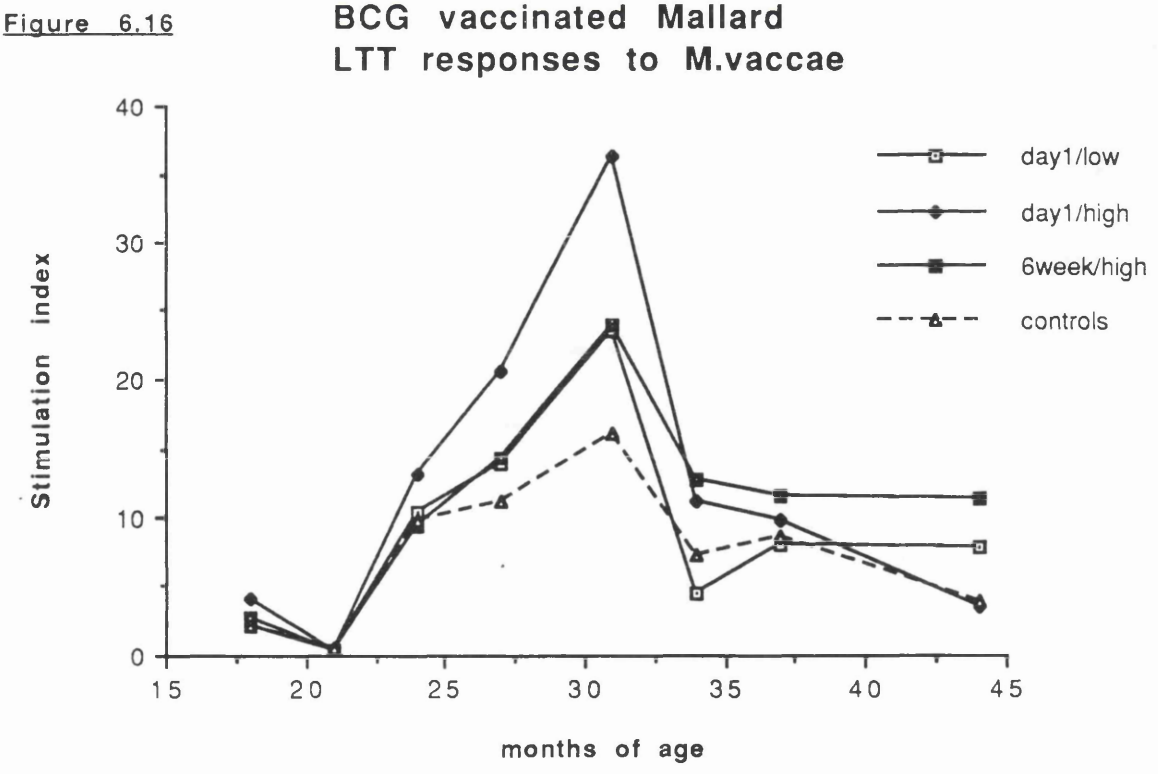


Figure 6.16



Skin Test.

Results of skin test reactions 41 hours post administration of skin test reagent are shown in figure 6.17. The results of the first three skin tests carried out when the birds were 18, 21 and 24 months of age show no difference in responses between any of the vaccine groups and the controls. However the following statistically significant responses were obtained:-

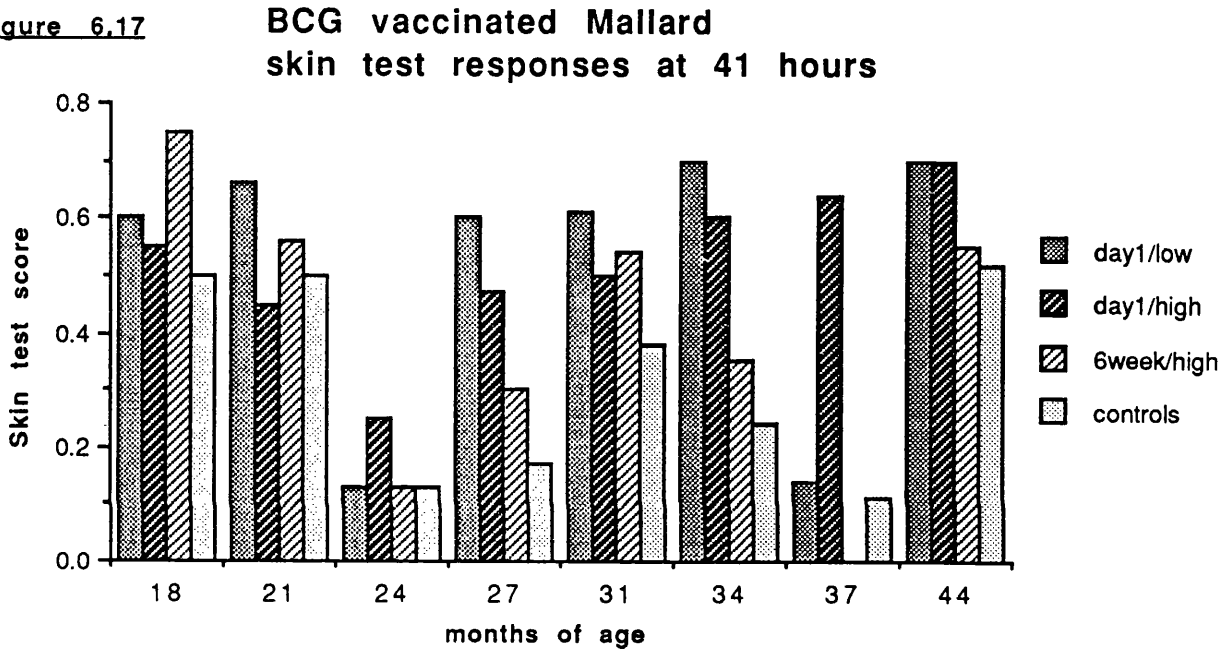
(i) the group vaccinated at one day old with the low dose of vaccine gave significantly greater skin test responses than the control group at 27 months ($p < 0.0002$) and 34 months ($p < 0.02$) using Vaccin and the avian skin test reagent respectively.

(ii) the group vaccinated with the high dose of vaccine at one day old gave greater responses than the control group at 27 months ($p < 0.0003$), 34 months ($p < 0.05$) and 37 months ($p < 0.0006$) at either one or both of the 41 and 48 hours skin test measurements.

(iii) the group vaccinated at six weeks of age with the high dose of vaccine, gave greater responses than the control group at 27 months ($p < 0.04$) and 34 months ($p < 0.002$) at the 41 and 48 hour skin test measurements respectively.

All skin test results from BCG vaccinated Mallard are summarised in appendix 6.8.

Figure 6.17


BCG vaccinated Mallard
skin test responses at 41 hours
Skin test score
0.0
0.2
0.4
0.6
0.8
18
21
24
27
31
34
37
44
months of age
day1/low
day1/high
6week/high
controls

ELISA.

Unlike the *M.vaccae* vaccinated Mallard there were no significant rises in antibody levels over time, in fact for the antibodies to BCG there was a statistically significant decrease in antibody levels comparing the first levels to the last ($p < 0.005$) as can be seen from figure 6.18.

Student's *t* tests show no significant differences in antibody levels to individual antigens between the vaccine groups and control group, and figures 6.18 - 6.23 show the marked similarity in antibody levels to all six antigens used. These results are summarised in appendix 6.9.

Like the ELISA results of the *M.vaccae* vaccinated Mallard there was a marked seasonal difference in antibody levels with raised levels in the summer and lower levels in the winter.

Figure 6.18

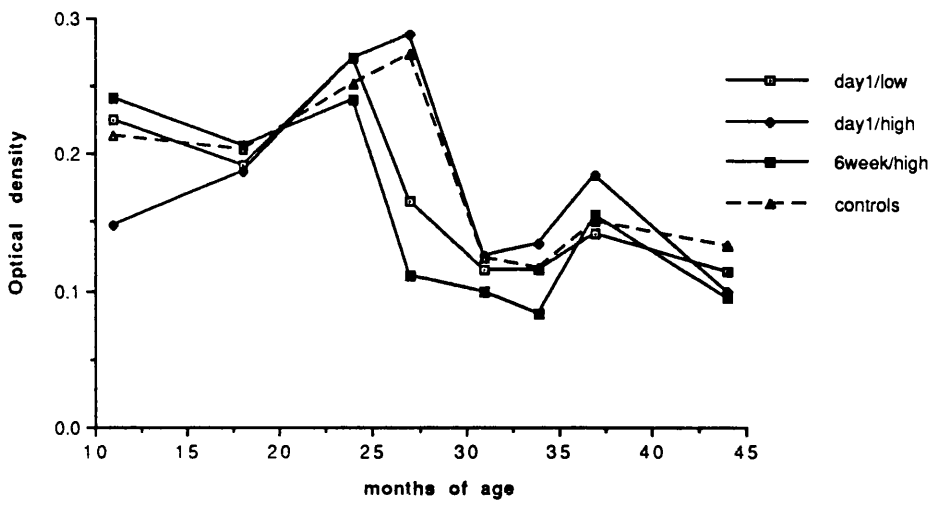


Figure 6.19

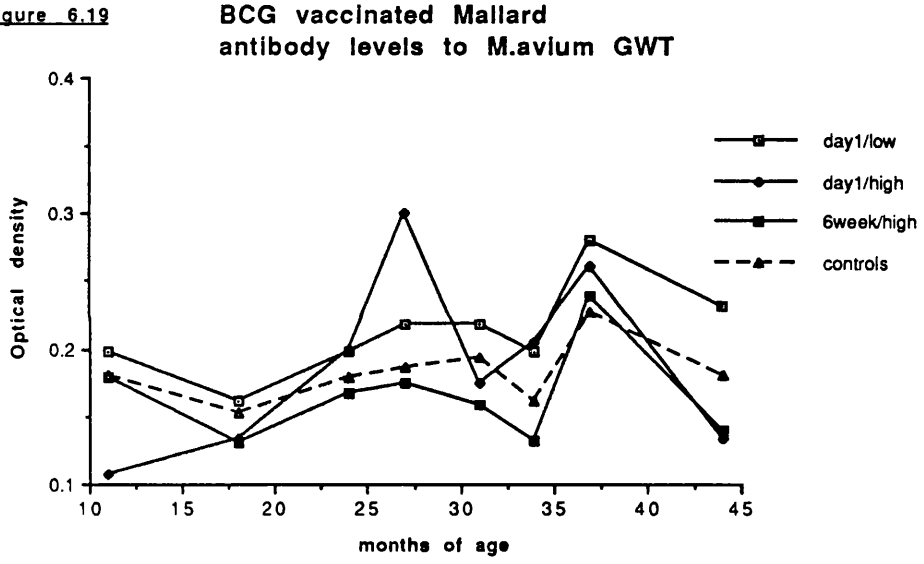


Figure 6.20

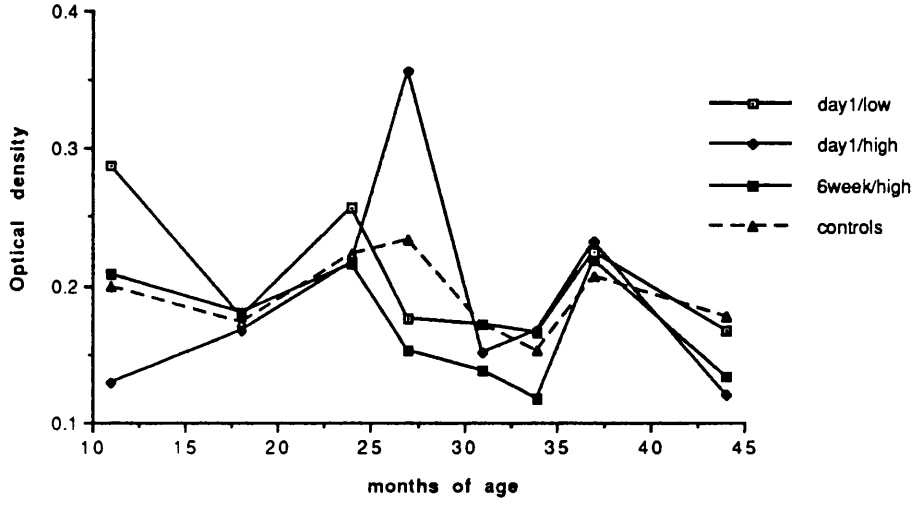


Figure 6.21

BCG vaccinated Mallard
antibody levels to M.gordonae

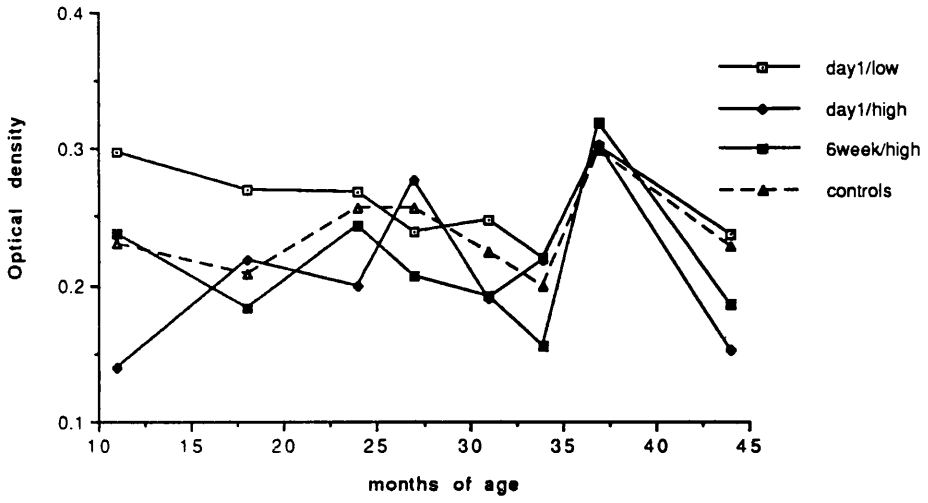


Figure 6.22

BCG vaccinated Mallard
antibody levels to M.vaccae

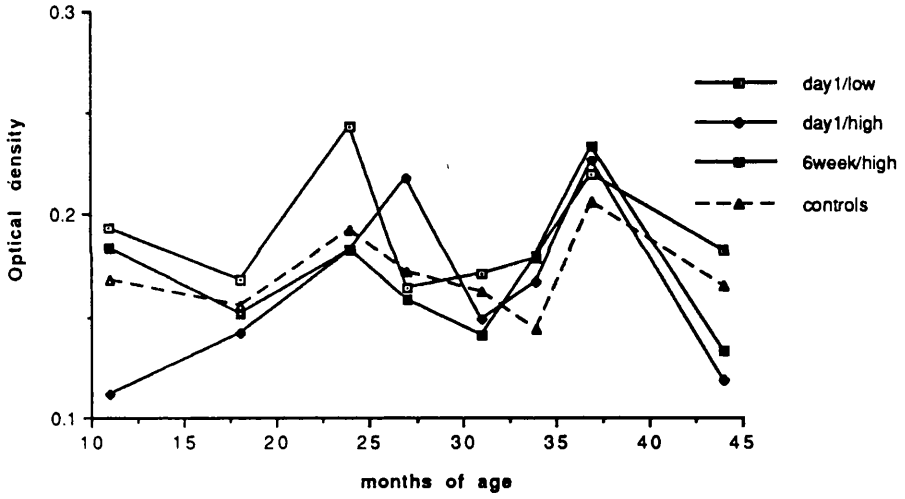
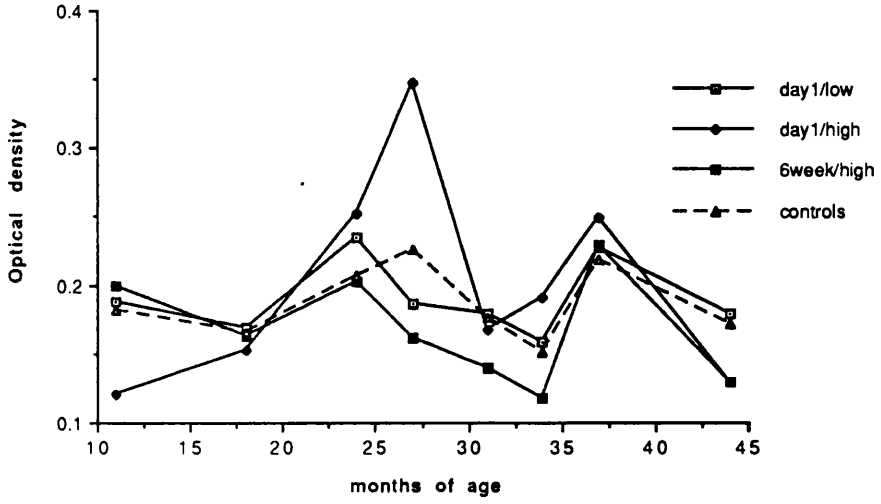


Figure 6.23

BCG vaccinated Mallard
antibody levels to M.avium RBP



Deaths caused by Tuberculosis.

The results of the tuberculous birds reveal a number of points. During the late stages of infection there were changes in immunological responses with an apparent suppression of cell mediated immunity resulting in low LTT responses, but a definite increase in humoral responses. This is typical of the situation in chronic tuberculosis and lepromatous leprosy in humans (Lenzini *et al*, 1977; Turk and Bryceson, 1971). This indicates an immunological spectrum in avian tuberculosis. This ranges from a state of immunity, through a period of variable cell mediated immune responses to a state of suppression of cell mediated immunity. In this latter stage there is an increase in humoral responses together, no doubt, with proliferation of the tubercle bacilli.

The lack of a reliable diagnostic test makes it impossible to determine at which stage the birds became infected although raised antibody levels appear to be indicative. These raised antibody levels during infection to all six of the antigens is a phenomenon encountered in both humans with pulmonary tuberculosis (Bahr *et al*, 1990a) and tuberculous badgers (Mahmood, 1985; Stainsby *et al*, 1989). Such a ubiquitous rise probably indicates responses to the shared mycobacterial antigens. In the absence of appropriate antibodies to the different classes of duck immunoglobulins it is impossible to say which classes are responsible for the rise.

In agreement with the work of Hawkey *et al* (1990) the haematology of the birds was often abnormal in the late stages of infection. Although thorough haematological examination was not carried out, simple

observations during cell separation for LTT's showed an increased total white cell count with an obvious increase in lymphocyte numbers. However, occasionally similar abnormal haematology could be found in non-tuberculous birds, possibly as a result of an unrelated infection.

Results from the *M.vaccae* vaccinated Mallard.

The results clearly show that birds vaccinated with *M.vaccae* produce stronger skin test responses and greater LTT responses than unvaccinated birds. The vaccine groups responded to both *M.vaccae* and *M.avium* skin test reagents and a variety of mycobacterial antigens in LTT's. Whilst this may be a reflection of the different mycobacterial species encountered in the environment, it may also be an indication of response to common antigens as LTT responses to all the antigens were very similar as seen from figures 6.1 - 6.6.

The vaccine group that repeatedly produced significantly greater responses in both tests was the group vaccinated at one day old with a high dose of killed *M.vaccae*. If indeed, this vaccine is acting as an immunopotentiating agent to stimulate cell mediated immunity, its effect is long lasting as significant skin test and LTT responses could still be gained at 43 months post vaccination.

In general, the groups that received the killed vaccine evoked greater skin test and LTT responses than the group receiving the live vaccine. Some possible reasons for the efficacy of killed vaccines have already been given at the beginning of this chapter. This study appears to confirm these ideas. The live *M.vaccae* appeared to be relatively incompetent at producing an increase in either LTT or skin test responses. Live vaccines are capable of producing secreted antigens *in vivo* and this ability is thought to be important for

immunogenicity. This is not born out from the results of these birds. It may even be possible that these secreted antigens are immunosuppressive, however the LTT and skin test results from the live vaccine group were no less than the control birds' so this is unlikely. The inclusion of an *M.vaccae* secreted antigen in LTT's and ELISA's may have provided some insight.

It would be thought that live *M.vaccae* may be killed or inhibited somewhat by the high avian body temperature and would therefore act in a similar way to the killed vaccine, but this is clearly not the case. Perhaps the structure of *M.vaccae* is altered during its killing with radiation. Although it is thought that the organisms remain whole during killing, some may be broken open or degrade in such a way as to expose internal antigens which then act as immunopotentiating agents. Indeed, killing mycobacteria would appear to have a qualitative effect. This was shown by Raffel (1948) who demonstrated guinea pig skin test responses were less 'Koch type' necrotic responses, following administration of a killed vaccine, compared to those responses of animals receiving a live vaccine.

The maximum skin test and LTT responses were given by those birds which were vaccinated at one day old. If these tests show a true reflection of cellular immunity it would imply that vaccination must be carried out prior to contact with environmental mycobacteria when the immune system is still fairly naive and even to some extent dependent upon maternal immunity from the egg. The effect of age on vaccination will be discussed further in the next chapter.

The significant rise in antibody levels over time is an indication of contact with mycobacteria, either pathogenic or non-pathogenic, in their environment. Such a finding was also made by Stainsby *et al*

(1989) in healthy badgers.

The relatively few statistical differences in antibody levels between the vaccine groups and controls could be viewed as encouraging with respect to a mycobacterial vaccine where humoral responses are considered inappropriate. Where there were differences, the birds vaccinated with the high dose of killed vaccine had lower levels than the control birds', indicating some degree of suppression of humoral responses rather than a mere non stimulation. The controls had particularly raised antibodies to both *M.fortuitum* and *M.gordonae* which may be an indication of these organisms being present in the environment. However, the raised antibody levels in some of the control birds may be indicative of early stages of *M.avium* infection, although in the absence of a diagnostic test this is difficult to ascertain.

The marked similarity in the antibody levels of the groups to the different antigens (figures 6.9 - 6.14) probably reflects an antibody response to the shared group i mycobacterial antigens.

Results from BCG vaccinated Mallard.

The LTT and skin test results are not as clear as the *M.vaccae* vaccinated birds. Many results showed no difference in responses from control and vaccinated birds. Where significantly different responses were given, these were to both *M.vaccae* and *M.avium* skin test reagents and BCG and *M.vaccae* in LTT's, again possibly to the common antigens. The similarity in the shape of the graphs of LTT results adds strength to this idea that it is the shared antigens that evoke cell mediated responses.

The group with the greatest number of significant differences was the group vaccinated with the high dose at one day old. As with the *M.vaccae* vaccinated birds it would appear that vaccination prior to contact with environmental mycobacteria is most capable of stimulating immune responses.

The lack of a rise in antibody levels over time is somewhat surprising, especially in view of the fact that the controls' antibody levels did not rise unlike the situation in the control birds used for the *M.vaccae* vaccinated Mallard. This may be due to the results of the tuberculous birds being removed from the data. Both of the controls that died were generally used as the controls for the BCG vaccinated Mallard.

The birds were already adults by the time the immunological monitoring began and therefore may have had antibody levels typical of those which have experienced an environment full of mycobacteria. Levels raised above this may be indicative of subclinical mycobacterial infection. The insufficient time course of this study makes this impossible to ascertain. BCG, like *M.vaccae* would appear to be ineffective at boosting humoral responses to a number of antigens. However it is possible that any differences in humoral responses were made earlier on in the time preceding this study period.

Seasonal Changes in Immunological Responses.

The results of both the BCG and *M.vaccae* vaccinated birds show a number of interesting seasonal variations. These indicate higher antibody levels in the summer and lower antibody levels in the winter. This may be as a result of the birds encountering greater numbers of

proliferating saprophytic and pathogenic environmental mycobacteria in the warmer summer months. However, the skin test responses were somewhat different from this in that responses were generally lower in the summer and higher in the winter. LTT results were less well defined with occasionally higher results in the summer and lower in winter.

CHAPTER SEVEN.

THE OPTIMISATION OF AGE FOR VACCINATION:

THE MANDARIN STUDY.

CHAPTER SEVEN.

THE OPTIMISATION OF AGE FOR VACCINATION: THE MANDARIN STUDY.

INTRODUCTION.

In the spring of 1987 the results of the Mallard study revealed no clear picture as to when vaccination should be given. Age for vaccination may be an important factor in immunising against mycobacterial infections as environmental contact prior to vaccination may be critical in how the immune system is primed. This study was carried out in the hope of defining an optimum age for vaccination.

The Mallard study indicated 10^7 killed *M.vaccae* as a potential immunising agent and so this was then used in this subsequent study.

METHODS AND MATERIALS.

Birds used for this Study.

The continuation of the use of Mallard in the studies may have provided more consistent results, however the collection at Slimbridge is already over populated with Mallard which breed very successfully and prolifically, including the study birds. If a vaccine is to be developed it must be able to afford protection in many different species. For these reasons a different species of bird was used in this study. The birds used were Mandarin Ducks *Aix galericulata*. These are members of the tribe Cairinini (perching ducks) which have been shown to be very susceptible to avian tuberculosis (Chapter three). They also provide a very colourful exhibit in the captive collection at Slimbridge.

Vaccination.

The vaccine was prepared in the same way as described previously in Chapter six. This was administered intradermally in the foot web to both one day old and six weeks old ducklings. All birds were considered healthy at vaccination. Each vaccine group contained 20 birds and 20 were left unvaccinated as controls. After vaccination the ducklings were pinioned and ringed before being released into the grounds at Slimbridge where they experienced a natural challenge.

Table 7.1. : Summary of Vaccines administered to Mandarins.

Number of Birds	Age	Vaccine	Killed	Dose
20	1 Day	*M.vaccae*	"	10^7
20	6 Weeks	"	"	"
20	_	Controls	_	_

Monitoring Vaccine Efficacy.

The birds were tested using the same three tests used in the Mallard study. These tests were performed every three months for 24 months post vaccination, and once more subsequently at 30 months. At each testing time, representatives from each of the vaccine groups and controls were caught and bled for LTT and ELISA prior to skin testing. The tests were carried out in this order for the same reasons as described for the Mallard.

As with the Mallard study, the birds were too young to take base line bleeds and skin tests as reference prevaccination points.

(i) Lymphocyte Transformation Test.

Those conditions found to be optimal for Mallard LTT also proved to be successful for Mandarin LTT's (Cromie *et al*, 1988; Cromie *et al*, 1989). The antigens used for the Mallard study were also used for the Mandarin study.

(ii) Skin Test.

The method described previously for the Mallard skin testing was also used in this study, employing the same skin test reagents.

Table 7.2 : Summary of Skin Test Reagents Used for Mandarins.

Months of age	**Skin Test Reagent**
3 months	Vaccin 20 ug/ml
6 "	"
9 "	Avumin A 2 ug/ml
12 "	*M.avium* GWT 20 ug/ml
15 "	Vaccin 20 ug/ml
18 "	"
21 "	"
24 "	"
30 "	"

(iii) ELISA.

The Mandarin sera was analysed using the same method and antigens used for the Mallard ELISA.

Statistical Tests used to Analyse the Results.

A two way analysis of variance was carried out on the SI's of the LTT results. This shows if there were any significant differences in responses to individual antigens; both over time and between groups. If there were any differences, the nature of the difference is seen from the figures and Student's *t* tests.

Fisher's exact tests were used to analyse the skin test data which had been scored as described previously for the Mallard study.

Student's *t* tests were used to analyse the ELISA data which was calculated as mean absorbances per group. Paired Student's *t* tests were used to calculate rises in antibody levels over time.

RESULTS.

Vaccination did not appear to affect the birds in any adverse way and the birds went through normal moult phases. Although the birds did not breed, this was due to nest boxes not being provided in their pens.

To date, none of the birds in this study have died of avian tuberculosis. Unlike the Mallard, there were few mortalities due to other causes. The few that did occur, were caused by intestinal obstruction and septicaemia following vaccination for duck virus enteritis.

LYMPHOCYTE TRANSFORMATION TEST.

The results of a two way analysis of variance of all the groups together showed that the responses to all the antigens differed significantly over time (BCG $p < 0.0003$; *M.vaccae* R877R $p < 0.0001$; *M.avium* RBP $p < 0.02$; *M.avium* GWT $p < 0.0001$ and *M.fortuitum* $p <$

0.01). This can also be seen from figures 7.1 - 7.3 which show the responses to BCG, *M.avium* GWT and *M.vaccae* respectively, over time.

Where the analyses showed significant differences between the groups, it was the group vaccinated at one day old that responded significantly differently than both the control group and the group vaccinated at six weeks of age to BCG 100 ug/ml ($p < 0.02$ and $p < 0.03$ respectively). However, there was interaction between this one day old vaccinated group and time in the response to *M.vaccae* R877R 100 ug/ml and *M.fortuitum* 100 ug/ml. Similarly there was interaction in the response from the six week old vaccinates to *M.fortuitum* 100 ug/ml.

When Student's *t* tests were used to analyse the data the most notable feature was the lack of significant differences in responses from the group vaccinated at six weeks of age when compared to the control group. In contrast, the group vaccinated at one day old gave significantly greater responses than the controls to a number of antigens and at different times. The earliest such response was at 12 months of age where greater responses were given to *M.vaccae* R877R 100 ug/ml ($p < 0.05$), BCG 50 ug/ml ($p < 0.05$) and *M.avium* GWT 50 ug/ml ($p < 0.05$). At 18 months of age this group gave significantly greater responses to BCG 50 ug/ml ($p < 0.05$), *M.fortuitum* 50 ug/ml ($p < 0.05$) and both concentrations of *M.avium* GWT ($p < 0.05$ and $p < 0.05$). At 24 months of age this group gave significantly greater responses to *M.fortuitum* 100 ug/ml ($p < 0.05$). However, it should be noted that the control group responded significantly more than this group to *M.avium* GWT 100 ug/ml at three months of age ($p < 0.05$), and to *M.fortuitum* 100 ug/ml at 21 months of age ($p < 0.025$).

LTT results are summarised in appendix 7.1.

Figure 7.1

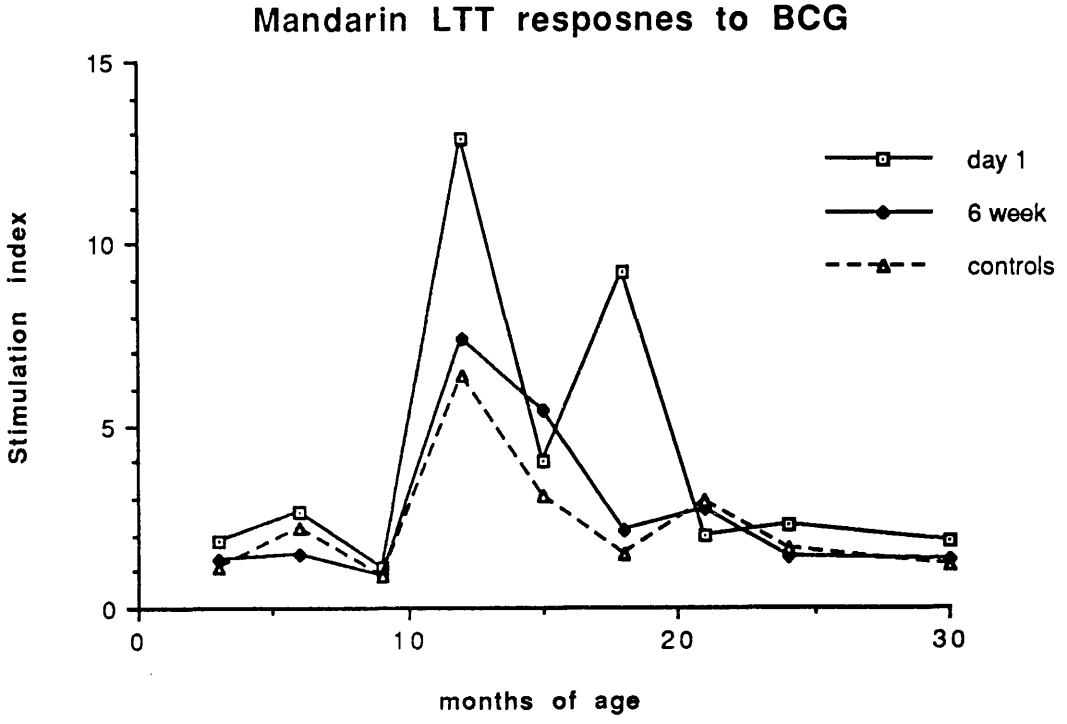


Figure 7.2

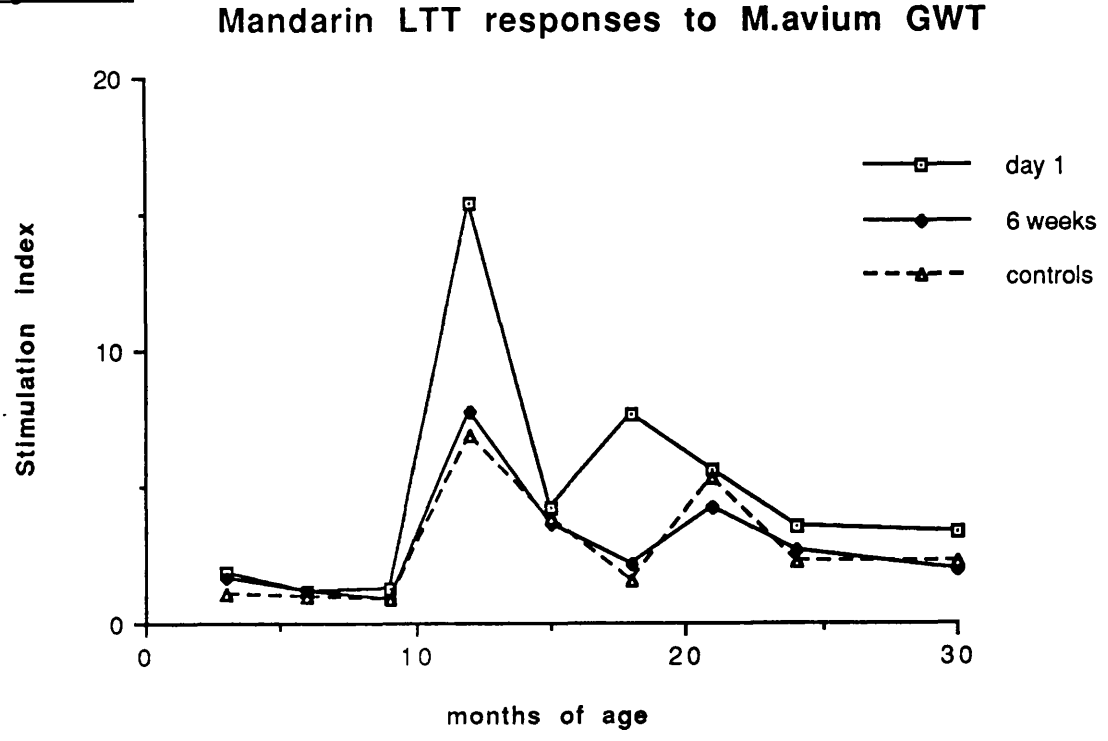
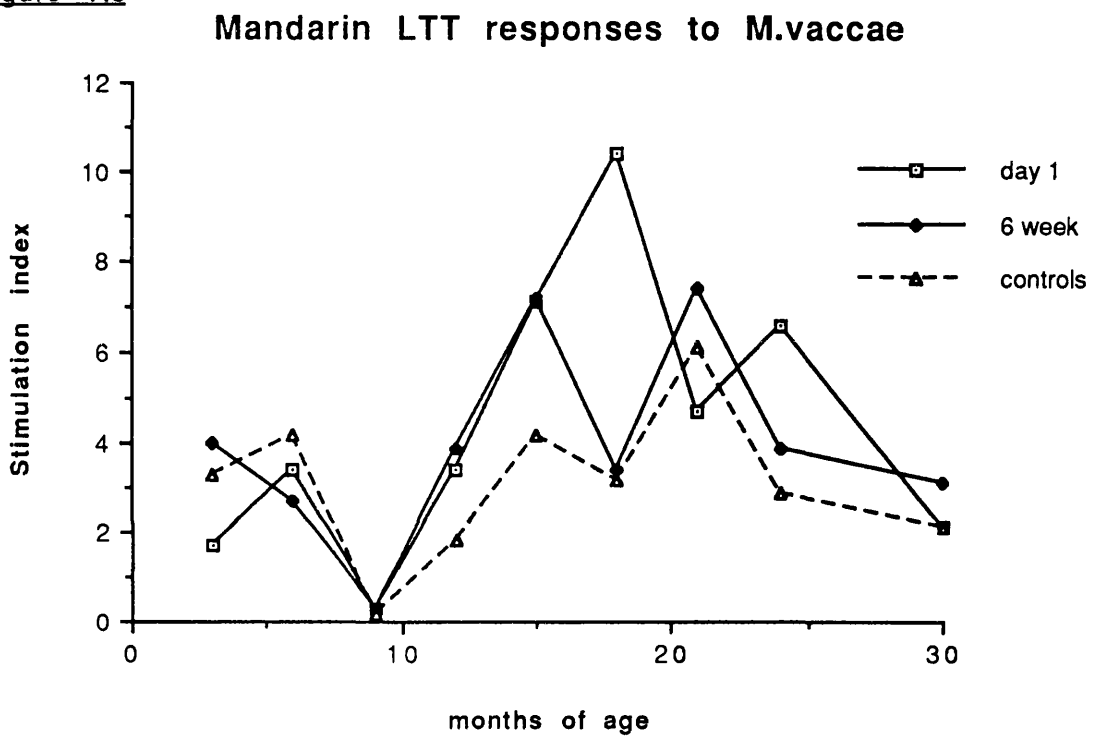


Figure 7.3


Mandarin LTT responses to M.vaccae
Stimulation index
12
10
8
6
4
2
0
0
10
20
30
months of age
day 1
6 week
controls

SKIN TEST.

The maximum responses in skin testing were obtained at 41 and 48 hours post administration of the skin test reagent. For this reason the statistical analysis has been carried out on the responses given at these two time points. Appendix 7.2 summarises the skin test results at these two, and the other time points.

Results from the Day Old Vaccinates.

(i) At 41 hours.

The most obvious feature of the results is the consistently significantly greater responses given by the day old vaccinates compared to the controls (figure 7.4). Using Vaccin 20 ug/ml, responses were greater at three months ($p < 0.004$), six months ($p <$ 05), 15 months ($p < 0.002$), 18 months ($p < 0.02$), 21 months ($p <$ 0.003) and 24 months ($p < 0.02$). At nine months a significantly greater response was given to the avian skin test reagent Avumin A 2 ug/ml ($p < 0.004$). There were only two skin tests which did not produce responses significantly higher than those of the controls. These were carried out at 12 and 30 months using the following skin test reagents: *M.avium* GWT (20 ug/ml) and Vaccin (20 ug/ml) respectively.

(ii) At 48 hours.

Fewer results gave significantly greater responses at 48 hours, those that did, include the results at nine and 21 months which were to Avumin A 2 ug/ml ($p < 0.02$) and Vaccin 20 ug/ml ($p < 0.003$) skin test reagents respectively.

Results from six week old vaccinates.

(i) At 41 hours.

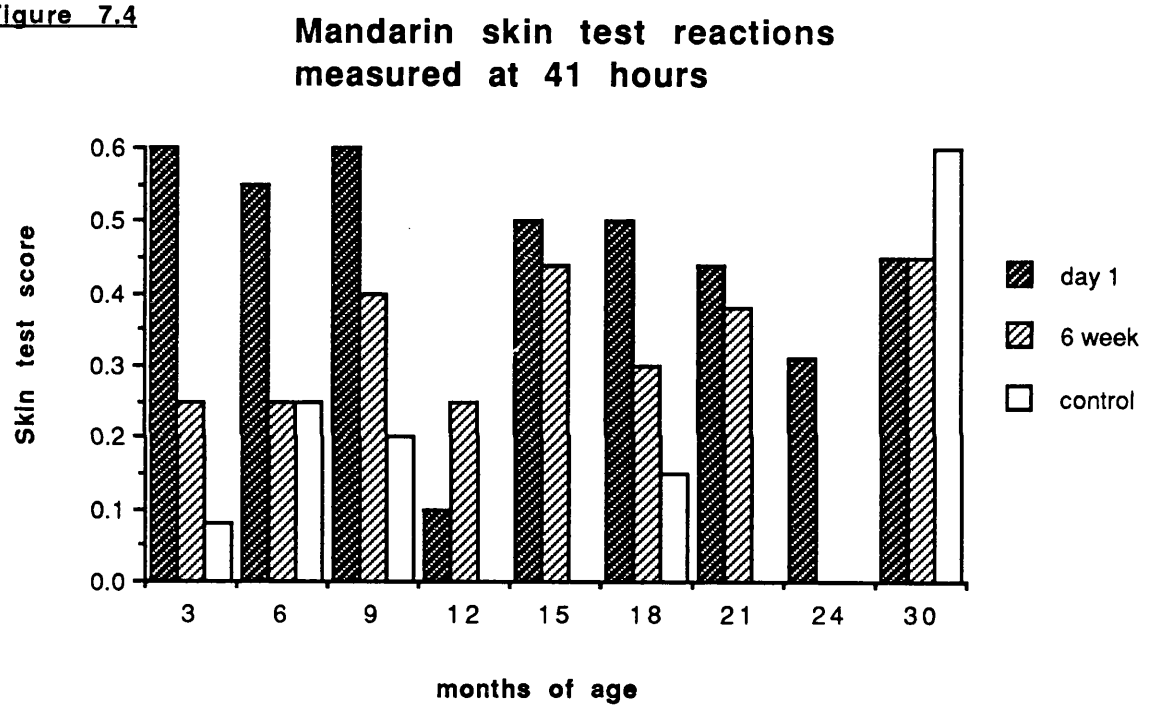
Although several of the results were significantly greater than those of the controls, they were not as consistent as the results from the one day old vaccinates (figure 7.4). Significantly greater responses were given at 12 months ($p < 0.02$), 15 months ($p < 0.003$), and 21 months ($p < 0.008$). The first of these was to the avian skin test reagents (20 ug/ml) and the latter two were to Vaccin (20 ug/ml).

(ii) At 48 hours.

The result at 15 months of age to Vaccin 20 ug/ml was the only response that was significantly greater than that of the controls ($p < 0.02$). The response at 18 months, to the same reagent, was significantly lower than that of the controls ($p < 0.004$).

When comparisons were made between the two vaccine groups, the day one vaccinates gave significantly greater responses to Vaccin at three months ($p < 0.04$), six months ($p < 0.05$), and 24 months ($p < 0.02$) at the 41 hour time point. At the 48 hour time point, the day one vaccinates responded significantly more at 18 months of age to the same skin test reagent ($p < 0.0000$).

Figure 7.4


Mandarin skin test reactions measured at 41 hours
Skin test score
0.0
0.1
0.2
0.3
0.4
0.5
0.6
3
6
9
12
15
18
21
24
30
months of age
day 1
6 week
control

ELISA.

The only rise in antibody levels over time to be demonstrated by a paired Student's *t* test was in the antibodies to *M.avium* GWT ($p < 0.05$)(figure 7.6). There were no such significant rises to the other antigens used. There were however, variations in antibody levels in relation to the seasons in both the vaccine and control groups, with higher summer levels which is shown in figures 7.5 - 7.10. ELISA results to all six antigens are summarised in appendix 7.3.

When a Student's *t* test was used to assess differences in antibody levels between groups, very few significant differences were found. The group vaccinated at one day old had significantly lower levels of antibodies to BCG than the control group at three months of age ($p < 0.05$). At six months, the group vaccinated at six weeks of age had lower antibody levels to *M.fortuitum* ($p < 0.025$) and *M.gordonae* ($p < 0.05$) than the control group. However at 24 months of age, this situation had been reversed and the vaccine group had higher antibody levels to almost all antigens with the exception of *M.avium* RBP (BCG $p < 0.01$; *M.fortuitum* $p < 0.01$; *M.gordonae* $p < 0.05$; *M.avium* GWT $p < 0.05$; *M.vaccae* $p < 0.05$).

Figure 7.5

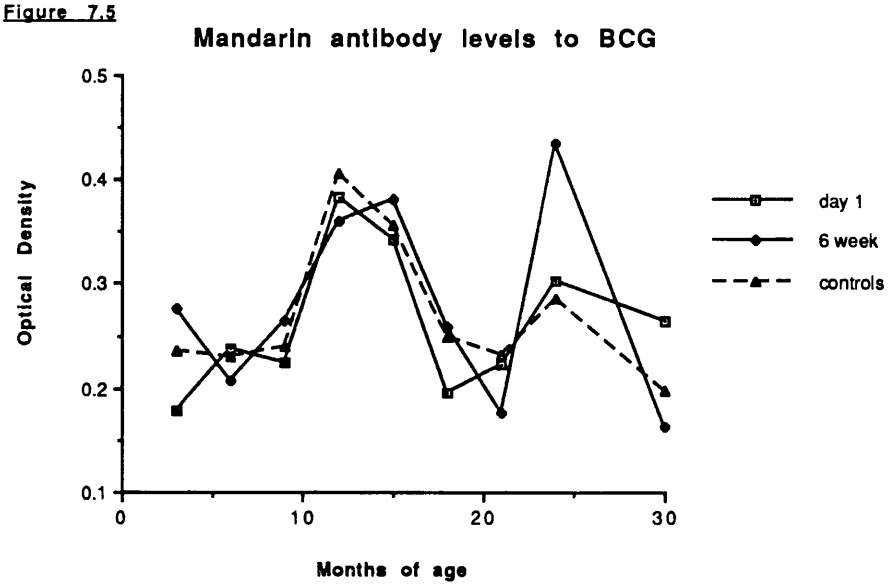


Figure 7.6

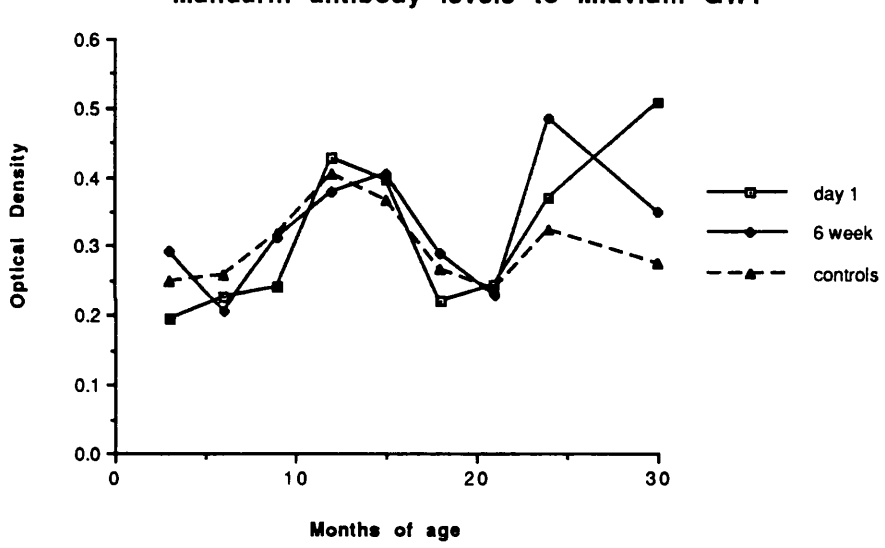


Figure 7.7

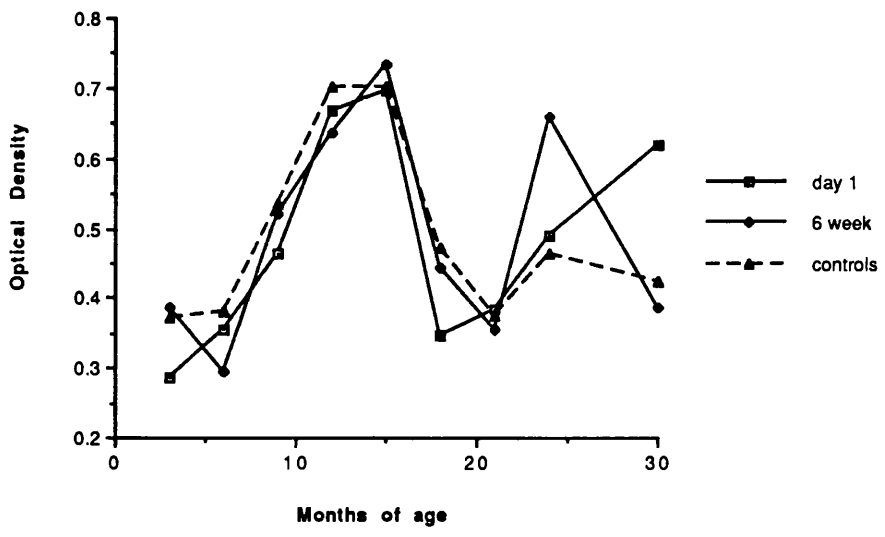


Figure 7.8

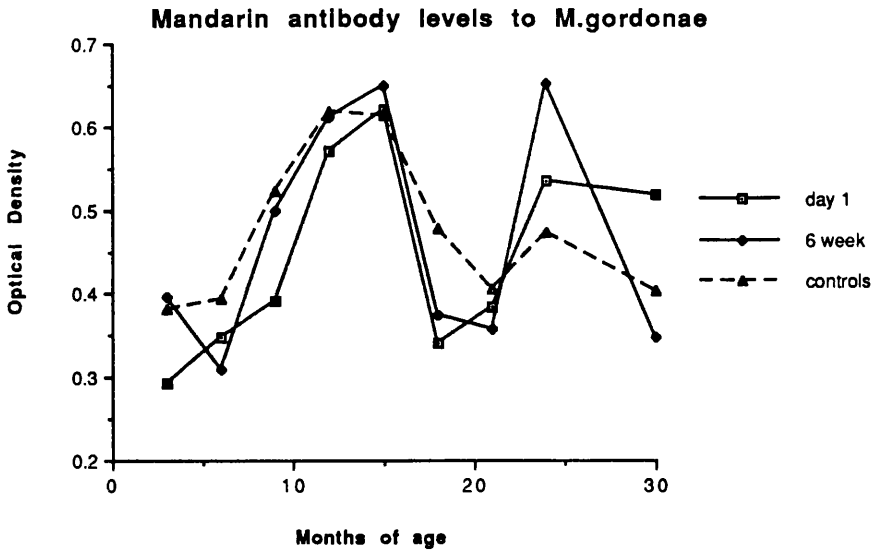


Figure 7.9

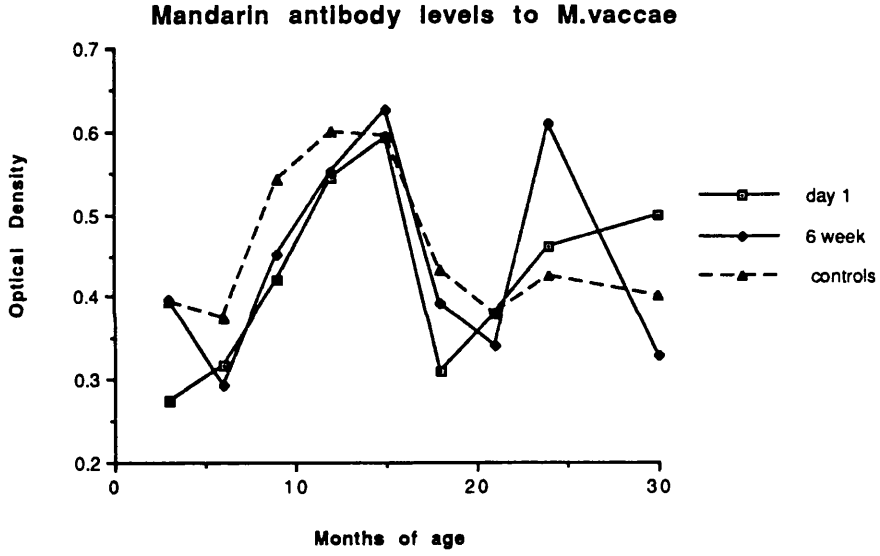
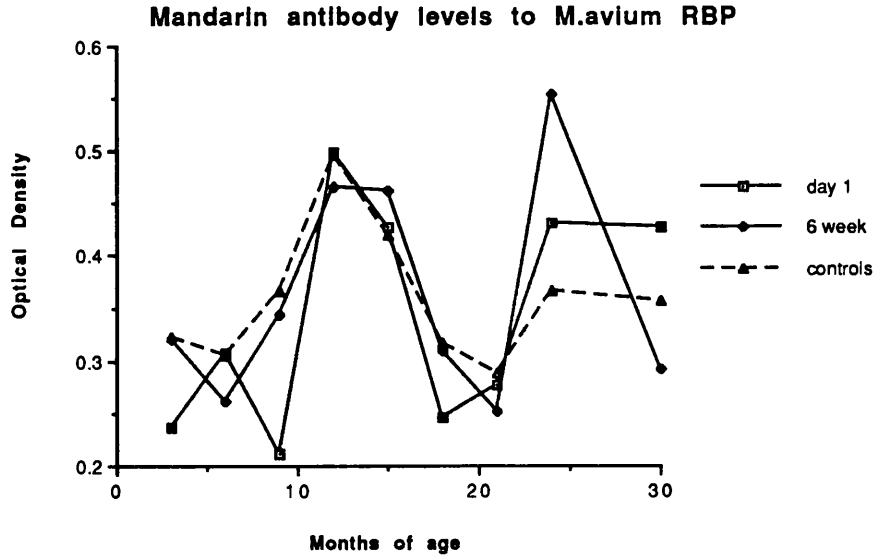


Figure 7.10



DISCUSSION.

Vaccination with killed *M.vaccae* at one day old significantly increases LTT and skin test responses to a number of mycobacterial antigens. Vaccination at six weeks of age does not produce such a marked effect. Significant differences in responses were recognised up to two years post vaccination. After this time the LTT and skin test responses by both the vaccine groups and the controls were equally positive which may be an indication of all the birds' exposure to frequent antigenic stimulation from environmental mycobacteria. Prior to this, strong recognition of the LTT antigens and skin test reagents had been restricted to the vaccinated birds.

It is perhaps surprising that there were no significant differences in LTT responses until 12 months of age and that the control birds had greater responses to *M.avium* GWT at three months of age.

The lack of a significant difference in skin test response of the one day vaccinates at 12 months of age, when using the avian skin test reagent (GWT 20 ug/ml), was unexpected as three months previously this group had responded strongly to the other avian skin test reagent (Avumin A 2 ug/ml).

The significant rise in antibody levels of both the vaccine and control groups to *M.avium* GWT, may be an indication of this organism's presence in the environment at Slimbridge. The lack of significant differences in antibody levels between the vaccine and control groups points to killed *M.vaccae* R877R being relatively incompetent at stimulating humoral responses. This however, is not discouraging with respect to a mycobacterial vaccine as antibody production in response to mycobacterial challenge is generally considered inappropriate.

The marked similarity in responses to the antigens used in both LTT's and ELISA's (figures 7.1 - 7.3 and 7.5 - 7.10; appendices 7.1 and 7.3) probably indicate responses to common antigens.

The seasonal variation in antibody levels may be an indication of the presence of high levels of both pathogenic and saprophytic mycobacteria in the warmer summer months due to their proliferation in the environment. This could then result in greater humoral responses at this time.

This study indicates that if cell mediated immune responses are to be stimulated, vaccination must be given prior to environmental contact with mycobacteria. In areas of high levels of endemic *M.tuberculosis* infection, vaccination of humans with BCG is usually carried out at birth whereas in areas of low risk of infection, vaccination is usually carried out at a later stage. However, there is some evidence that BCG vaccination may be more effective in humans that are a few weeks old than in newborns. This may be associated with carry over of maternal immunity, which would not complicate the situation to such an extent, in ducklings. The level of *M.avium* in the environment at Slimbridge is almost certainly very high and the results from this study advocate vaccination early in life.

Discrepancies in the protection afforded to humans by BCG have been put down to a number of factors including this apparent 'vaccination' by other environmental mycobacteria prior to the administration of the BCG vaccine. This effect has been blamed for the failure of the BCG trial in South India (Tuberculosis prevention trial, Madras, 1980). Various trials involving the skin testing and subsequent vaccination of children have revealed similar situations. These results showed that protection was afforded to children vaccinated in Ahmednagar,

India, an area of low levels of sensitisation (Stanford *et al*, 1987a). Conversely, vaccination of children in Agra, India, an area of high contact with environmental mycobacteria, was considered ineffective (Stanford *et al*, 1987b). However, the most encouraging results from this latter trial came from the youngest age groups, i.e. those with least environmental sensitisation. Differences in the protection afforded by BCG and skin test responses were obtained even within the same city of Bombay, depending upon the patients previous contact with mycobacteria (Stanford *et al*, 1988b). The mouse model proposed by Brown *et al* (1985) also showed the effect of previous sensitisation with mycobacteria (oral *M.vaccae* in this case) to adversely influence the results of BCG vaccination.

Although the group of birds vaccinated at six weeks of age were not out in the grounds at Slimbridge for their first six weeks of life, they would have had contact with environmental mycobacteria, mainly from their water supply. Subsequent vaccination may then mobilise inappropriate immune responses (Stanford, 1983b). This may first, account for the reduced responses in this group of birds and second, if one can extrapolate from the human situation, be an indication of protective responses in those birds with little prior contact with mycobacteria i.e. the one day old vaccinates. The lack of deaths from avian tuberculosis in this study means that it is impossible to say whether or not these responses are indeed protective and this will only be shown in time.

Vaccination of ducks against other diseases seems to be most effective when administered within the first weeks of life. The work of Weingarten (1989) on a potential vaccine for duck virus enteritis, suggests that if birds are going to be exposed to infection from early

on in life, then they should be vaccinated when newly hatched. This is very similar to the situation at Slimbridge where the birds may be in contact with pathogenic or saprophytic mycobacteria from the moment they hatch. Weingarten's work also indicated that the protection afforded from duck virus enteritis was not necessarily due to neutralising antibodies as immune birds had very low levels of these immunoglobulins. If this was instead, an indication of cell mediated immune responses this would agree with the results obtained from this Mandarin study and may be linked to the presence of the largest relative size of the thymus in the newly hatched bird.

Other infections in which antibody production is closely related to immune status also advocate vaccination early in life. Timms and Marshall (1989) report protection from experimental *Pasteurella anatipestifer* infection being afforded to ducklings when vaccinated at 14 days old, although at five weeks old the ducklings had developed natural resistance to infection. Protection from *P.anatipestifer* is dependent upon antibody production and vaccination prior to 14 days led to interference with maternal antibodies. Floren *et al* (1988) carried out similar work on a vaccine for the same infection and found solid immunity in those ducklings vaccinated at ten days old. Vaccination prior to this resulted in lower levels of detectable serum antibodies. Perhaps vaccination of the Mandarins at six weeks old leads to antibody production whereas vaccination at one day old stimulates alternative cell mediated responses. ELISA results do not confirm this but differences in antibody levels may have been found if the birds had been bled prior to the first three month bleed. However, Davis and Hannant (1987) demonstrated protective humoral responses in ducklings four days post vaccination, when vaccinated at two days old with live duck hepatitis virus vaccine.

CHAPTER EIGHT.

THE OPTIMISATION OF A VACCINE DOSE FOR DUCKS AND GEESE:

THE GADWALL .....

.....AND NENE STUDY.

CHAPTER EIGHT.

THE OPTIMISATION OF A VACCINE DOSE FOR DUCKS AND GEESE: THE GADWALL AND NENE STUDY

INTRODUCTION.

From both the Mallard and Mandarin studies an optimum age for vaccination has been established. However, it is possible that those doses of vaccine used to date may have been inappropriate or at least sub-optimal. The *M.vaccae* vaccinated Mallard study indicated the 10^7 bacilli dose as superior to the lower 10^6 bacilli dose at stimulating skin-test and LTT responses. The higher of these two doses was used in the Mandarin study but it is possible that this was still too low.

The work of Potter (1942; cited by Weiss, 1959) suggests that larger doses of bacilli may be necessary when using a killed vaccine. This seems feasible as the presence of antigens of live vaccines may persist in the host for some time due to the vaccine multiplying and/or secreting antigens. Such an effect will not be true in the case of killed vaccines. It is therefore possible that a similar quantitative effect is only achieved when larger doses are used.

If a vaccine is to be used in all species of wildfowl it may be necessary to alter the dose in view of the enormous difference in body size. This study aims to determine an optimum dose of vaccine in both ducks and geese.

The previous vaccine studies in Mallard and Mandarins, have used *M.vaccae* killed by γ-irradiation. Both Weiss (1959) and Rook (1980) report the use of killed vaccines being immunogenic regardless of the manner in which they are killed. Heat has been used extensively as a

method of killing vaccines, as has autoclaving (Petroff, 1923). Results from vaccination of badgers have shown autoclaved *M.vaccae* to be immunogenic (Stainsby, 1989). The vaccines used in this Gadwall and Nene study were killed by autoclaving.

MATERIALS AND METHODS.

Birds used in the Study.

A species of duck and goose were used in this study as representatives of the Anatidae as a whole. The ducks used were Gadwall *Anas strepera*. These are small European dabbling ducks in the tribe Anatini and like the Mallard, are at risk of *M.avium* infection.

The goose chosen for this study was the rarest goose in the world: the Hawaiian Goose or Nene *Branta sandvicensis*. These rather unusual, largely terrestrial, geese have a population of about 400 on their native Hawaii. Their numbers were as low as about 50 birds during the 1940's but they have bred well in captivity at Slimbridge since 1952 and their captive numbers have increased dramatically. Although they are members of the Anserini they are at greater risk of infection due to their feeding habit of cropping vegetation close to the ground. They also may be relatively more susceptible to avian tuberculosis as they have evolved for a hot climate and a barren volcanic environment where mycobacterial immunity may be of less importance than in more freshwater habitats.

Vaccination.

The vaccine was prepared as for the previous trial, but instead of γ-irradiation being used to kill the organisms, the vaccines were diluted to the appropriate concentration and then autoclaved at 121°C

at a pressure of 15 lbs per inch^2 for 15 minutes to kill the bacilli. The vaccines were stored at 4°C until they were required.

All the birds were vaccinated intradermally in the foot web at one day old. Both the Gadwall and the Nenes were given one of three doses of killed *M.vaccae* R877R, either 10^7, 10^8 or 10^9 bacilli. Each vaccine group contained 10 birds with 10 birds left unvaccinated as controls. As for the birds in the other trials, they were pinioned and ringed after vaccination, and then released into the grounds at Slimbridge.

It was decided to monitor these birds for six months following vaccination and then to observe their subsequent fates. This decision was made as a compromise as there is limited space within the collection for suitable pens to house the large numbers of birds being used in the studies. Latterly, the Gadwall were kept for a further three months. After these monitoring periods, all the birds were released within the grounds. As all were individually ringed, any birds that died would hopefully be recovered for PM examination.

Immunological Tests.

The same three tests used previously for assessing the immune responses to the vaccines were used together with the same antigens. As in the prior studies, representatives from each group were tested every three months post vaccination. Likewise, the birds were too young to skin test or take blood for baseline references.

(i) Lymphocyte Transformation Test.

The conditions developed for Mallard lymphocyte transformation were satisfactory for the Gadwall lymphocytes (Cromie *et al*, 1989). This is not surprising as these birds are closely related and both belong to

the genus *Anas*. However, when these conditions were tried for the Nene lymphocytes, thymidine uptake was low in both stimulated and unstimulated cells. A variety of methods were tried to overcome this problem.

Blood was collected and transported as described for the Mallard blood. The same method was used for lymphocyte separation but the cells were cultured in 10% CVPDS and 10% autologous sera. Cells were also cultured at two different cell concentrations; 4 x 10^5 cells/well and 8 x 10^5 cells/well in 96 well microtitre trays (Nunc). From observations of the cells in culture, the culture medium became very yellow after three days culture indicating actively metabolising cells causing acidic conditions. Microscopic observations also showed a large amount of cell proliferation within the first two and three days. For this reason a three day incubation was tried as well as four days, to see if the kinetics of Nene lymphocyte transformation were different from those of ducks'.

Results.

The cells cultured at 4 x 10^5 cells/well either died or produced very low counts per minute. The results of the culture of cells at 8 x 10^5 cells/well, in different sera (table 8.1) showed that culture in 10% autologous sera gave higher SI's due to higher counts per minute in stimulated wells. Microscopic observations of the cells cultured in CVPDS showed that the cells were 'crinkled' and unhealthy, and the low n values in table 8.1 are a result of many of the cells dying. Radiolabelling the cells after three days' incubation gave higher counts per minute than four days' incubation.

Table 8.1 : LTT results of Nene lymphocytes in 10% CVPDS and autologous sera at 8 x 10^5 cells/well.

Stock Antigen Concentration ug/ml	10% Autologous sera Mean cpm (Mean SI)	SD	n.	10% CVPDS Mean cpm (Mean SI)	SD	n.
M.vaccae						
400	676 (1.8)	346 (0.4)	5	149 (0.9)	26 (0.1)	2
100	796 (1.7)	799 (1.6)	5	172 (0.9)	47 (0.4)	3
BCG						
400	540 (1.3)	494 (1.0)	4	170 (1.0)	58 (0.2)	2
100	1088 (2.6)	950 (2.0)	5	121 (1.1)	104 (0.4)	3
M.fortuitum						
400	585 (1.2)	583 (1.1)	4	155 (0.9)	49 (0.4)	3
100	1557 (2.9)	1521 (3.0)	4	211 (1.2)	37 (0.4)	3
M.avium						
400	222 (0.7)	113 (0.7)	5	126 (0.7)	19 (0)	3
100	1248 (2.7)	1437 (2.7)	5	250 (1.4)	42 (0.4)	3
Control unstimulated wells	528	235	5	189	28	3

(See footnote from table 5.1)

Discussion.

The poor results obtained from the culture of the Nene lymphocytes in CVPDS is probably a consequence of this sera being obtained from White Pekin ducks which are genetically very different from Nenes. Like the duck LTT, the Nene LTT requires a high cell concentration, although goose lymphocytes are somewhat larger than ducks' they still require close cell to cell contact to ensure transformation in response to antigenic stimulation. The only difference in lymphocyte transformation responses appears to be the kinetics, and thereafter

Nene lymphocytes were harvested after three days of incubation (Cromie *et al*, 1989).

(ii) Skin Test.

The same method of skin testing developed for the Mallard was used for both the Gadwall and the Nenes. An unexpected practical problem in skin testing Nenes was discovered when reading the reactions, as their feet are black and not very translucent this obscured any area of erythema. This was overcome using a strong lamp which is usually used for candling eggs. When the Nenes' feet were placed over this lamp the skin test reactions were illuminated.

Table 8.2 : Summary of Skin Test Reagents Used in Gadwall and Nene.

(i) Nene.

Months of age	Skin Test Reagent
3 months	*M.avium* GWT 40 ug/ml
6 "	Vaccin 20 ug/ml

(ii) Gadwall.

Months of age	Skin Test Reagent
3 months	Vaccin 20 ug/ml
6 "	"
9 "	"

(iii) ELISA.

The same method developed for the Mallard ELISA was used for both species of bird. The conjugated anti-duck antibody worked satisfactorily in the Nene ELISA and it was considered that raising a specific anti-goose antibody was unnecessary.

Statistical Tests used to Analyse the Results.

As before, a two way analysis of variance was carried out on the SI's of LTT results. Student's *t* tests were also used to compare mean SI's.

Skin test results were scored as before and Fisher's exact tests were used to analyse the data.

Student's *t* tests were used to analyse the ELISA data calculated as mean absorbances per group. Paired Student's *t* test were used to measure any overall rise in antibody levels over time.

RESULTS.

There were no deaths of any of the birds in this study due to either tuberculosis or other cause of mortality.

Vaccination did not appear to affect the birds in any adverse way.

LYMPHOCYTE TRANSFORMATION TEST.

From the two way analysis of variance of the Nene results, there were no significant differences between the responses of vaccine and control groups to any of the antigens over time, with the exception of the responses to *M.avium* RBP 100 ug/ml ($p < 0.04$). No significant differences between the vaccine and control groups were observed to any of the antigens.

Unlike the Nenes, the two way analysis of variance showed that there were significant differences in the responses of the Gadwall vaccine and control groups to all the antigens over time ($p < 0.0001$ for all the antigens except the response to *M.avium* RBP $p < 0.02$).

Between individual vaccine and control groups the following statistically significant differences were found:-

High dose.

The only significant difference from the two way analysis of variance between individual Gadwall vaccine and control groups, was that of the response of the high dose vaccinated group to *M.vaccae* R877R 100 ug/ml ($p < 0.005$).

Student's *t* tests of Nene results showed that at six months post vaccination, this vaccine group responded significantly better than the control group to all the antigens, at either one or both concentrations (*M.vaccae* 100 ug/ml $p < 0.05$; BCG 100 ug/ml $p < 0.01$; BCG 50 ug/ml $p < 0.05$; *M.fortuitum* 100 ug/ml $p < 0.01$; *M.fortuitum* 50 ug/ml $p < 0.05$; *M.avium* GWT 100 ug/ml $p < 0.05$ and *M.avium* RBP 100 ug/ml $p < 0.005$).

With Student's *t* tests at six months in the Gadwall experiment, the high dose vaccine group responded significantly more than the control group to:- *M.vaccae* R877R 100 ug/ml ($p < 0.05$); *M.vaccae* R877R 50 ug/ml ($p < 0.025$); BCG 100 ug/ml ($p < 0.05$); *M.fortuitum* 100 ug/ml ($p < 0.025$) and *M.avium* GWT 100 ug/ml ($p < 0.025$).

Middle Dose.

The following statistically significant results were obtained from Student's *t* tests:-

At three months of age, the Nene middle dose vaccine group gave significantly greater responses than the control group to all the antigens, with the exception of *M.fortuitum*, at one or both concentrations (*M.vaccae* R877R 100 ug/ml $p < 0.05$; *M.vaccae* R877R 50 ug/ml $p < 0.025$; BCG 50 ug/ml $p < 0.05$; *M.avium* GWT 50 ug/ml $p < 0.025$ and *M.avium* RBP 100 ug/ml $p < 0.05$). At six months of age this vaccine group gave significantly greater responses than those of the controls to both *M.vaccae* R877R 100 ug/ml ($p < 0.05$) and *M.fortuitum* 100 ug/ml ($p < 0.025$).

At the three month experiment, the response of the Gadwall middle dose vaccine group was significantly greater than the control group to the *M.avium* RBP 100 ug/ml antigen ($p < 0.05$). Three months later, at the six month experiment this group gave significantly greater responses to *M.avium* GWT 100 ug/ml ($p < 0.05$).

Low dose.

The following statistically significant results were obtained from Student's *t* tests:-

The Nene low dose vaccine group did not differ significantly from the controls for any antigen or at any time. However, the Gadwall low dose vaccine group responded significantly more than the control group at the six month experiment to:-*M.vaccae* R877R 100 ug/ml ($p < 0.025$); *M.vaccae* R877R 50 ug/ml ($p < 0.01$); BCG 100 ug/ml ($p < 0.01$); *M.fortuitum* 100 ug/ml ($p < 0.025$); *M.avium* GWT 100 ug/ml ($p < 0.025$) and *M.avium* GWT 50 ug/ml ($p < 0.05$).

Nene LTT responses to *M.avium* RBP and *M.vaccae* over time are shown in figures 8.1 and 8.2. Gadwall LTT responses to *M.avium* GWT and *M.vaccae* over time are shown in figures 8.3 and 8.4.

The Nene and Gadwall results are summarised in appendices 8.1 and 8.4 respectively.

Figure 8.1

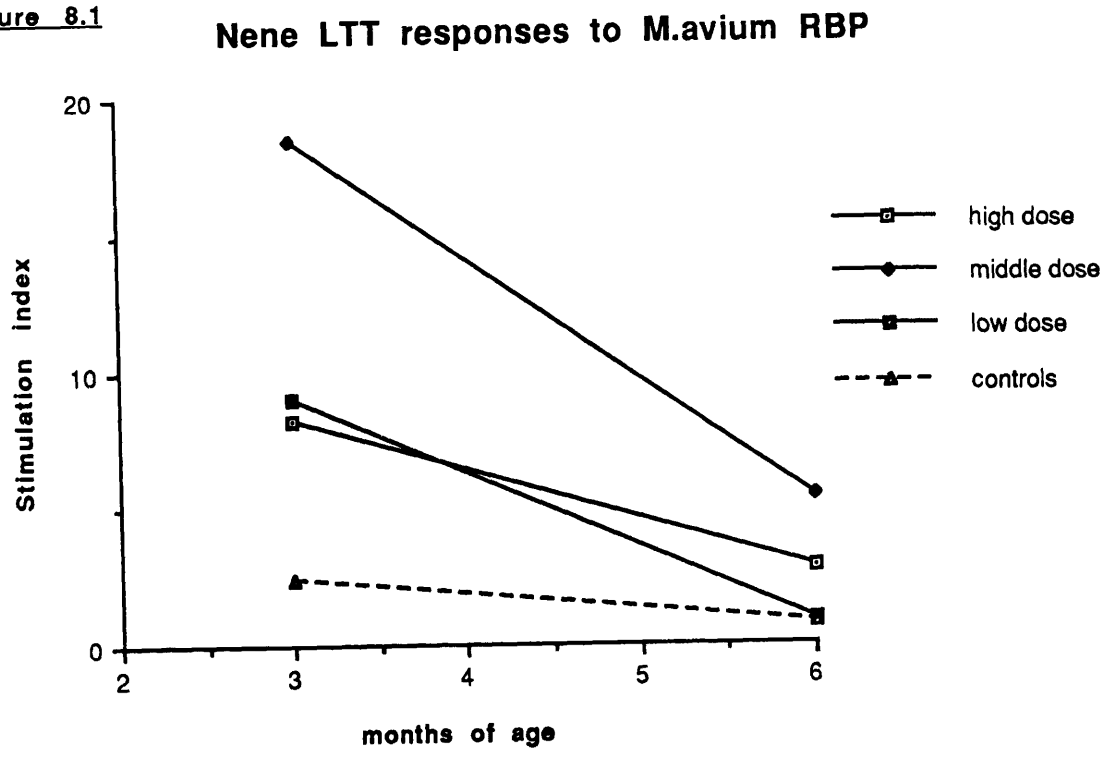


Figure 8.2

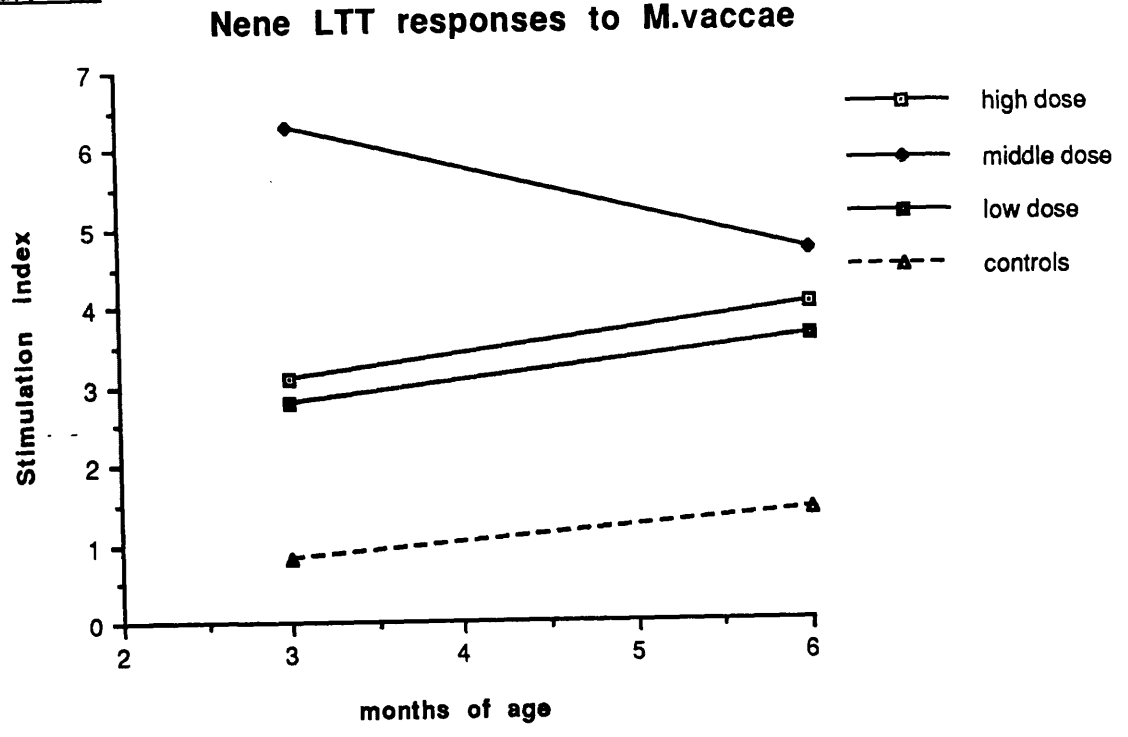


Figure 8.3

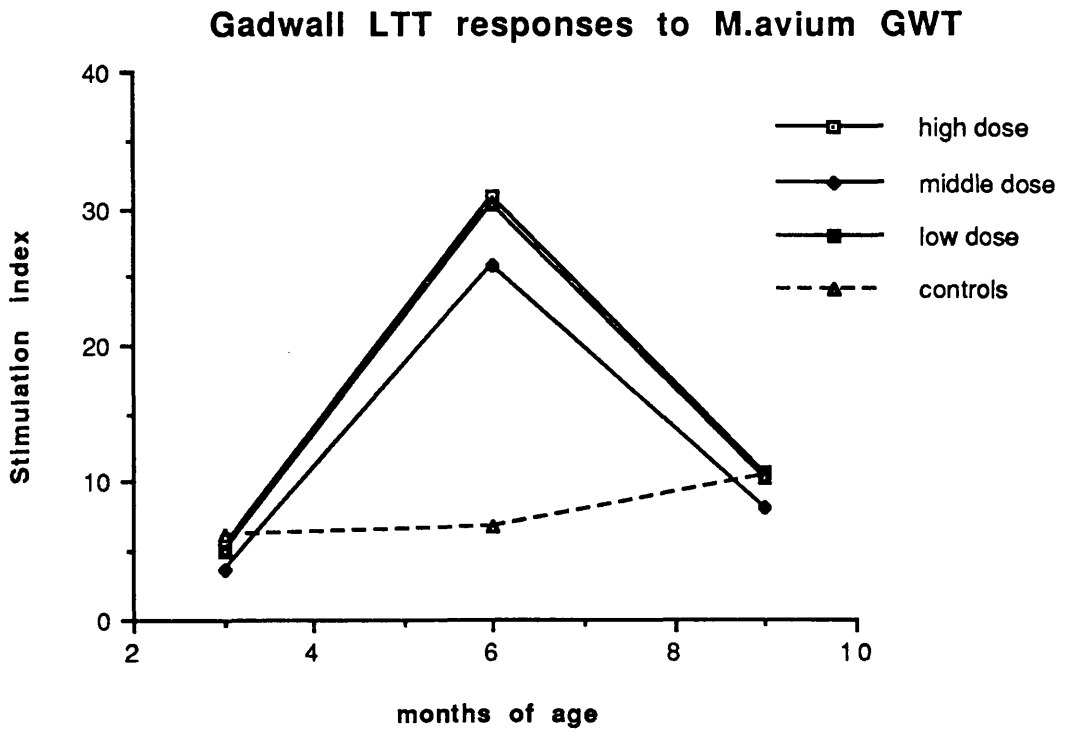
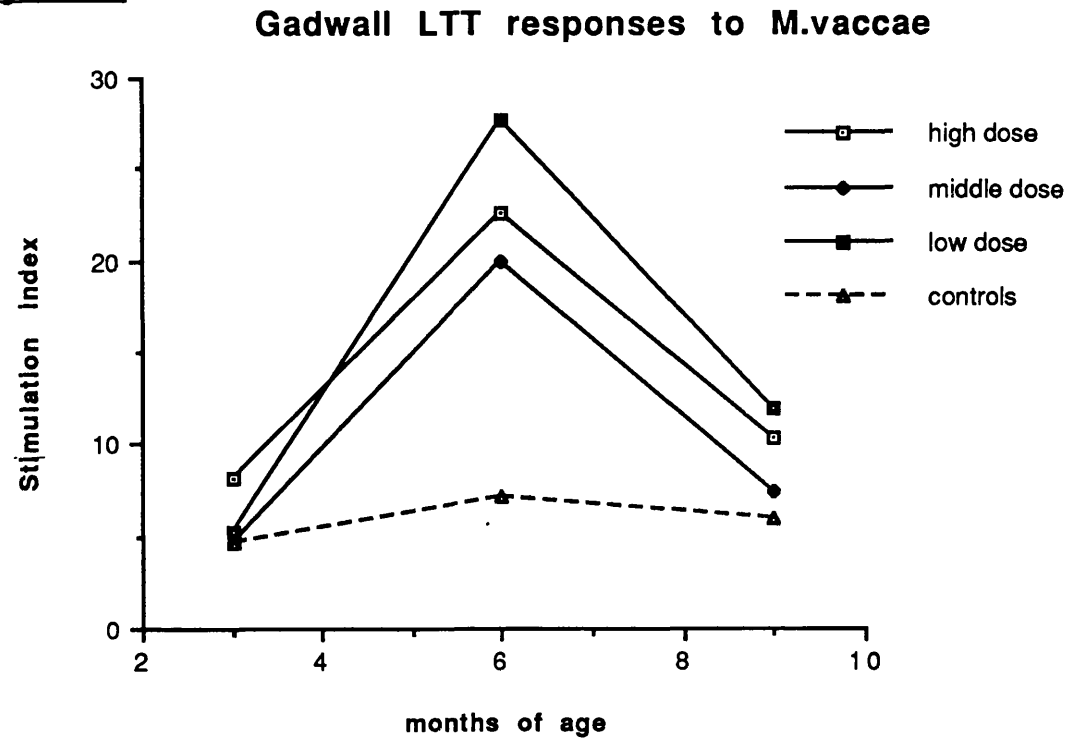


Figure 8.4



SKIN TEST.

High dose.

At the three month experiment the Nene high dose vaccine group gave responses significantly greater than the controls' to the avian skin test reagent at the 41 and 48 hour measurements ($p < 0.01$ and $p < 0.0000$ respectively). The responses at these two measurements were also greater than those of the middle and low dose vaccine groups (41 hours: $p < 0.0003$ in both cases; and 48 hours: $p < 0.0003$ and $p < 0.0007$ respectively).

The responses of the Gadwall high dose vaccine group did not differ significantly from the control birds at any of the three skin tests with the exception of the response measured at 48 hours at the three month experiment, where responses were less than the unvaccinated birds ($p < 0.02$). However significantly greater responses than those of the low dose group, were elicited at three months old to Vaccin 20 ug/ml ($p < 0.02$) at the 41 hour measurement.

Middle dose.

The Nene middle dose vaccine group did not differ significantly from the control group at either of the skin tests.

The Gadwall middle dose vaccine group responded significantly more than the control group at the first skin test at the 41 hour measurement ($p < 0.01$) and at nine months at the 48 hour measurement ($p < 0.01$). At the first test this groups' responses were also greater than the responses of the low dose group ($p < 0.0004$) at the 41 hour measurement, and at 48 hours, the responses were greater than both the high and the low dose vaccine groups ($p < 0.0008$ and $p < 0.007$

respectively).

Low dose.

The Nene low dose vaccine group responded significantly less than the control group to the avian skin test reagents at three months old at the 41 and 48 hour measurements ($p < 0.04$ and $p < 0.01$ respectively).

At the nine month skin test the Gadwall low dose vaccine group responded significantly more than the control group ($p < 0.006$) at the 48 hour measurement.

The results of Nene skin tests at both three and six month time points are shown in figure 8.5 and Gadwall skin test results over time are shown in figure 8.6.

Nene and Gadwall skin test results are summarised in appendices 8.2 and 8.5 respectively.

Figure 8.5

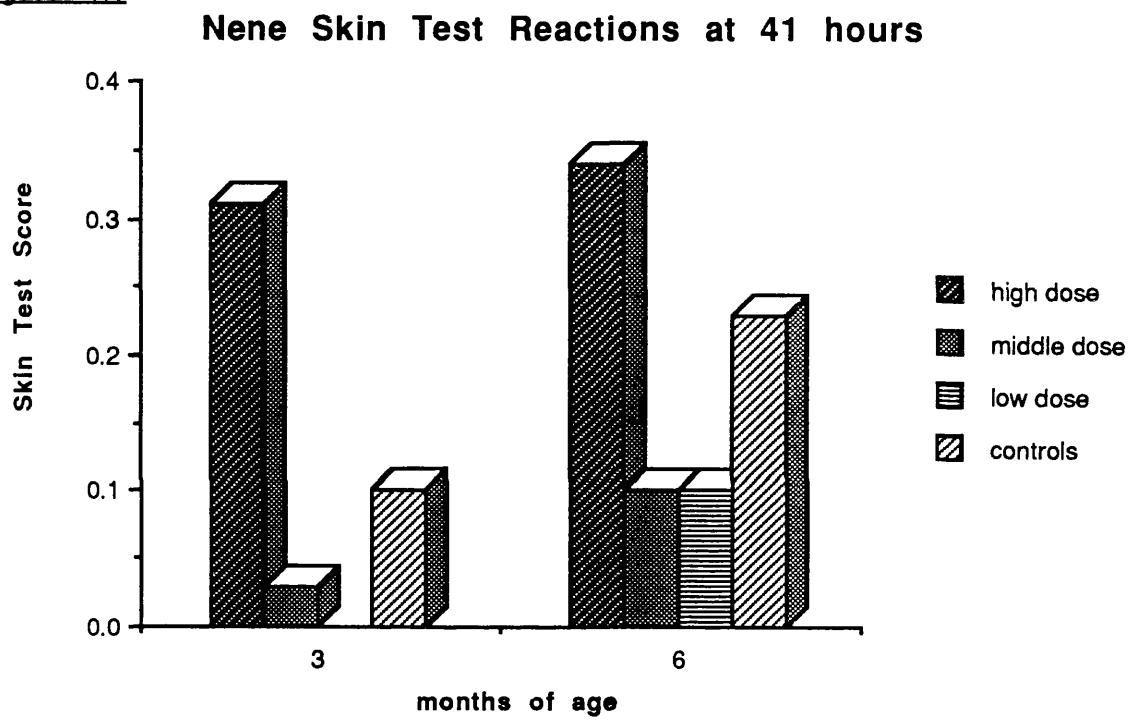
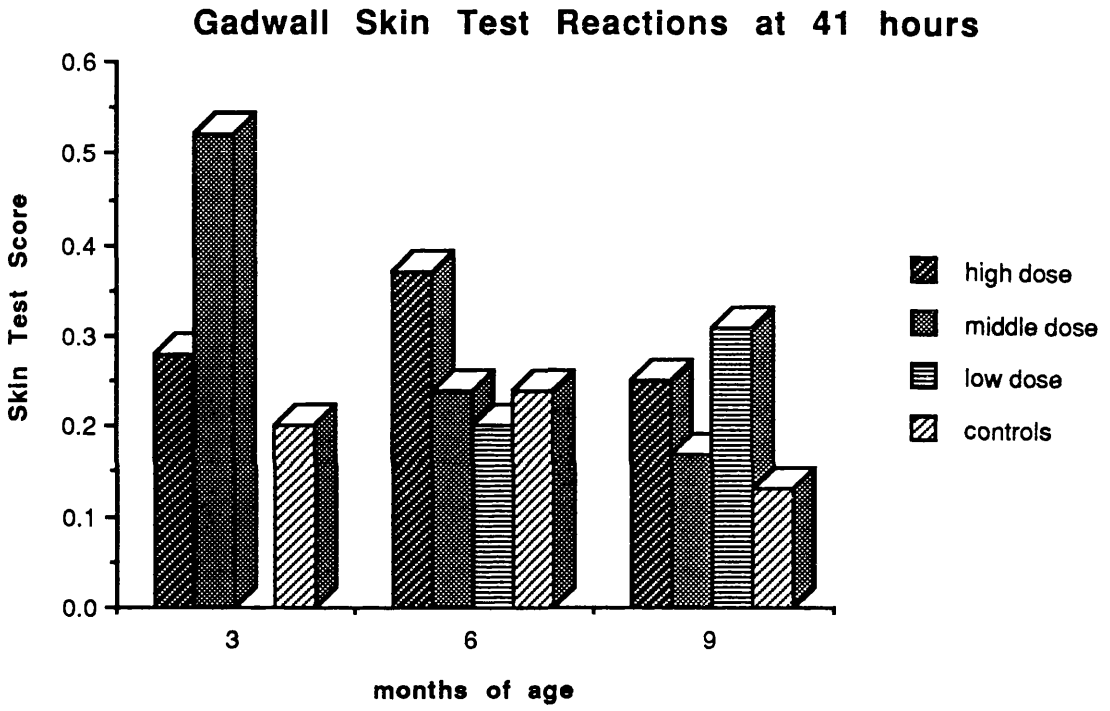

Nene Skin Test Reactions at 41 hours
Skin Test Score
0.4
0.3
0.2
0.1
0.0
3
6
months of age
high dose
middle dose
low dose
controls

Figure 8.6


Gadwall Skin Test Reactions at 41 hours
Skin Test Score
0.6
0.5
0.4
0.3
0.2
0.1
0.0
3
6
9
months of age
high dose
middle dose
low dose
controls

ELISA.

The paired Student's *t* test of Nene results showed that there was a statistically significant rise in antibody levels to all antigens over time regardless of vaccine group ($p < 0.005$ for all the antigens). There was also a statistically significant rise in Gadwall antibody levels over time to BCG ($p < 0.025$), *M.gordonae* ($p < 0.05$), *M.vaccae* ($p < 0.05$)(figure 8.9) and both *M.avium* GWT ($p < 0.01$)(figure 8.10) and RBP ($p < 0.025$). Antibody levels to *M.fortuitum* did not rise significantly over time, although the results show an upward trend (appendix 8.6).

The results of Student's *t* tests on the Gadwall antibody levels of the different vaccine groups at different time points show no significant differences between vaccine and control groups to any of the antigens at any of the time points. Similarly, the Nene results showed that there were no differences between any of the antibody levels of the vaccine and control groups at three months old.

However, at the subsequent test the following significant differences were found:-

High dose.

The Nene high dose vaccine group had significantly higher antibody levels than the controls to five of the six antigens (BCG $p < 0.025$, *M.gordonae* $p < 0.025$, *M.avium* GWT $p < 0.025$, *M.vaccae* R877R $p < 0.05$ and *M.avium* RBP $p < 0.05$).

Middle dose.

At the six month test, the Nene middle dose vaccine group had higher levels than the controls to BCG ($p < 0.025$), *M.gordonae* ($p < 0.05$), *M.avium* GWT ($p < 0.025$), *M.vaccae* R877R ($p < 0.025$) and *M.avium* RBP ($p < 0.05$).

Low dose.

Nene antibody levels between the low dose group and the control group did not differ significantly.

Nene and Gadwall ELISA results are summarised in appendices 8.3 and 8.6 respectively.

Nene and Gadwall antibody levels to *M.avium* GWT and *M.vaccae* over time are shown in figures 8.7 - 8.10.

Figure 8.7

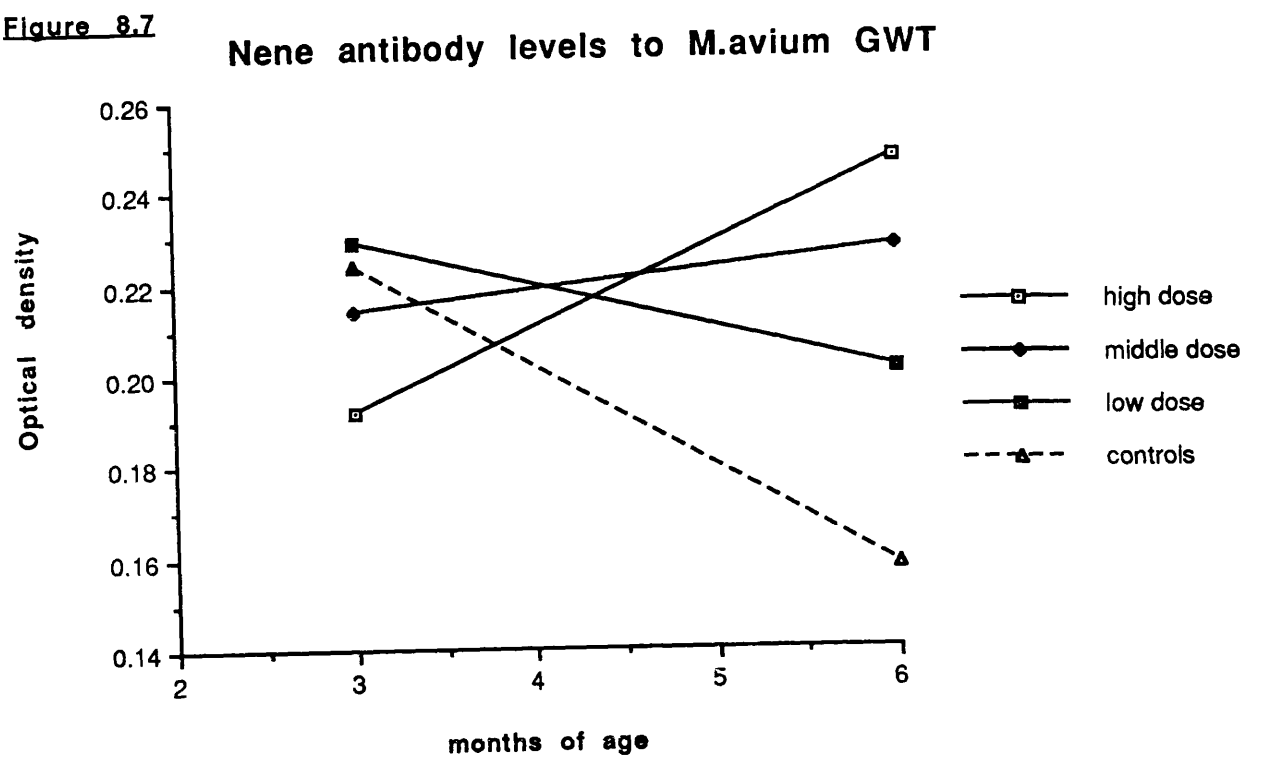


Figure 8.8

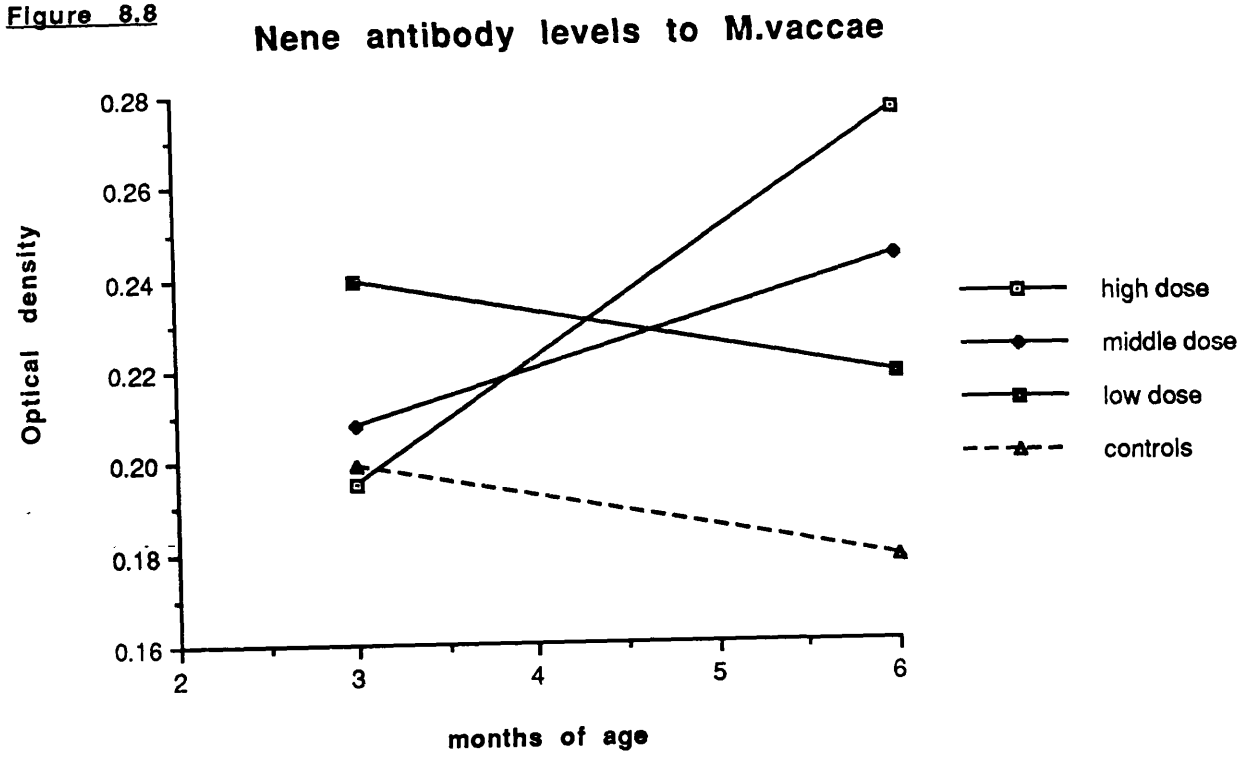


Figure 8.9

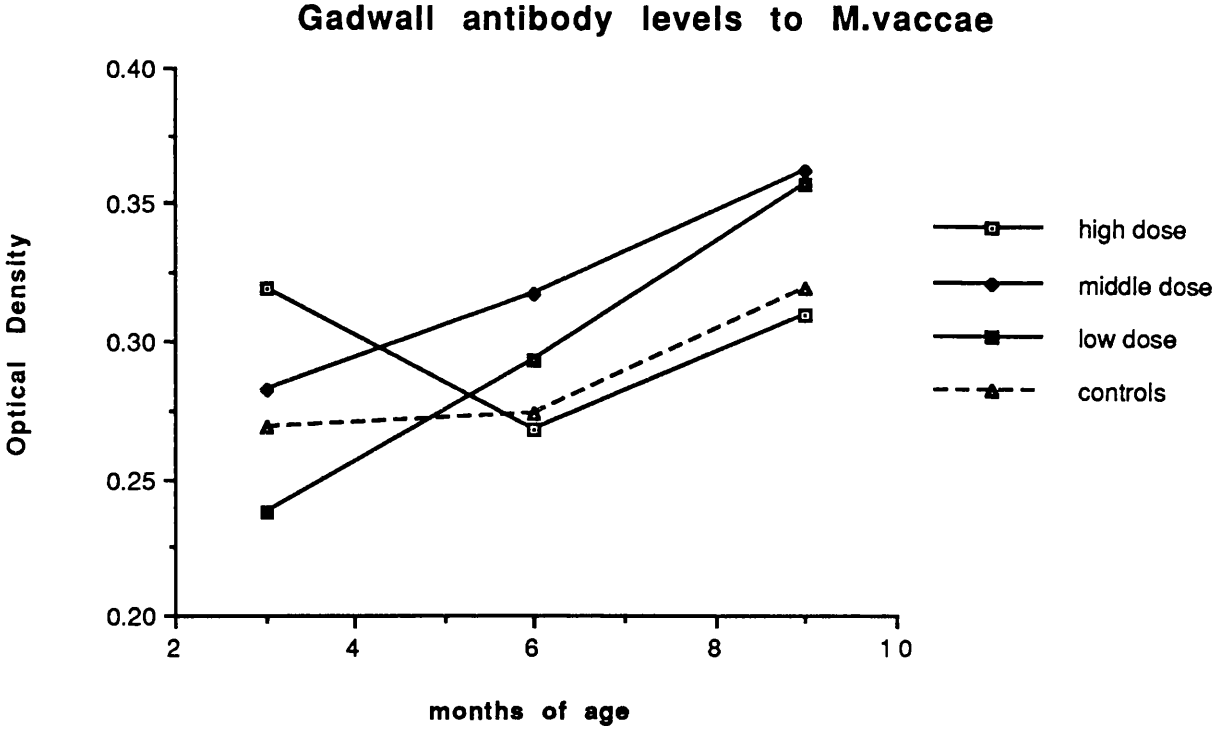
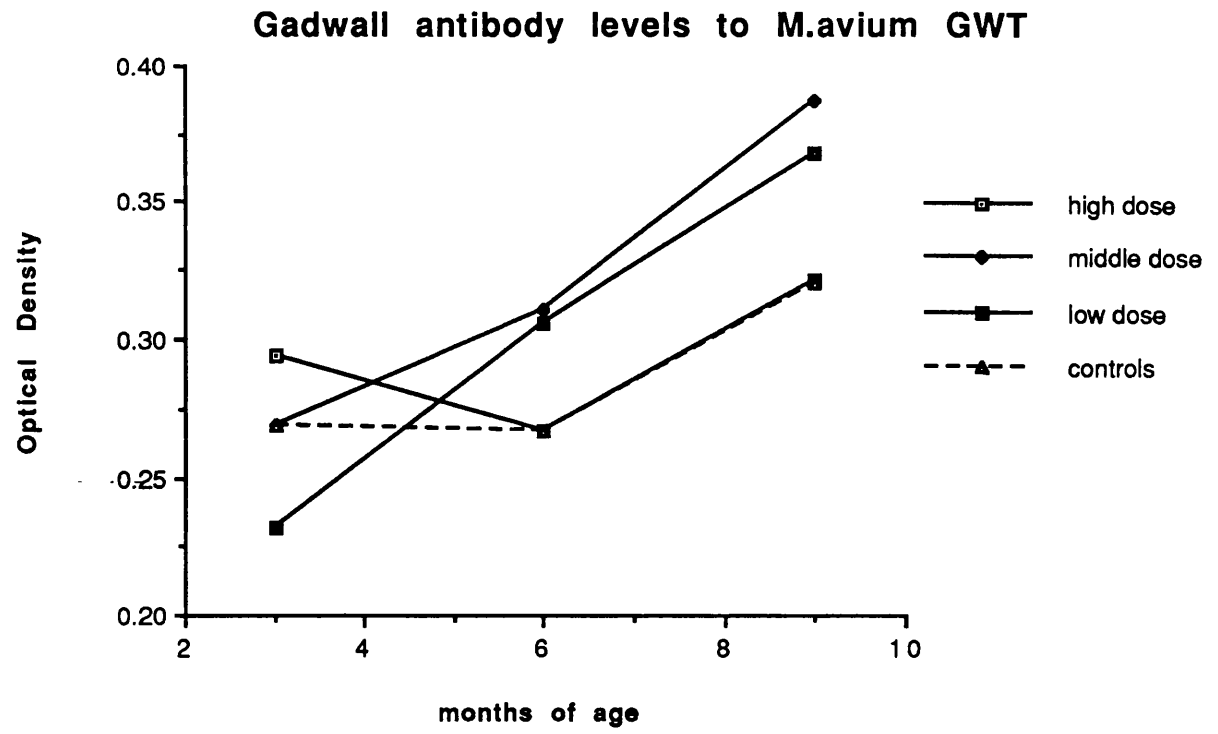


Figure 8.10



DISCUSSION.

This study was limited somewhat by its short time span, however sufficient results were gained to provide an overall picture showing that the high dose of 10^9 killed *M.vaccae* was required for optimal boosting of cell mediated responses in both Gadwall and Nene. This high dose of vaccine gave the most consistently encouraging responses. This study also shows that autoclaved *M.vaccae* has similar immunogenicity to γ-irradiated bacilli.

The lack of significant differences from the two way analysis of variance of the Nene LTT results may be due to the small number of time points, reducing the likelihood of significant differences. However, the Student's *t* tests of these results indicate both the high and middle doses of vaccine as capable of inducing greater responses than unvaccinated birds to a number of antigens. The low dose of vaccine appeared to have no effect in that responses did not differ significantly from those of the controls'. This situation is reflected to some extent in the skin test results with the exception that the middle dose group did not differ from the control group. A high dose of vaccine would therefore seem to have the greatest immunopotentiating ability.

The results from the Gadwall are similar to those of the Nenes'. As with the Nenes, there were few time points in the two way analysis of variance which reduces the likelihood of significant differences being found. However, the high dose was shown by both the two way analysis of variance and Student's *t* tests to be capable of increasing *in vitro* responses in LTT's to a number of antigens. Unlike the Nenes, the low dose of vaccine also increased lymphoproliferative responses.

Surprisingly, the skin test responses did not reflect the LTT results, instead the middle dose of vaccine group produced the greatest skin test reactions although the LTT responses from this group were relatively poor.

The significant rise in antibody levels to almost all the antigens in both species of bird is probably a reflection of the naive immune systems encountering both saprophytic and pathogenic environmental mycobacteria for the first time. A similar situation was seen in personal unpublished work carried out with Dr. D.A. Higgins, of Hong Kong University. The results of successive ELISA's of sera from newly hatched White Pekin ducklings showed a drop in anti-mycobacterial antibodies during the first ten days of life, followed by a gradual increase over time in both control birds and those vaccinated with 10^9 killed *M.vaccae* R877R. The initial drop was probably due to a decrease in maternal antibody levels and then the rise would be due to the individual bird producing its own antibodies in response to environmental antigenic stimuli.

ELISA results to individual antigens at each time point from both the Nene and the Gadwall were somewhat different. None of the vaccines produced measurable humoral responses from the Gadwall. The same was true at the first test of the Nenes but subsequently at six months of age both the high and middle doses of vaccine appeared to boost antibody responses to all the antigens with the exception of *M.fortuitum*. This is somewhat surprising but may be explained to some extent by the different times at which the birds hatched. Nenes breed earlier in the year and the birds used in this study were three months older than the Gadwall. From ELISA results of the other ducks in these trials, seasonal variations have been found with peak antibody levels

in the summer. It was late summer when the Nenes were six months old and gave increased antibody responses (it was winter when the Gadwall were six months old). Perhaps the vaccine did boost humoral responses during this time when it seems that there could be a proliferation of environmental mycobacteria.

The similarity in the antibody levels to the different antigens shown in figures 8.7 - 8.10 and is probably a response to common antigens.

Rook (1980) reviews the use of killed vaccines and emphasises the need for higher doses than of live vaccines, to promote protective responses. The Gadwall and Nene study reinforces this and agrees with the work of Potter (1942; cited by Weiss, 1959). Petroff and Stewart (1925) reported requiring large doses of killed mycobacteria administered intraperitoneally to guinea pigs to provide protection from *M.tuberculosis* infection. Similar protection was afforded to rabbits if repeated doses of killed mycobacteria were given (Freund and Opie, 1938). Mackaness also showed the need for higher doses of killed mycobacteria, than comparable live vaccines, to afford protection to rabbits and guinea pigs (1967; 1968).

In mice *M.vaccae* is capable of evoking 'Listeria like' responses when given live, but also when given killed in a dose of at least 2 x 10^7 organisms (Rook and Stanford, 1979). This is a somewhat smaller dose than that which has been found to be optimal in wildfowl but emphasises the requirement for high doses. In humans, prior to the wide use of BCG vaccine, heat-killed *M.tuberculosis* was used. However the necessity for 30-50 times the dose of the live vaccine, often resulted in distressing lesions, which led to its abandonment in favour of the live vaccine (Rook, 1980).

High doses of antigen have been shown to abolish evidence of delayed-type hypersensitivity in a number of situations or infections such as schistosomiasis or Leishmaniasis. Suppression of such a response may be due to a number of factors, one of which is route of injection. An intravenous route requires relatively low doses of antigen to inhibit delayed-type hypersensitivity responses, thought to be due to complexing of the antigen with antibody. The work of Lagrange *et al* (1974) showed that in intravenously injected CD-1 mice, the optimum dose of antigen (sheep red blood cells) for maximal delayed-type hypersensitivity responses was 100 fold lower than the dose required for similar responses in those injected subcutaneously. Those injected subcutaneously were also less susceptible to the blocking effects of excess antigen. The intradermal vaccines used in this Gadwall and Nene study may allow the presentation of a large amount of antigen without stimulating splenic humoral responses and without interfering with appropriate lymphocytic responses.

The 'fear' that different species may require different doses of vaccine appears to be unfounded as the results from both the Gadwall and the Nenes point to the high dose of vaccine being optimal. However, perhaps it should be noted that the results from the Nenes receiving the low dose of vaccine did not differ very significantly from the results of the controls. Perhaps so low a dose to a gosling is insufficient to boost appropriate responses. Although no swans have been included in the studies it is hoped that their immune functioning will be analagous to that of geese at least, and the dose of vaccine determined from this study will be appropriate for species as different in size as Hottentot Teal *Anas punctata* and Mute Swans *Cygnus olor*.

CHAPTER NINE.

VACCINATION IN ADULT LIFE:

THE WHITE-WINGED WOOD DUCK STUDY.

CHAPTER NINE.

VACCINATION IN ADULT LIFE: THE WHITE-WINGED WOOD DUCK STUDY.

INTRODUCTION.

The importance of the disease avian tuberculosis in the captive breeding programme of White-winged Wood Ducks has already been stressed in Chapter two. After the Mallard study began in 1986, it was decided to vaccinate half of the adult White-winged Wood Ducks both present, and brought into, Slimbridge. The other half were left as a control group. This study was an attempt to establish if vaccination, after contact with environmental mycobacteria, is of any benefit, and/or if vaccination in adult life is superior to vaccination soon after hatching.

MATERIALS AND METHODS.

Vaccination.

Adult White-winged Wood Ducks, some of which had recently arrived from The Jersey Wildlife Preservation Trust and Arundel Wildfowl and Wetlands Trust centre, were vaccinated intradermally in the foot web. Each bird received 10^7 killed (by γ-irradiation; 2.5 M. rads. emitted from a ^{60}Cobalt source) *M.vaccae* R877R, prepared as described for the Mallard study.

The birds were over two years old but of unknown exact age. In all, 28 birds were vaccinated and 28 were left as control birds. Although the birds appeared in good condition at vaccination, the lack of a reliable diagnostic test for the disease meant that they could not be considered free from avian tuberculosis, unlike the young birds used

in the other studies. After vaccination they were released into the Asian side-pens and later the newly built boardwalk pen (previously known as the Decoy side-pens) in the grounds at Slimbridge where they experienced a natural challenge.

Monitoring the Effects of Vaccination.

At the time of vaccination the conditions for duck LTT were still being developed. The following experiments were carried out to find the optimal conditions for White-winged Wood Duck lymphocyte transformation responses (Cromie *et al*, 1988; Cromie *et al*, 1989).

(i) Lymphocyte Transformation Test.

Blood for the LTT was collected and transported, and lymphocytes were separated, using the same techniques as for Mallard blood (Chapter five). Back at the laboratory in London, the cells were cultured at two concentrations: 4×10^5 cells/well and 8×10^5 cells/well in 96 well microtitre trays (Nunc). Cell cultures in the following sera were compared: 10% autologous sera; 10% Cherry Valley pooled duck sera (CVPDS); and 10% pooled White-winged Wood Duck sera (WwWdPDS) taken from the same group of birds. The pooled sera were heat inactivated for one hour at 56°C to destroy complement activity.

The antigens used were the same as for the Mallard LTT, and after four days of culture the cells were harvested in the same way.

Results.

The results of cell concentration on lymphocyte transformation (table 9.1) showed that although the cells generally did not die at the lower cell concentration, thymidine uptake and SI's were lower than at the higher concentration. As a result of this, the higher cell

concentration, which was also found to be optimal for the Mallard lymphocytes, was used subsequently.

Culture of cells in different sera showed that counts per minute from cells cultured in CVPDS tended to be higher than those in WwWDPDS but SI's were lower due to higher backgrounds (table 9.2). There were also many dead cells in cultures in CVPDS.

Responses to the non-specific mitogen PHA were greater in the WwWDPDS but the highest SI's to the mycobacterial antigens were obtained in 10% autologous sera, and this was adopted for subsequent work.

Discussion.

The conditions for White-winged Wood Duck LTT have been shown to be the same as those optimised for the Mallard LTT. Once again high cell concentrations appear to be essential for *in vitro* duck lymphocyte transformation. As with the Mallard LTT, the highest SI's to mycobacterial antigens were given when the cells were cultured in 10% autologous serum. The high number of dead cultures and increased background counts of cells cultured in the White Pekin duck derived CVPDS were almost certainly due to the birds belonging to different species and tribes.

Table 9.1 : LTT responses of White-winged Wood Duck lymphocytes at different cell concentrations in 10% autologous sera.

Stock Antigen Concentration ug/ml	8×10^5 cells/well Mean cpm (Mean SI)	SD	n.	4×10^5 cells/well Mean cpm (Mean SI)	SD	n.
PHA						
500	11370 (62.8)	5625 (29.4)	4	956 (5.2)	1069 (5.7)	4
100	61222 (300.0)	43924 (179.0)	3	17378 (111.4)	25576 (178.4)	4
10	5399 (27.0)	5228 (22.2)	4	4217 (25.3)	5625 (40.7)	4
M.vaccae						
1000	875 (3.6)	903 (3.5)	5	898 (3.5)	717 (3.0)	4
100	698 (2.4)	657 (1.2)	3	536 (2.2)	353 (1.2)	4
10	524 (3.3)	217 (2.4)	3	598 (2.3)	450 (1.2)	4
Control unstimulated wells	218	91	5	223	256	4

(See footnote from table 5.1)

Table 9.2 : LTT responses of White-winged Wood Duck lymphocytes in 10% CVPDS, WwWDPDS and autologous sera at 8 x 10^5 cells/well.

Stock Antigen Concentration ug/ml	10% Autologous sera			10% WwWdPDS			10% CVPDS		
	Mean cpm (Mean SI)	SD	*n.*	Mean cpm (Mean SI)	SD	*n.*	Mean cpm (Mean SI)	SD	*n.*
PHA									
500	11370 (62.8)	5625 (29.4)	4	19824 (115.4)	19620 (113.4)	4	1842 (8.5)	2348 (9.5)	3
100	61222 (300.0)	43924 (179.0)	3	63040 (395.5)	46406 (317.8)	4	18990 (133.5)	12418 (177.1)	3
10	5399 (27.0)	5228 (22.0)	4	5925 (40.1)	5631 (46.7)	4	15648 (69.3)	8169 (65.6)	3
M.vaccae									
1000	875 (3.6)	903 (3.5)	5	204 (1.2)	105 (0.3)	3	793 (3.3)	572 (3.5)	3
100	698 (2.4)	657 (1.2)	3	417 (2.2)	324 (0.8)	3	791 (1.3)	809 (0.5)	2
10	524 (3.3)	217 (2.4)	3	252 (1.4)	140 (0.4)	3	487 (0.9)	376 (0.1)	2
M.avium									
1000	578 (2.9)	175 (0.6)	3	197 (1.7)	47 (0.4)	2	115 (0.3)	9 (0.2)	2
100	1304 (6.3)	1118 (4.7)	3	227 (1.9)	102 (0.8)	2	444 (0.8)	349 (0)	2
10	1379 (6.5)	1858 (8.3)	3	148 (1.2)	4 (0)	2	457 (1.0)	282 (0.2)	2
Control unstimulated wells	218	91	5	168	71	4	400	414	3

(See footnote from table 5.1)

(ii) Skin Test.

The method used for skin testing the Mallard also worked satisfactorily in the White-winged Wood Ducks. The skin test reagents were prepared using the same method described previously (Chapter five).

Table 9.3 : Summary of Skin Test Reagents used for White-winged Wood Ducks.

Months post beginning of study	Skin Test Reagent
9 months	Vaccin 20 ug/ml
14 "	*M.avium* GWT 20 ug/ml
17 "	Vaccin 20 ug/ml
20 "	"
23 "	"
26 "	"
29 "	"
33 "	"

(iii) ELISA.

The same method for ELISA as described for the Mallard was found to be successful and the same antigens as before were used.

After optimisation of these three techniques, representatives from both groups were caught every three months, bled for the *in vitro* test and ELISA, and then skin tested.

Statistics Used to Analyse the Data.

The same statistical methods employed in the Mallard study were used to analyse the results obtained. SI's from LTT's were analysed using a two way analysis of variance and Student's *t* tests. Skin test data was scored as described previously and was analysed using Fisher's exact test. ELISA data was analysed using Student's *t* tests.

RESULTS.

Deaths caused by Avian Tuberculosis.

During the 33 months of this study there were 19 deaths from avian tuberculosis and four due to other causes, including aortic rupture, lead poisoning and tumour of the gizzard. Table 9.4 shows the birds that died of avian tuberculosis. Of these 19 birds, 11 were vaccinated and eight were controls (a non significant difference). There was no overall sex predilection as ten males and nine females died.

In the first year post vaccination, the only bird to die of the disease was a vaccinated female. In the next year, seven birds died; four vaccinated and three controls. In the last year of the study 11 birds died; six vaccinated and five controls. There was no significant difference in the time post-vaccination at which vaccinates and controls died.

In the late stages of infection (three months prior to death) the tuberculous birds often gave low SI's, due to high background control cultures or 'sick' cells responding weakly or often dying (appendix 9.1). Although there were 19 deaths from avian tuberculosis in this study, there were few birds from which complete data has been obtained due to the random sampling from populations, i.e. at each three

monthly testing, different birds may have been selected. Consequently, to analyse the data, results from both vaccinates and controls have been pooled and from this the following analyses have been carried out.

Using a paired Student's *t* test to compare the first SI given by a particular bird to the last SI obtained within three months of its death, there was a statistically significant decrease in the response to *M.vaccae* ($p < 0.01$) but a significant increase in the response to *M.avium* GWT ($p < 0.05$)(figure 9.1). LTT results from those still alive, to date, do not show any significant differences in SI's.

There was a trend showing positive skin test reactions in vaccinated birds and negative or reduced skin test reactions in control birds (appendix 9.2). No statistically significant results were obtained however, due to sample size being even smaller than for the LTT's, as moribund tuberculous birds often died between bleeding for LTT and skin testing the following week, no doubt due to the stress of handling and bleeding.

Of the birds from which data are available, all had high antibody levels to all antigens used in the ELISA's within six months of death (appendix 9.3). Using the paired Student's *t* test as for the LTT results, there were statistically significant increases to all six ELISA antigens ($p < 0.001$ for all antigens). The dramatic rise in antibody levels to *M.avium* GWT and *M.vaccae* over time are shown in figures 9.2 and 9.3. No such significant increase was seen in those birds still alive.

Table 9.4 : White-winged Wood Ducks dying of Tuberculosis.

Vaccine Group Bird (ring number)	Sex	Months post beginning of study at which bird died
Vaccinated		
S1747	Female	9 months
S1742	Female	14 months
S1765	Female	18 months
S1743	Female	20 months
S1748	Male	20 months
S1746	Male	29 months
S1763	Male	30 months
WA6732	Male	31 months
S1764	Male	31 months
S1741	Female	33 months
S1739	Male	35 months
mean time post beginning of study = 24.5 ± 8.7		
Controls		
S1771	Female	16 months
S1754	Female	20 months
S1755	Female	22 months
S1758	Female	24 months
S1753	Male	24 months
S1749	Male	24 months
S1757	Male	29 months
S1761	Male	31 months
mean time post beginning of study = 23.8 ± 4.7		

Figure 9.1

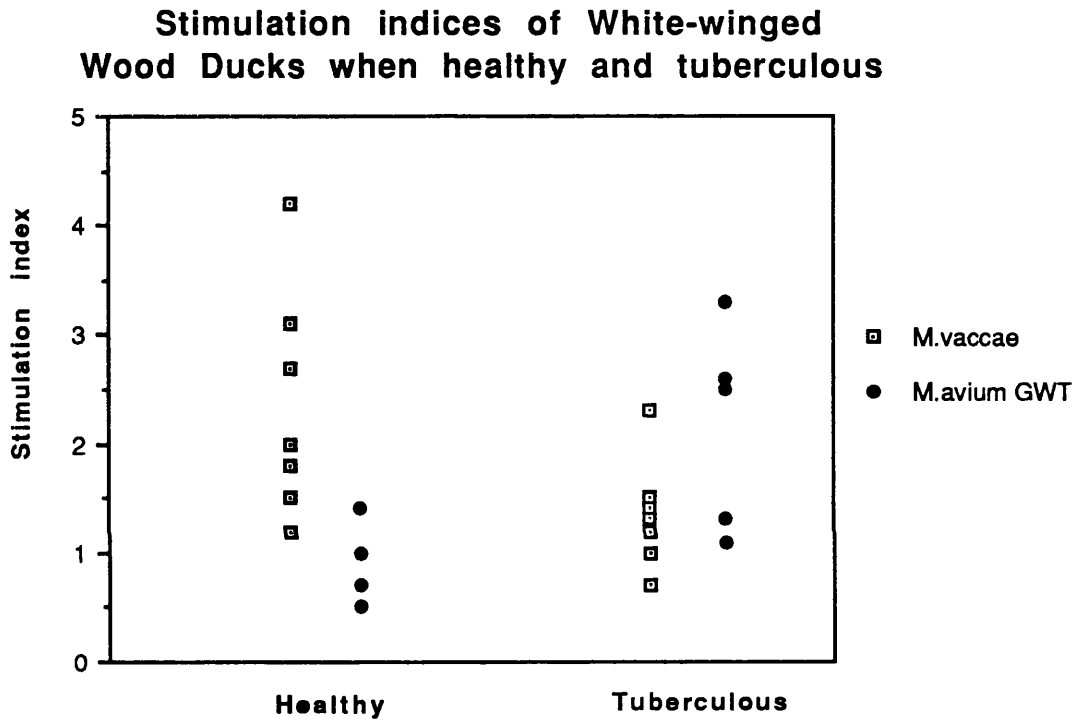

Stimulation indices of White-winged Wood Ducks when healthy and tuberculous
Stimulation index
5
4
3
2
1
0
Healthy
Tuberculous
M.vaccae
M.avium GWT

Figure 9.2

White-winged Wood Duck antibody levels to M.avium GWT during M.avium infection

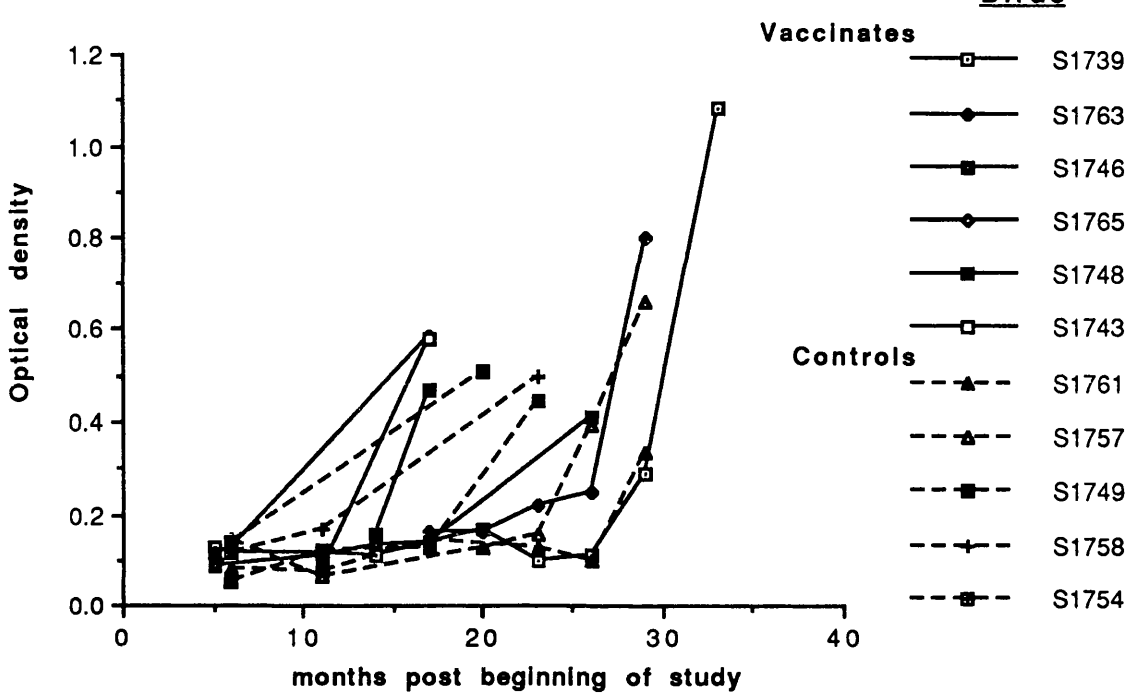
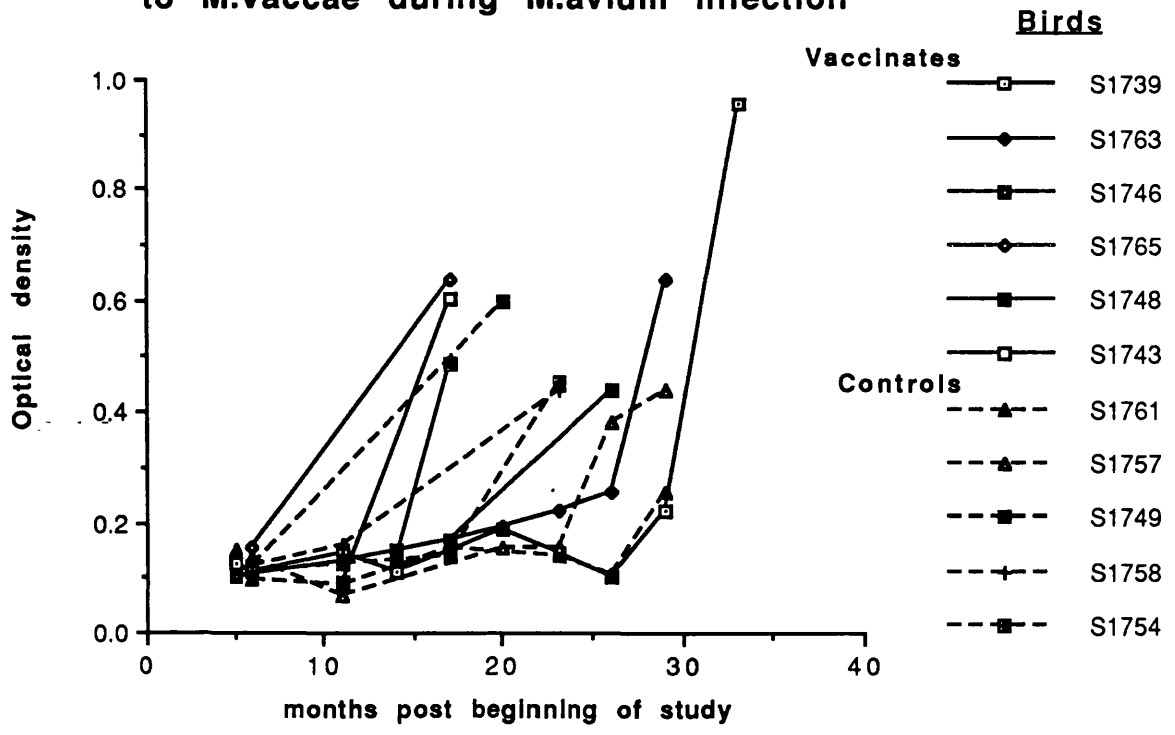


Figure 9.3

White-winged Wood Duck antibody levels to M.vaccae during M.avium infection



Pooled Results from the Vaccine Studies.

The results from tuberculous birds have not been removed from the data below. This would have left too few birds in each group. Although 56 birds were involved in this trial (of which 19 have died to date), very few of these are now left at Slimbridge as many have been moved to other Wildfowl and Wetland Trust centres to continue the captive breeding programme. However, attempts were made to move equal numbers of vaccinates and controls to other centres to leave a balanced group at Slimbridge for this study.

Lymphocyte Transformation Test.

Results from a two way analysis of variance showed that the responses of both groups differed significantly with time to BCG 100 ug/ml ($p < 0.0002$), *M.vaccae* R877R 100 ug/ml ($p < 0.04$) and *M.avium* GWT 100 ug/ml ($p < 0.0005$). However no difference between the groups' responses to any of the antigens could be found.

Similarly when Student's *t* tests were used to analyse the data, no significant differences could be found between the two groups to any of the antigens, at any of the time points.

LTT responses over time to *M.avium* GWT and *M.vaccae* are shown in figures 9.4 and 9.5. LTT responses to these and the other antigens are summarised in appendix 9.4.

Figure 9.4

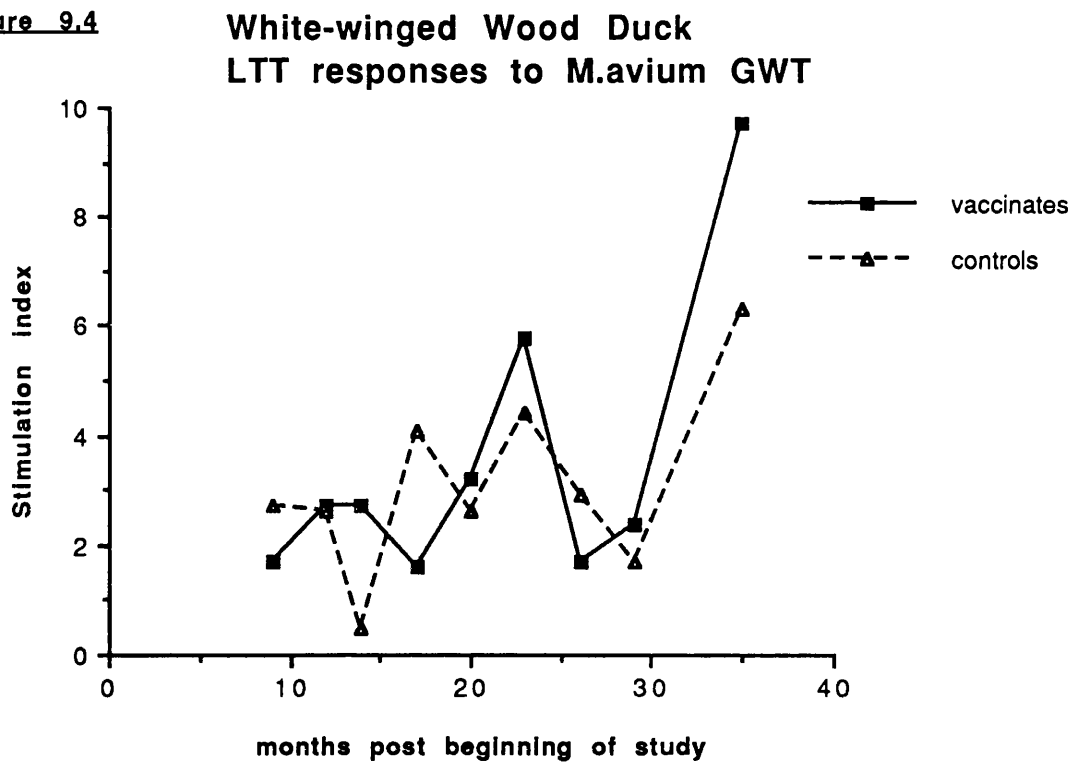
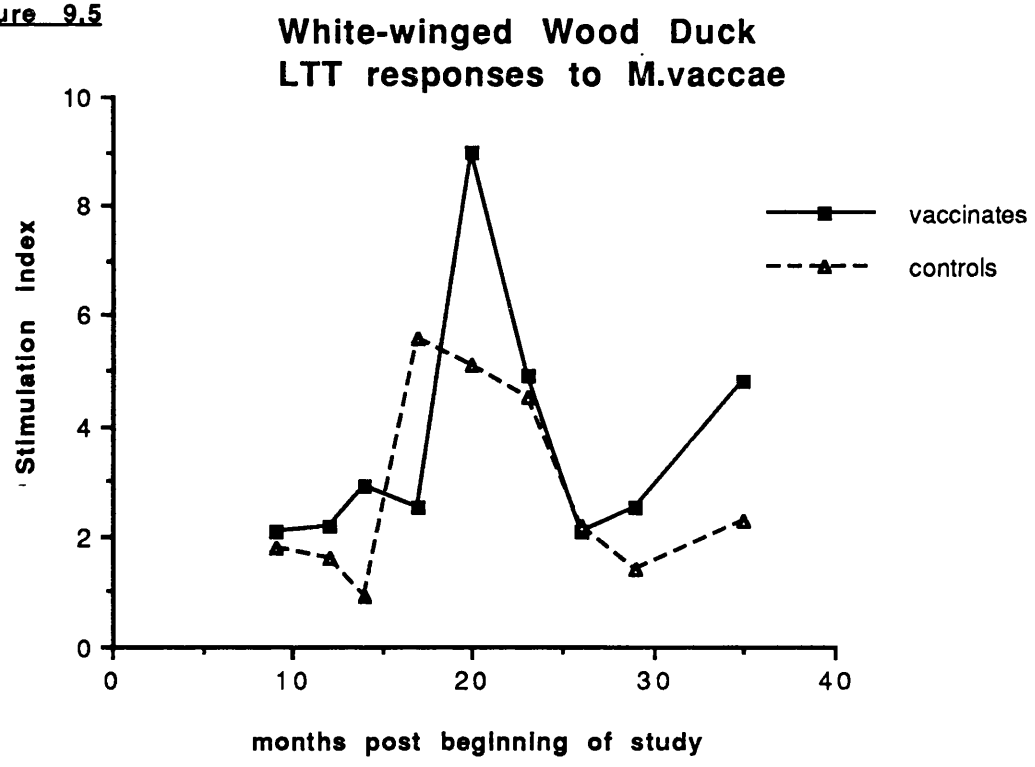


Figure 9.5



Skin Test.

(i) 41 Hours.

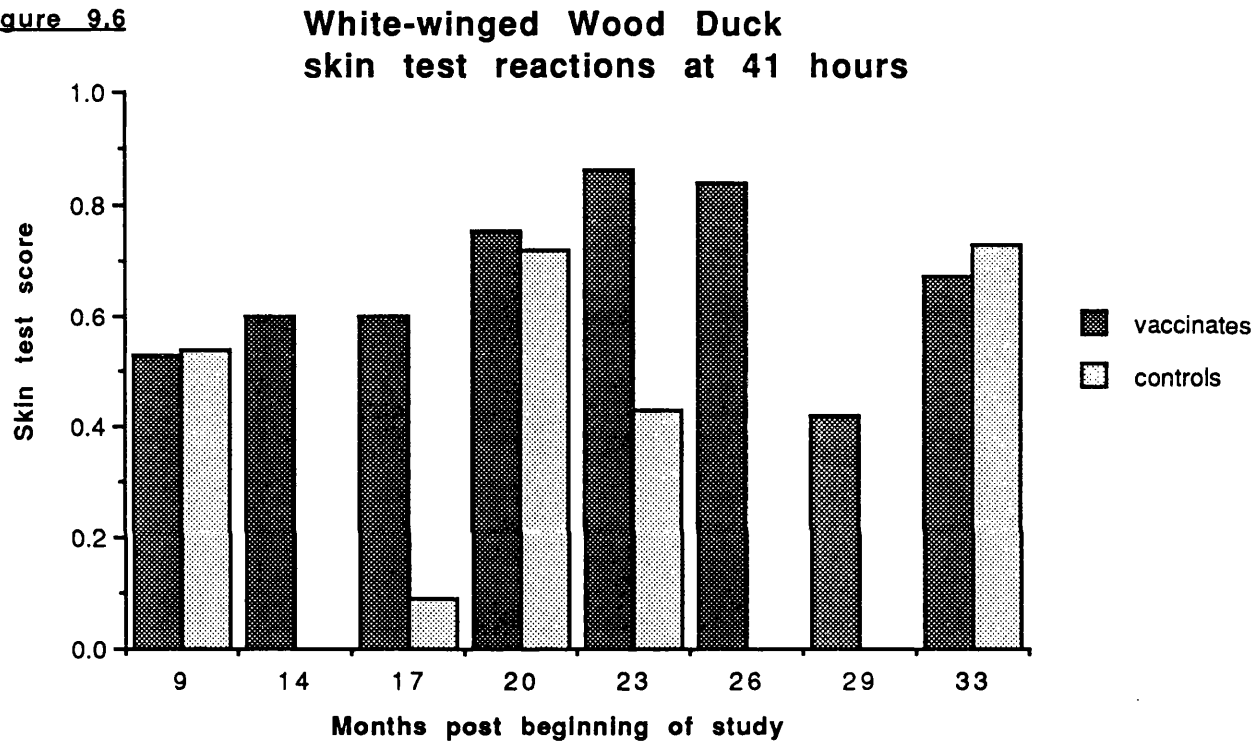
Of the eight different skin tests carried out, five of them resulted in the vaccinated birds giving significantly greater responses than the control birds regardless of skin test reagent used (figure 9.6). These results included the responses at 14, 26 and 29 months after the beginning of the study, to the avian skin test reagent GWT 20 ug/ml ($p < 0.0000$, $p < 0.0000$ and $p < 0.0004$ respectively). The significantly greater responses to Vaccin 20 ug/ml were given at the 17 and 23 month experiments ($p < 0.0000$ and $p < 0.0004$ respectively). Non significant differences were obtained at 9, 20 and 33 months after the beginning of the study.

(ii) 48 Hours.

The responses at the 48 hours skin test measurement differed very little from those at 41 hours, and similar levels of significance were obtained at each different skin test: at 14 months $p < 0.002$; 17 months $p < 0.0000$; 23 months $p < 0.002$; 26 months $p < 0.0000$ and 29 months $p < 0.0007$.

Skin test results over time are summarised in appendix 9.5.

Figure 9.6


White-winged Wood Duck
skin test reactions at 41 hours
Skin test score
1.0
0.8
0.6
0.4
0.2
0.0
9
14
17
20
23
26
29
33
Months post beginning of study
vaccinates
controls

ELISA.

Using a paired Student's *t* test no significant rise in antibody levels over time, to any of the antigens could be demonstrated.

The only significant differences in antibody levels between the vaccinated and control groups was at the 17 month experiment when the vaccinated group had higher antibodies to all six antigens (BCG $p < 0.025$; *M.fortuitum* $p < 0.05$; *M.gordonae* $p < 0.05$; *M.vaccae* R877R $p < 0.05$; *M.avium* GWT $p < 0.05$ and *M.avium* RBP $p < 0.025$). Antibody levels over time to *M.avium* GWT, *M.vaccae* and BCG are shown in figures 9.7 - 9.9. ELISA results to these and the other three antigens used, are summarised in appendix 9.6.

Figure 9.7

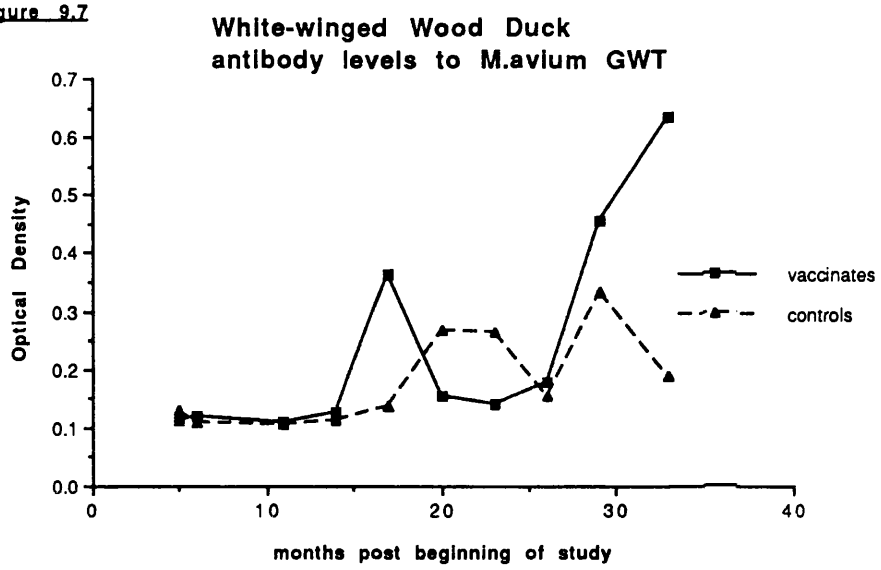


Figure 9.8

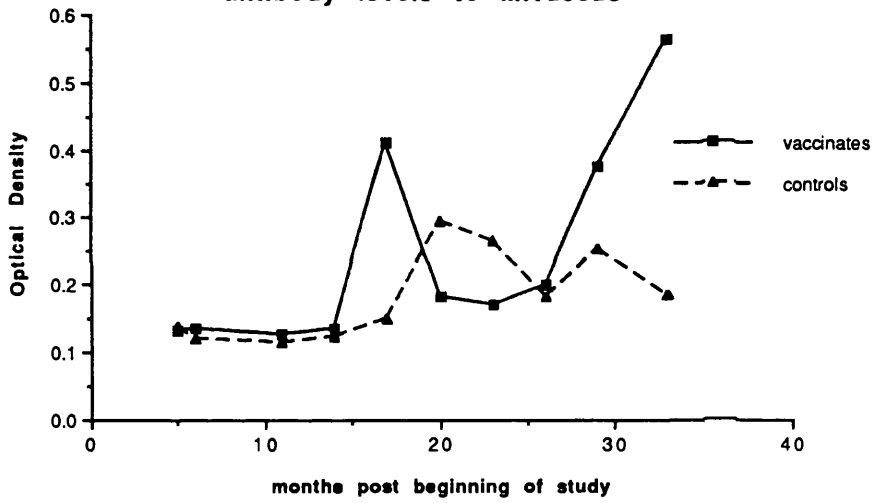
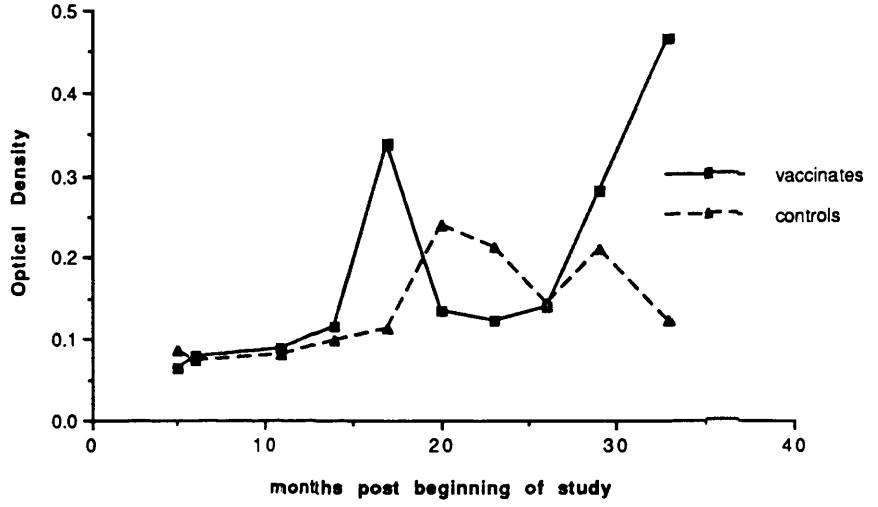


Figure 9.9



DISCUSSION.

The results of LTT's would indicate no differences in cell mediated immune responses between the vaccine and control group. This is born out in the results of mortalities with similar numbers of each group dying. The differences in skin test results were interesting in that unvaccinated birds tended to respond less than the vaccinated birds.

Both the vaccinates and controls recognised the *M.vaccae* skin test reagent which may indicate response to the shared antigens of the preparation. The similarity in responses to the LTT antigens may also indicate common antigen responses.

The vaccine appears not to have had any effect on antibody levels, as there were so few differences between the two groups. Where there was a difference at the 17 month experiment, it was to all the antigens. This is indicative of the similarities in antibody levels to the different antigens which again is probably a response to the common antigens. The similarity in the shapes of figures 9.7 - 9.9 shows this clearly.

Although there was an increase in antibody levels of the vaccinated group at the last two tests, this was not a statistically significant rise. It was due to one or two individual birds having raised antibody levels which may be indicative of avian tuberculosis. The lack of a statistically significant rise in antibody levels of both groups over time may be due to the fact that as adults, the birds had experienced many environmental mycobacteria already and as such, had appropriate antibody levels to reflect this. Any raised levels above this could be indicative of mycobacterial infection.

There was a slight suggestion of a seasonal variation in antibody levels with highest levels during the summer. This is distorted a little by the high antibody levels of the vaccinates at the last two tests.

Deaths caused by Avian Tuberculosis.

Vaccination of these birds in adult life appears to have afforded no protection from the disease, as a similar number of vaccinates and controls died. The increasing numbers of deaths from avian tuberculosis as the study progressed follows the pattern of deaths that would be expected, i.e. the incidence of the disease increases with the age of the birds.

The increased skin test responses of the tuberculous vaccinates were obviously not indicative of protective immunity. Instead, LTT results seemed to reflect immunological status in that both vaccinated and control tuberculous birds generally had low SI's. The significant reduction in the LTT response to *M.vaccae* and increased response to *M.avium* in the final stages of the disease, is a phenomenon encountered in mammals, where Listeria-like responses directed against group i, ii or iv antigens are suppressed in favour of Koch-like responses directed against group ii or iv but not group i antigens (Stanford and Rook, 1983).

The negative skin test responses of the tuberculous control birds is a phenomenon encountered in chickens in advanced stages of *M.avium* infection (Thoen and Karlson, 1978). In these chickens, skin test responses are switched off, resulting in false negative diagnostic tuberculin tests.

The raised and similar antibody levels to all six antigens suggests

response to the common mycobacterial antigens. Such a finding has been made in human patients with pulmonary tuberculosis (Bahr *et al*, 1990a) and tuberculous badgers (Stainsby *et al*, 1989). Which classes of antibody are responsible for this increase are unknown, but could be determined by the raising of specific anti-immunoglobulins.

The optimisation of a dose of vaccine has been discussed in Chapter eight. The apparent failure of the vaccine to provide protective immunity to the White-winged Wood Ducks may have been due to the dose of vaccine being too small for use in adult birds.

As was mentioned earlier in the chapter, the birds appeared healthy at vaccination. However, the slow insidious nature of the disease allows birds to remain in an asymptomatic condition for considerable periods of time. Thus, it is possible that the birds were suffering from sub-clinical infection at the time of vaccination. Therefore, the dose of vaccine used neither afforded protection nor acted as an immunotherapeutic agent, as *M.vaccae* has been shown to be in tuberculosis patients (Stanford *et al*, 1990; Bahr *et al*, 1990b).

In addition to contact with pathogenic *M.avium*, the birds would also be exposed to sensitisation from other saprophytic mycobacteria. Schaefer *et al* (1973) isolated free-living mycobacteria from 22% (14/65) of soil, mud or water samples from Slimbridge. Those birds with great experience of, or subclinical infection with, environmental mycobacteria may have little immunity to pathogenic *M.avium* and succumb to infection more rapidly than those with little prior experience (Stanford and Rook, 1983). Vaccination following excessive contact with environmental mycobacteria may result in less protective responses being mobilised. This phenomenon is discussed more fully in Chapter eleven.

CHAPTER TEN.

A BACTERIOLOGICAL STUDY OF *M.AVIUM* ISOLATED FROM TUBERCULOUS ANATIDAE.

CHAPTER TEN.

A BACTERIOLOGICAL STUDY OF *M.AVIUM* ISOLATED FROM TUBERCULOUS ANATIDAE.

INTRODUCTION.

This bacteriological study has been carried out in an attempt to gain a better understanding of the epizootiology of the disease and its causative organism within the collections of birds of The Wildfowl and Wetlands Trust.

The survey carried out by Schaefer *et al* (1973) found two separate epizootics in the birds at Slimbridge, one in the Anatidae and the other in the chickens used for brooding and incubation, caused by *M.avium* serotypes 1 and 2 respectively. As mentioned in Chapter four, there has been a move away from the use of broody chickens and so this study analyses only strains pathogenic for the Anatidae. Although most of the isolates have been made from birds from Slimbridge, a number of isolates were from birds of The Wildfowl and Wetlands Trust Centre at Arundel, Sussex. There were also a small number of isolates from wild Anatidae. No environmental isolates were examined.

Mycobacterium avium.

As discussed in Chapter one, *M.avium* has a complex variety of sub-species and variants. However, it is only *M.avium avium* that is typically pathogenic for birds. As with all mycobacteria *M.avium* is acid-fast. Its size varies from 1 um to 3 um in length. It is non-motile and reproduces by simple binary fission. Its ability to grow at 40-45°C allows it to exploit birds as a host and distinguishes it from most other mycobacterial species. The egg based medium Löwenstein

Jensen allows adequate growth after several weeks, and as a slow grower its primary isolation may take up to eight weeks of culture before the colonies become visible. *M.avium* typically grows in culture as raised discrete white or cream colonies on the surface of the medium, although scotochromogenic strains are occasionally found. *M.avium* is aerobic and colonies of organisms do not penetrate the medium on which they grow and are hence easily removed from its surface.

Biochemically, *M.avium* differs from other mycobacteria in that it does not hydrolyse Tween 80, produce niacin, or reduce nitrate, it is peroxidase negative but does produce catalase.

METHODS AND MATERIALS.

All PM's were carried out by the pathologist Martin Brown of The Wildfowl and Wetlands Trust. Diagnosis was made by macroscopic examination of the dissected body supported by the presence of acid fast bacilli in Ziehl-Neelsen stained smears. Organs considered to be tuberculous were sent to the laboratory in London for analysis.

Recipes for the buffers and solutions are summarised in appendix 5.1.

Retrieving *Mycobacteria* from Tissue Samples.

Tissues were ground in homogeniser tubes with a few mls of saline. Decontamination of non-mycobacteria was done by adding one volume of 5% oxalic acid (BDH 10174). After 15 minutes (or longer in more heavily contaminated samples) two volumes of 2% sodium hydroxide (BDH 10252) were added. An excess volume of sterile phosphate buffer (pH 6.8) was then added to ensure neutrality.

A film of the suspension was put onto two Löwenstein Jensen slopes and a smear was made for Ziehl-Neelsen staining.

The Ziehl-Neelsen (ZN) Method of Staining.

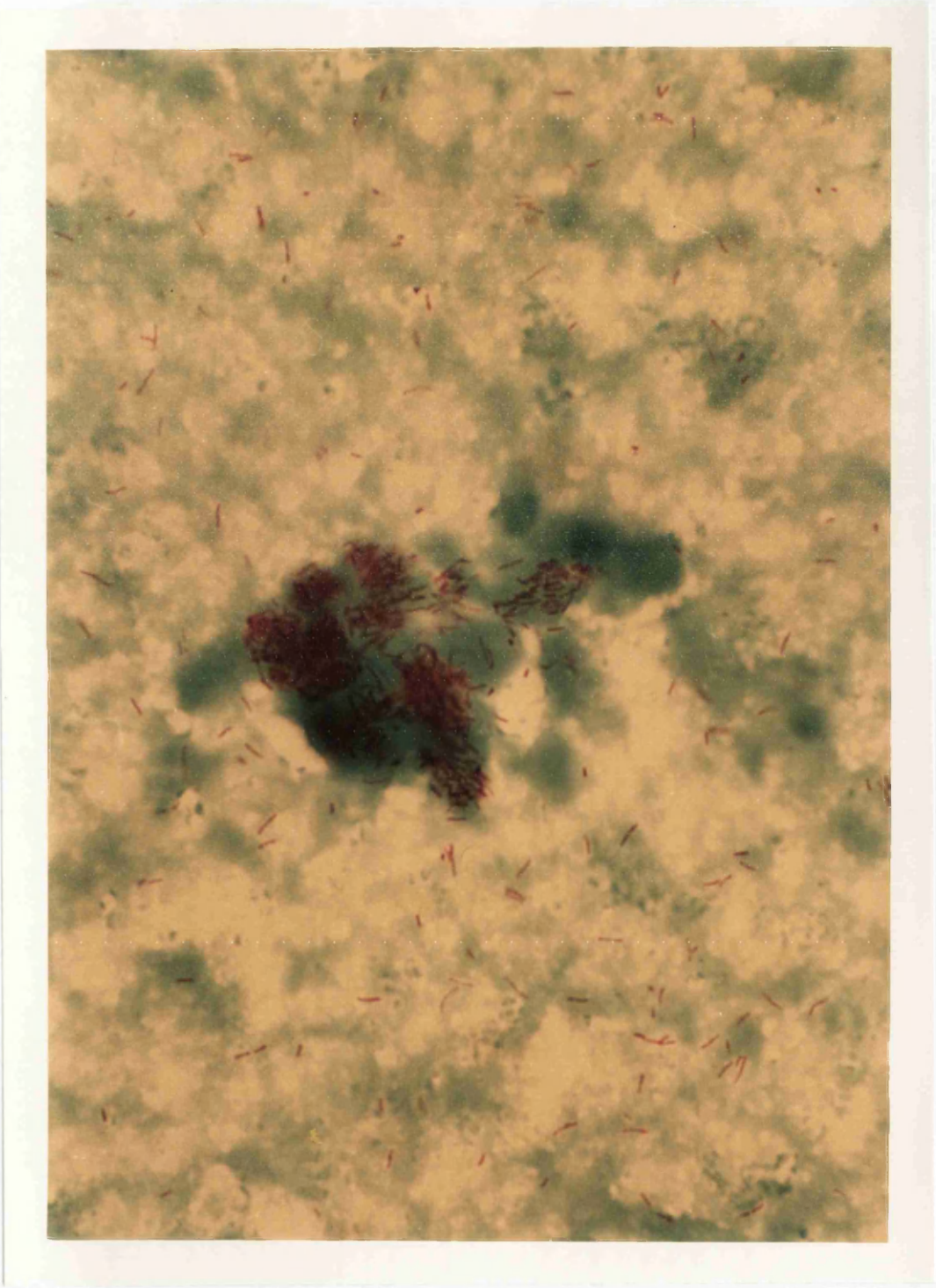
Smears were fixed on a slide by passing through a bunsen flame quickly several times, never allowing the smear to become greater than hand-hot. The smear was then flooded with carbol fuchsin stain. This was heated gently from below using the flame from an alcohol soaked cloth for about three minutes until steaming. (The slide should not be heated more than once, nor boiled as this will dislodge the smear. If the stain is left too long it may be precipitated or if it is allowed to dry the preparation is spoiled).

The slide was washed in running water and drained. It was flooded with decolourising solution (12.5% sulphuric acid, BDH 10276) and left for two minutes before being washed off with running water. This was repeated twice.

The slide was blotted with a fresh piece of filter paper and the counterstain, 0.5% Malachite green (Sigma M-6880), was poured on and left for 30 seconds. This was washed off with running water and the slide was blotted and allowed to air-dry.

A Ziehl Neelsen stained liver homogenate from a White-winged Wood Duck is shown in figure 10.1. The pink acid fast *M.avium* rods are somewhat longer than those of *M.tuberculosis*.

Figure 10.1 : *M.avium* in a Ziehl Neelsen stained liver homogenate from a White-winged Wood Duck.



Even if the sample appeared to be ZN negative, the results of culture were awaited.

The inoculated Löwenstein Jensen slopes were incubated at 32°C for several weeks. After this time negative cultures were discarded but positive cultures were subjected to various techniques including lipid, DNA, and protein pattern analysis. Descriptions were made of colonial morphology and pigmentation, as well as ability to grow at a range of temperatures.

Lipid Analysis.

The lipids found in mycobacteria have two important roles when considered in relation to disease. They may be involved in the virulence of the strain and they may also influence the immunological response to that strain. Mycobacterial lipids consist of mycolic acids, phospholipids, glycolipids and mycosides. Together these may form up to 40% of the mass of the bacilli being found mainly in the cell wall (Grange, 1980).

Schaefer (1965) demonstrated stable serotypes of *M.avium* which could remain so, even after years of artificial growth. This allowed a classification of the organism which has been a major contribution to the understanding of *M.avium* infection and epidemiology.

Marks *et al* (1971) showed that the agglutination serotypes of *M.avium* could be distinguished by their lipid patterns obtained by thin layer chromatography. This therefore provided a system of classification for the bacilli without the need for raising antisera. In this study techniques have been used to analyse the lipids from the Anatidae strains as well as from reference strains.

Thin-Layer Chromatography of Mycobacterial Lipids.

This method was described by Jenkins (1980).

The mycobacteria were grown on Sauton's medium solidified with 1% agar, unlike Löwenstein Jensen medium it contains no egg lipids and so produces clearer lipid patterns in thin layer chromatography. The bacteria were harvested from the medium and freeze dried over phosphorous pentoxide (BDH 33167) in a partial vacuum overnight.

Lipids were extracted by adding to each mg of dried bacilli, 16 ul of ether (BDH 28132); ethanol (BDH 45101); water mixture (17;17;6 v/v). Approximately 10 mg of dried bacilli of each culture were extracted overnight at room temperature.

Whatman 20 cm x 20 cm silica gel 60 A plates were cleaned with acetone (BDH 45100) and allowed to air dry. The plates were then activated by heating to 120°C for 30 minutes. To these, 20 ul of the lipid extracts were added in discrete spots 2 cm from the bottom of the plate and 15 mm apart. The extracts were added in 2.5 ul aliquots allowing each to dry before applying the next, to conserve the spot dimension. The chromatograms were run using *n*-propanol (BDH 29679); water; ammonia (specific gravity 0.88)(BDH 27141) mixture (75;22;3 v/v) (Dr. P.A. Jenkins, personal communication) until the solvent front had moved 10 cm.

The plates were dried at 100°C for 30 minutes. They were then sprayed lightly with a mixture of two volumes of 60% sulphuric acid (BDH 10276) and one volume of freshly prepared 0.1% orcinol (Sigma 07875) in water. The lipid spots were revealed by heating the plates at 140°C for about five minutes or until the spots appeared. The lipid patterns were photographed as they fade on the plates after some time.

Analysis of Protein Patterns of *M.avium* using SDS-Polyacrylamide Gel Electrophoresis SDS-PAGE.

Analysis of bacterial proteins using polyacrylamide gel electrophoresis has been widely used for taxonomic purposes, for example for Enterobacteriaceae (Sacks *et al*, 1969) and Yersiniae (Hudson and Quan, 1975). Haas *et al* (1972) used this system as a tool for classification and identification of mycobacteria. They concluded that such a technique is a good measure of genetic relatedness of strains. This method has also been used widely as an aid to assess which mycobacterial components are immunogenic (Roszman *et al*, 1968; Abou-Zeid *et al*, 1987).

Polyacrlyamide gel electrophoresis of mycobacterial proteins can be used to determine intra-specific variations (Haas *et al*, 1974) and hence was used to analyse the strains isolated from tuberculous Anatidae and reference strains, in this study. Such differences may relate to the genetics of the host and thus relate to the epizootiology of the disease, as has been shown in *M.tuberculosis* infections in man (Collins *et al*, 1982).

Preparation of Mycobacteria for running on SDS-Polyacrylamide Gel Electrophoresis.

Bacterial extracts were prepared using a modified method of Stanford *et al* (1975a).

The bacteria were grown to log growth phase on Sauton's medium solidified with 1% agar. This medium contains no egg proteins unlike Löwenstein Jensen medium, and so no egg protein standards had to be run on the gels.

The bacteria were harvested and a large loopfull was put into 1.5 ml of distilled water. The different strains were harvested at the same growth phase to ensure that they were in the same physiological state and hence not secreting different proteins. The bacilli were then sonicated in an MSE 100 Watt ultrasonic disintegrator for 15 minutes with an amplitude of 6-8 um.

The resultant mixture of any remaining whole bacteria, broken cell walls and cytoplasm was centrifuged at 3000 rpm for 10 minutes.

The protein concentration of the supernatant was measured spectrophotometrically based on absorption of ultra-violet light at 260 nm and 280 nm by the method described by Warburg and Christian (1941).

SDS-Polyacrylamide Gel Electrophoresis.

ExcelGel gradient 8-18 polyacrylamide gels (Pharmacia, LKB Biotechnology, Sweden 2351-001) were used to analyse the protein patterns of the sonicated mycobacteria. These gels were used as they can separate up to 26 samples on one gel and therefore facilitate easy comparisons. They are cast on a plastic support to enable the gel to be handled easily. A stacking gel is incorporated at the cathodic end of the gel which is 8% acrylamide. This region increases its concentration gradually until it reaches the final separating zone which has 18% acrylamide. A discontinuous buffer system is used in the form of precast buffer strips (ExcelGel SDS buffer strips, Pharmacia 2351-100) which supply the ions for the electrophoresis.

Methods and Materials.

The gel containing unit, the Multiphor II (LKB Electrophoresis Unit 2117), was connected to the Multitemp II thermostatic circulator (LKB) and the temperature was adjusted to 15°C. This temperature control was switched on 20 minutes before starting the experiment.

Approximately 1 ml of light paraffin oil (BDH 29436) was pipetted onto the cooling plate to act as an insulator. The gel was placed on top of this ensuring that there were no air bubbles trapped beneath. The buffer strips were applied to the appropriate ends of the gels with their narrow edge in contact with the gel.

Samples were freshly prepared by diluting 1:1 in sample buffer. These were then heated to 95°C for 3 minutes in a water bath. Samples were loaded onto the gel using a 26 well sample application piece (Pharmacia 1850-901) which was placed 1 cm from the cathodic buffer strip. To each well 20 ul of sample, containing 20 ug of protein, was added. Molecular weight markers (Sigma MW-SDS 200) were also loaded in the outside and centre wells. These markers represent 29, 45, 66, 97, 116 and 205 kDa proteins.

The electophoresis unit was connected up so that the appropriate electrodes were in contact with the buffer strips and a constant current of 50 mA was applied. After approximately 20 minutes, the application strip was removed. The run was stopped when the Bromophenol Blue front from the sample buffer had reached the anodic buffer strip.

The buffer strips were removed and the gel was immediately immersed in fixing solution. Fixing was allowed to continue for at least 30 minutes, or overnight. The gel was then stained in Coomassie solution

for 15 minutes. After this, the gel was rinsed in distilled water. The gel was destained with several changes of destaining solution until the background became clear. The gel was photographed and then dried to preserve it.

DNA Analysis.

Several strains were analysed by Zubair Kunze, of The University of Surrey. They were typed by restriction fragment length polymorphism (RFLP) using the DNA probe pMB22.

Reference Strains.

The strains shown in table 10.1 were used as reference types and have been taken from the collection of strains of known Schaefer serotype. Others include strains isolated from tuberculous Slimbridge Anatidae as part of the study carried out by Schaefer *et al* (1973). These were supplied in freeze dried form by Mr. E. Boughton of The Central Veterinary Laboratory, Weybridge. Two miscellaneous strains have also been analysed: M1 , supplied by Mr. E. Boughton who isolated this type 2 from a tuberculous Mute Swan *Cygnus olor* in the Bristol area; and 14330 an *M.avium* strain isolated from an AIDS patient.

Table 10.1 : Reference strains of M.avium.

Serotype	Reference Number (origin/ comments)	Colour of colony	Type of colony
Schaefer serotypes:			
1	1220 (Human)	Cream	Smooth
1	1222 (Woodpigeon)	Cream	Smooth
1	1226 (Nene)	Cream	Smooth/Rough
1	1235	Peach	Rough
1	1235	Cream	Smooth
2	1236	Cream	"
2	1237	Pale yellow	"
3	1239	Cream	"
8	1248	Yellow	"
10	1252	Cream	"
10	1253	Cream	"
43	1280	Pale yellow	"
	1086 (*M.intracellulare*)	Cream	"
From Schaefer *et al* (1973) study:			
1	M364[1]	Cream	Smooth
1	M366[2]	Cream	Rough
1	M368[3] (Scotochromogen)	Orange	Smooth
1	M373[4]	Cream	Smooth/Rough
1	M443[5]	Cream	Smooth
2	M372[6]	Colourless	"
Miscellaneous:			
2	M1 (Mute Swan)	Yellow	"
	14330 (AIDS patient)	Orange	"

[1] This strain (termed as B15 in Schaefer *et al*, 1973) was rough on primary isolation but after passage through chickens smoothness was improved and it was typed as type 1.

[2] This strain (B23) was a rough type 1 with low virulence and no specific lipid pattern.

[3] This strain (B38) was pigmented but otherwise a typical type 1.

[4] This strain (B59) showed no lipid pattern on primary isolation but was later shown to be a rough type 1.

[5] This strain (B94) was rough even after passage through chickens and lacked specific lipids although it was serotyped as type 1.

[6] This strain (B56) was serotyped as type 2 but had no characteristic lipid spots.

Other Mycobacterial Species.

Several other species of sonicated mycobacteria were prepared in the same way as the study strains and then analysed on SDS-PAGE. These strains were: BCG (Glaxo), *M.vaccae* R877R, *M.fortuitum* and *M.gordonae*.

Table 10.2 : Results of Morphological Examination and Lipid Analysis of Strains Isolated from Tuberculous Anatidae.

PM Number	Species isolated from.	Colour of colony	Type of colony	Type
	Tribe: Anserini			
88/4X	Whooper Swan *Cygnus cygnus cygnus*	Cream	Smooth	1*
87/221	Black Swan *Cygnus atratus*	Cream	"	1
87/222	Black Swan *Cygnus atratus*	Cream	"	1
87/65A	Trumpeter Swan *Cygnus buccinator*	Pale Yellow	"	1*
87/135	Emperor Goose *Anser canagicus*	Cream	"	1
87/214	Nene *Branta sandvicensis*	Cream	"	1*
88/12	Barnacle Goose *Branta leucopsis*	Colourless/cream	"	1
88/31	Barnacle Goose *Branta leucopsis*	Cream	Rough	no spots
88/91	Barnacle Goose *Branta leucopsis*	Cream	Smooth	1
88/8A	Red-breasted Goose *Branta ruficollis*	Cream	"	1*
	Tribe: Anatini			
86/136	Green-winged Teal *Anas crecca carolinensis*	Cream	"	1*
86/154	Bahama Pintail *Anas bahamensis bahamensis*	Pale yellow	"	1*
87/65	Red-billed Pintail *Anas erythrorhyncha*	Dark cream	"	1*

Table 10.2 continued.

87/138	African Yellowbill *Anas undulata undulata*	Pale yellow	Smooth	1*
87/120	African Yellowbill *Anas undulata undulata*	Cream	"	1*
87/231	Chilean Pintail *Anas georgica spinicauda*	Pale yellow	"	1*
87/211	Falcated Duck *Anas falcata*	Cream	"	1*
87/248	Blue-winged Teal *Anas discors*	Pale yellow	"	1*
88/12X	Mallard *Anas platyrhynchos platyrhynchos*	Pale yellow	"	1*
86/179	Meller's Duck *Anas platyrhynchos melleri*	Cream	"	no spots
86/180	Meller's Duck *Anas platyrhynchos melleri*	Cream	"	1
	Tribe: Cairinini			
87/12A	Carolina *Aix sponsa*	Pale yellow	"	1*
87/124	Carolina *Aix sponsa*	Cream	"	1*
87/160	Carolina *Aix sponsa*	Cream	"	1*
	Carolina (Juvenile) *Aix sponsa*	Dark cream	"	1*
88/8	White-winged Wood Duck *Cairina scutulata*	Cream	"	1*
88/133	White-winged Wood Duck *Cairina scutulata*	Dark cream	"	1*
	Tribe: Oxyurini			
88/3	African White-backed Duck *Thalassornis leuconotus*	Yellow	"	1

Table 10.2 continued.

	Tribe: Aythyini			
88/9	Scaup *Aythya marila marila*	Cream	Smooth	1*
	Tribe: Mergini			
87/114	Smew *Mergus albellus*	Pale yellow	"	1*
88/85	Smew *Mergus albellus*	Cream	"	1
88/127	Smew *Mergus albellus*	Cream	"	1
87/223	Red-breasted Merganser *Mergus serrator serrator*	Cream	"	1*
87/114A	Goosander *Mergus merganser merganser*	Pale yellow	"	1*
88/119	Goosander *Mergus merganser merganser*	Pale yellow	"	1*
87/61A	Long-tailed Duck *Clangula hyemalis*	Dark cream	"	1*
87/74A	Long-tailed Duck *Clangula hyemalis*	Yellow	"	no spots
86/181	Barrow's Goldeneye *Bucephala islandica*	Cream	"	1
	Tribe: Tachyerini			
87/113A	Steamer Duck *Tachyeres brachypterus*	Pale yellow	"	no spots

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* : these strains were subjected to DNA analysis.

no spots: indicates no pattern on lipid analysis.

A : those with "A" after the PM number are isolates from birds of The Wildfowl and Wetlands Trust centre at Arundel.

X : the two birds with "X" after the PM number are isolates from wild birds. Mallard 88/12X was found dead in the grounds at Slimbridge

and Whooper Swan 88/4X was found at Caerlaverock Wildfowl and Wetlands Trust centre.

Results of Morphological Characteristics.

Decontamination of non-mycobacteria from samples sometimes proved difficult, particularly in those samples that had come from birds in a fair state of decomposition. However, those strains of *M.avium* isolated, were all originally smooth colonies and tended to be either cream or pale yellow in colour. The strain isolated from the Barnacle Goose 88/31 was originally smooth but became rough after some months of culture.

There appeared to be no correlation between host and colour of isolate, i.e. the scotochromogenic strains were found in birds from several tribes and various areas within the grounds at both Slimbridge and Arundel.

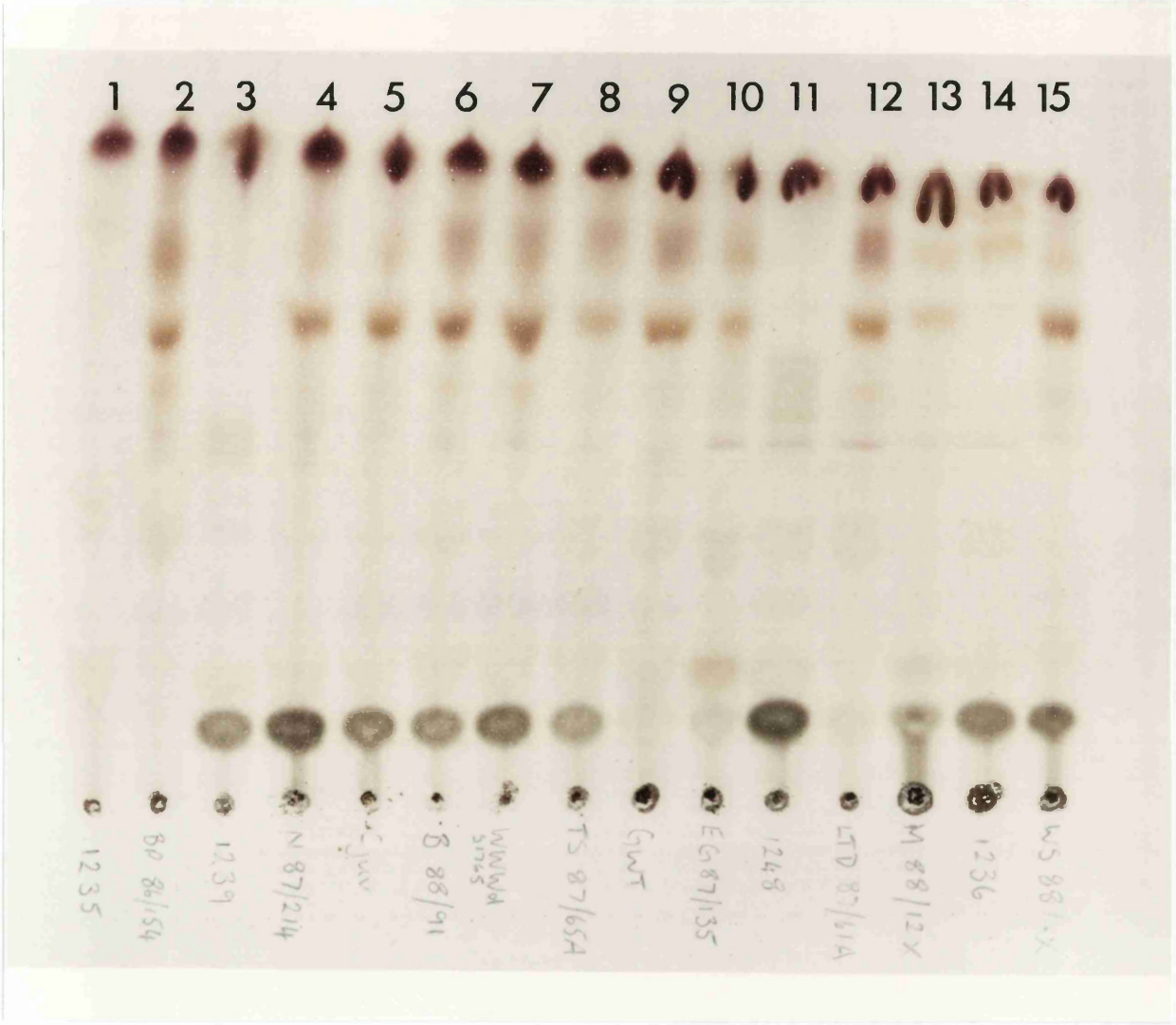
Results of Growth at Different Temperatures.

All the strains from the tuberculous Anatidae grew at 32°C, 37°C and 43°C.

Results of Lipid Analysis.

All the strains isolated from Slimbridge Anatidae were type 1 with the exception of four which had no pattern of lipid spots. Figure 10.2 shows some of the results on a chromatography plate of typical type 1 lipid patterns together with reference strains of various serotype. This experiment was carried out with Dr. P.A. Jenkins of the Mycobacterium Reference Unit, Cardiff.

Figure 10.2 : A thin-layer chromatography plate showing typical type 1 patterns plus reference strains.



Key:

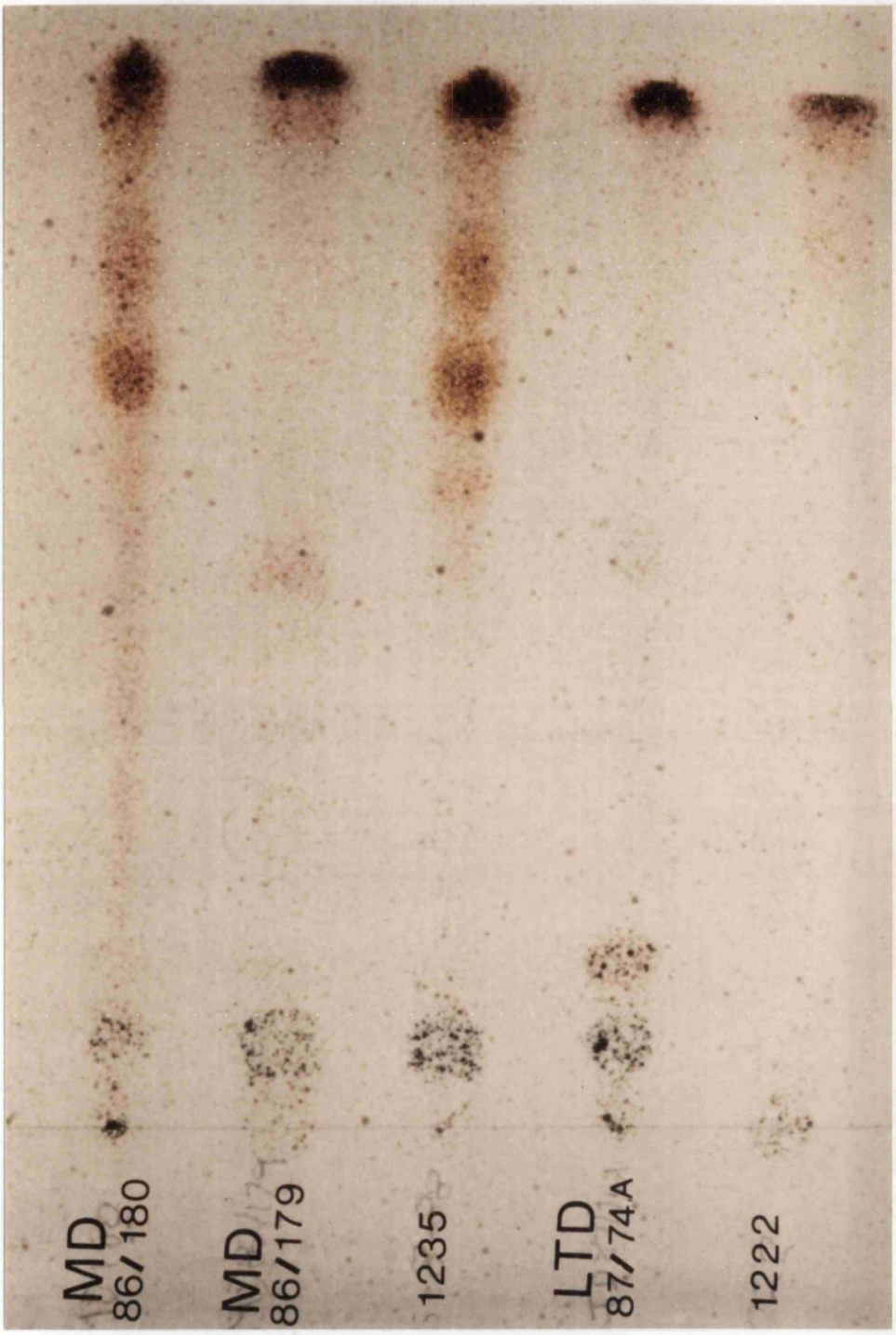
1. 1235 (rough strain)
2. Isolate from Bahama Pintail 86/154
3. 1239 (type 3)
4. Isolate from Nene 87/214
5. " " Carolina (juvenile)
6. " " Barnacle Goose 88/91
7. " " White-winged Wood Duck 88/133
8. " " Trumpeter Swan 87/65A
9. " " Green-winged Teal 86/136
10. " " Emperor Goose 87/135
11. 1248 (type 8)
12. Isolate from Long-tailed Duck 87/61A
13. " " Mallard 88/12X (wild bird)
14. 1236 (type 2)
15. Isolate from Whooper Swan 88/4X (wild bird)

The four aberrant strains with no lipid spots were isolated from: Barnacle Goose 88/31, Meller's Duck 86/179, Long-tailed Duck 87/74A and Steamer Duck 87/113A.

Figure 10.3 shows isolates from Meller's Ducks 86/179 and 86/180 (which were a pair, and died of tuberculosis at the same time); the isolate from the Long-tailed Duck 87/74A; strain 1222 (Woodpigeon bacillus); and the smooth strain of 1235, a reference serotype 1. The figure shows the typical type 1 appearance of the smooth 1235 and the isolate from Meller's Duck 86/180, and the lack of lipid spots from the other three.

Of the six strains isolated from the study of Schaefer *at al* (1973) M366 and M372 showed no lipid pattern at all as was originally reported. The other four strains all showed the lipid pattern of type 1. The strain M1 produced a very weak lipid pattern which was probably type 2.

Figure 10.3 : Lipid Patterns of isolates from Meller's Ducks 86/179 and 86/180; Long-tailed Duck 87/74A; strain 1222 isolated from a Woodpigeon; and strain 1235, a reference serotype 1.



Key:

MD 86/180 : Isolate from Meller's Duck 86/180 (typical type 1)
MD 86/179 : " " " " 86/179 (no lipid pattern)
1235 : Reference type 1
LTD 87/74A: Isolate from Long-tailed Duck 87/74A (no lipid pattern)
1222 : Woodpigeon bacillus (no lipid pattern)

Results of Protein Analysis by Polyacrylamide Gel Electrophoresis.

All the strains isolated from the tuberculous birds in this study showed a very similar protein pattern on SDS-PAGE which is shown in figure 10.4, with the exception of four strains. These four strains were alike and differed only slightly from the others in that they lacked one heavy band (approximately 70 kDa) and had an extra lighter band (approximately 60 kDa). These four strains were isolated from: Mallard 88/12X, Carolina 87/160, Steamer Duck 87/113A and Meller's Duck 86/180. The aberrant pattern of the first two is shown in figure 10.5 along with the typical patterns from 12 other strains.

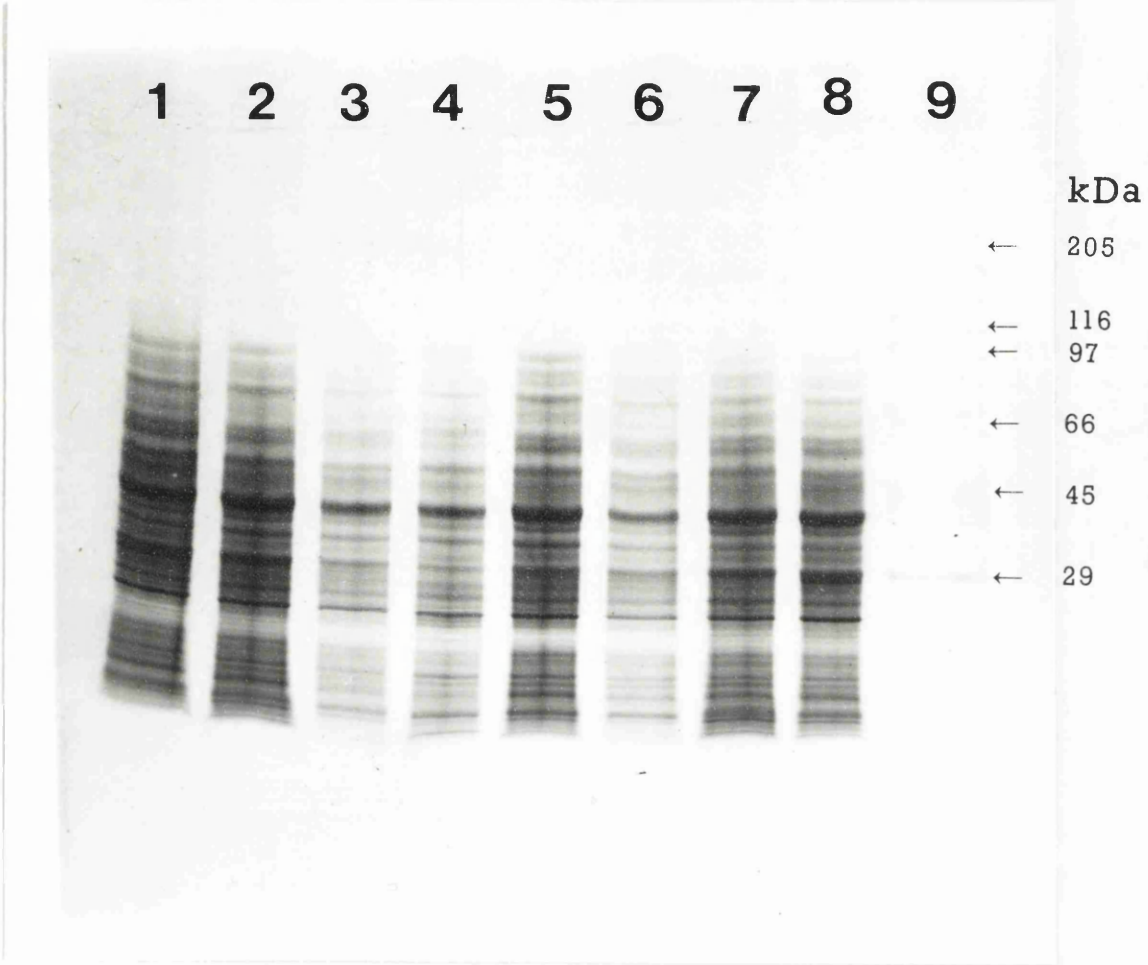
Of the reference strains of known serotype, all showed the typical protein pattern with the exception of five:
1222 (type 1, Woodpigeon bacillus), M364 (type 1, rough on primary isolation), M1 (type 2, from the Mute Swan), 1280 (type 43) and 14330 (isolated from an AIDS patient).

These five strains lacked the predominant band (approximately 38 kDa) which seemed to most readily identify *M.avium* from the other mycobacterial species run on the gels. Figure 10.6 shows the aberrant patterns from M364 and 1222 together with five typical patterns.

Results of DNA analysis.

All the strains analysed were found to contain an identical RFLP, type A6/I, when probed with pMB22. They also contained an identical insertion sequence which is typical for the avian tubercle bacilli.

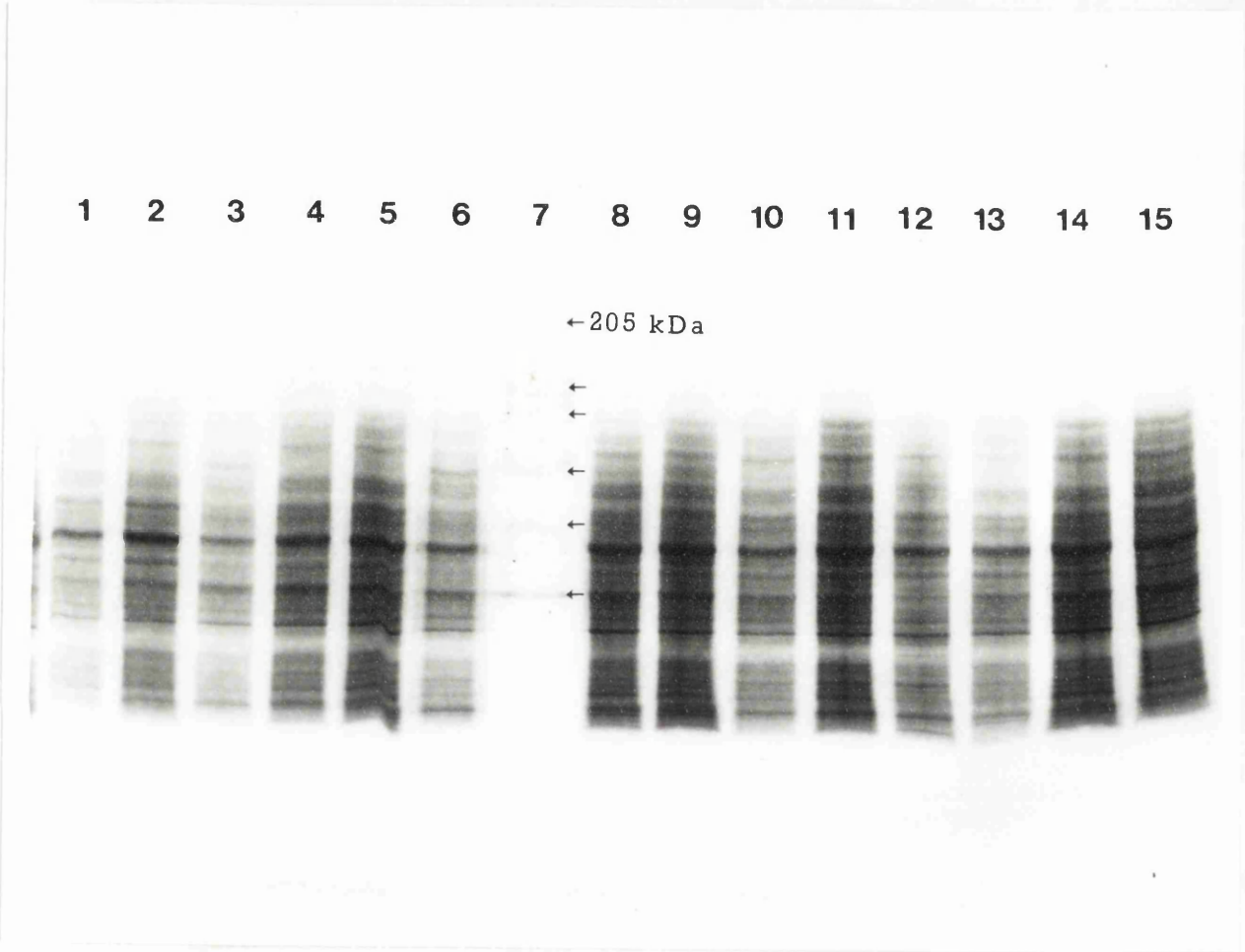
Figure 10.4 : Typical *M.avium* protein patterns on SDS-PAGE.



Key:

1. Isolate from Black Swan 87/221
2. " " " " 87/222
3. " " Whooper Swan 88/4X
4. " " Meller's Duck 87/179
5. " " Bahama Pintail 87/154
6. " " Chilean Pintail 87/231
7. " " African Yellowbill 87/138
8. " " " " 87/120
9. Protein marker (29, 45, 66, 97, 116 and 205 kDa)

Figure 10.5 : Aberrant protein pattern on SDS-PAGE of the isolates from Mallard 88/12X and Carolina 87/160, together with typical patterns.

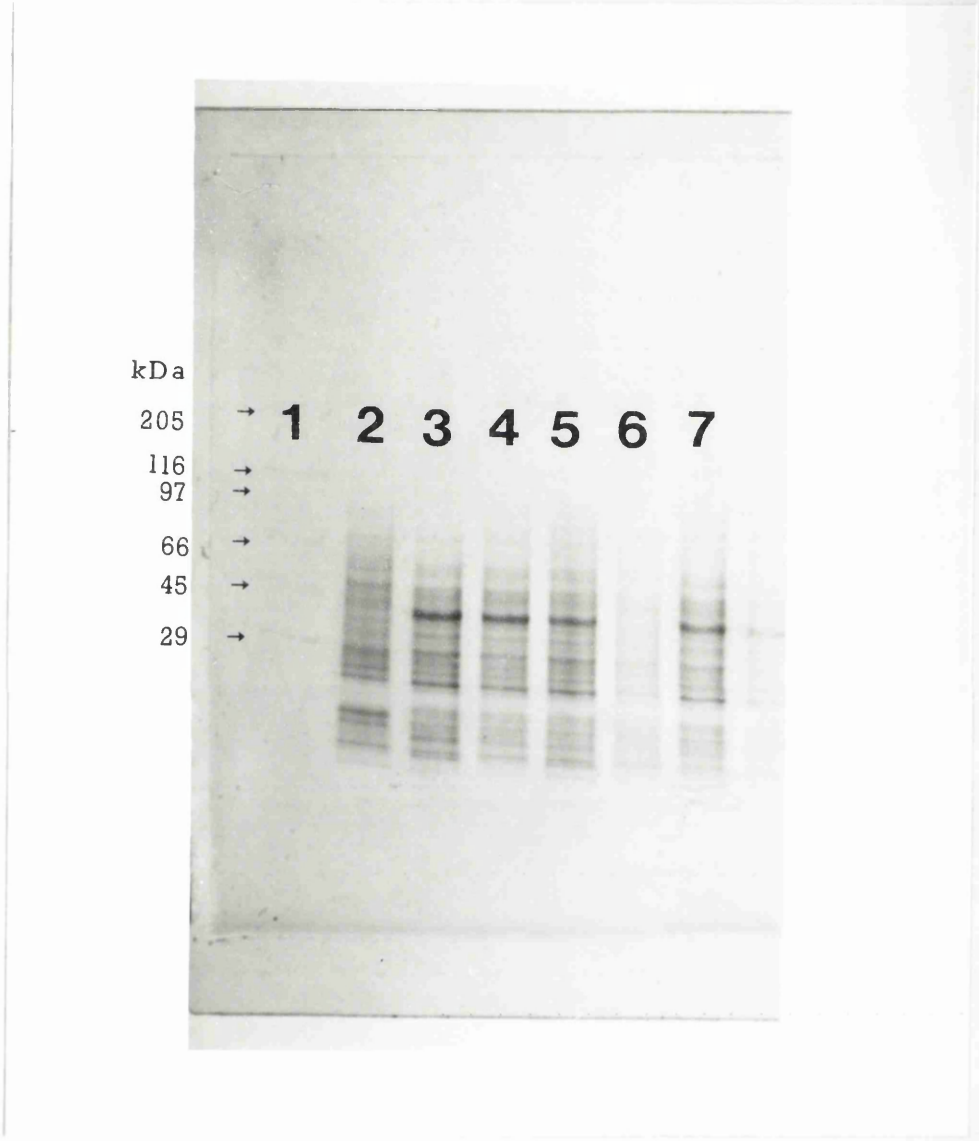


Key:

1. Isolate from White-winged Wood Duck 88/133
2. " " " " " " 88/8
3. " " Carolina 87/160*
4. " " " 87/124
5. " " " 87/12A
6. " " Mallard 88/12X*
7. Protein marker (29, 45, 66, 97, 116 and 205 kDa)
8. Isolate from African Yellowbill 87/120
9. " " " " 87/138
10. " " Chilean Pintail 87/231
11. " " Bahama Pintail 86/154
12. " " Meller's Duck 86/179
13. " " Whooper Swan 88/4X
14. " " Black Swan 87/222
15. " " " " 87/221

* Aberrant strains

Figure 10.6 : Aberrant protein patterns on SDS-PAGE of M364 and 1222 together with typical patterns.



Key:

1. Protein marker (29, 45, 66, 97, 116 and 205 kDa)
2. Strain M364*
3. " M368
4. " M373
5. " 1086 (*M.intracellulare*)
6. " 1222 (Woodpigeon bacillus)*
7. " 1226 (Isolate from a Nene)

* Aberrant strains

DISCUSSION.

Morphological Characteristics.

The great majority of the isolates from both the Slimbridge and Arundel birds were morphologically typical of pathogenic *M.avium*. Pigmented strains, caused by carotenoids, were isolated from different species of birds from both Slimbridge and Arundel. There have been several reports of pigmented *M.avium* strains from water birds (Chalquest and Matsuoka, 1962; Schaefer, 1965; Schaefer *et al*, 1973) leading Schaefer *et al* (1973) to suggest some association between pigmentation and water as a source of infection. However, such pigmentation does not correlate with virulence as in staphylococci (Grange, 1980).

Lipid Analysis.

The study clearly shows that *M.avium* type 1 is highly pathogenic to wildfowl which agrees with the work carried out by Schaefer *et al* (1973). Wildfowl are a very diverse group of birds yet all exhibit this susceptibility to type 1. Even the birds from the Arundel Wildfowl and Wetlands Trust centre were infected with this type. This is perhaps not surprising as the introduction of infection would probably have come from infected birds being moved to Arundel from Slimbridge.

The wild Mallard 88/12X found at Slimbridge was also infected with this type. This is probably an indication of its association with the infected grounds and birds at Slimbridge, rather than wild birds providing the source of infection, although a wild source may have been responsible for introducing the disease initially.

This present study may be an indication of either the pathogenicity of type 1 or an indication that it is far more prevalent in the environment at Slimbridge and therefore infecting many birds. Its apparent abundance at Slimbridge must be however, indicative of its pathogenicity, as it has no doubt built up in the environment after being excreted by successive generations of tuberculous birds.

In the absence of environmental isolates it is impossible to say whether or not other serotypes of *M.avium* are present at Slimbridge and whether or not the birds are susceptible to them. Schaefer *et al* (1973) isolated both types 1 and 2 from the environment at Slimbridge and suggested that this represented a large reservoir of infection. If other pathogenic serotypes were present at Slimbridge it would be expected that some of the birds would have become infected and developed disease accordingly. Type 2 was not isolated from any of the wildfowl in this study. Although this may be a reflection of its lower pathogenicity in wildfowl, the level of type 2 in the environment would have been reduced by the move away from the use of chickens. There is also a spacial separation between where the chickens and wildfowl are housed, with an effectively contained unit used for former.

As discussed in Chapter one, the most common isolate from British Anatidae outside The Wildfowl and Wetlands Trust collections is type 2 (Schaefer *et al*, 1973). The type 2 isolated from the Mute Swan *Cygnus olor* from the Bristol area reflects the presence of type 2 in wild populations. These reports, and those mentioned in Chapter one, indicate that wildfowl are susceptible to types 1, 2 and 3 of the avian tubercle bacillus.

Protein Analysis.

As with the lipid analysis there was a high degree of similarity between the strains from both Slimbridge and Arundel isolates, no doubt indicating a common source of infection. However, it would be incorrect to assume that this is the protein pattern of type 1 *M.avium* as many of the reference strains of different serotypes exhibited this pattern. The pattern does however, appear to be species specific and as such is useful for mycobacterial classification at the species level.

DNA Analysis.

The uniformity of the results of DNA analysis ties in with the findings of the lipid and protein analyses, and is therefore not surprising. The use of other probes could provide a more detailed study of the causative organisms.

Aberrant Strains.

Schaefer *et al* (1973) found 16/69 (23%) of isolates from Anatidae to be aberrant, compared to none of the isolates from the chickens or the environment. Of the 39 isolates reported here, seven were aberrant in at least one aspect (18%). Schaefer *et al* (1973) suggest that the tendency towards roughness of isolates from Anatidae, creates these problems. This was shown in the isolate from the Barnacle Goose 88/31 which was rough and hence untypable by lipid analysis. These authors also suggest that aberrant strains may be related to birds having prior immunity to this type of *M.avium* and either relapsing or becoming infected again at a later challenge. This may account for the difference in the strains isolated from the two Meller's Ducks. These birds were a pair, and died together. They would have been exposed to

the same environment and yet the isolate from one differed in its lipid pattern (86/179), and the other in its protein pattern (86/180). The strain isolated from the vaccinated White-winged Wood Duck (88/133) was not aberrant in any way, suggesting that vaccination had no effect on its susceptibility to the typical *M.avium* strain at Slimbridge. (The lipid pattern of this isolate can be seen from lane 7 in figure 10.1 and the protein pattern can be seen from lane 2 in figure 10.5).

The pigmentation of the strains from the Long-tailed Duck 87/74A, Steamer Duck 87/113A and Mallard 88/12X may have resulted in the lack of lipid spots in the first two and aberrant protein pattern in both the Steamer Duck and Mallard. The aberrance of the latter strain may be related to this bird being wild and possibly becoming infected elsewhere. Only one strain was aberrant in both its lipid and protein pattern, namely that from the Steamer Duck 87/113A, which may be related to the Tachyerini being very resistant to *M.avium* infection.

Aberrant strains were isolated from both Arundel and Slimbridge birds. Three out of the seven aberrant strains were isolated from birds of the Anatini, although more isolates were made from this tribe than any other. Those strains that were difficult to type could have been passaged through chickens in an attempt to allow easier typing, although the ethics of such a procedure are highly questionable.

The major differences in protein patterns were found in some of the reference strains. Those with an obvious lack of the characteristic 38 kDa band tended to be those aberrant or unusual in other ways too, or those of different serotype. Even the strain from the Mute Swan (M1), serotyped by Mr. E. Boughton and described as "a completely typical *M.avium* serotype 2" (personal communication) produced a poor pattern

in lipid analysis and lacked the 38 kDa band. Although this bird was found in the Bristol area and as such may have been to Slimbridge, it would seem unlikely that it had become infected there. Thus, this method of protein analysis is not in itself a method for identifying the source of infection, but would appear to be specific enough to indicate that some sources are unlikely.

Summary.

In general very few differences have been found between isolates of *M.avium*. The findings made in the epizootiological study hence show susceptibilities to the same organism. Whether or not the same pattern of susceptibility would be seen with a different type of *M.avium* is open to question.

The presence of only one major type of *M.avium* is encouraging with respect to control of infection in that measures can be directed against just one causative agent.

The Case of *M.avium* type 1 Infection in a wild Whooper Swan.

The case of avian tuberculosis infection in the wild Whooper Swan *Cygnus cygnus cygnus* (PM number 88/4X) is of interest. This juvenile female was picked up from The Wildfowl and Wetlands Trust centre at Caerlaverock on the Solway Firth in February 1988. The Whooper Swans at Caerlaverock breed on Iceland and winter on the Solway Firth. Return rate to this area of South West Scotland is very high, especially amongst families of birds (Black and Rees, 1984).

The first point of interest about this bird was its age. It was a juvenile bird, and cases of avian tuberculosis in juvenile wildfowl in captivity are very rare (Cromie *et al*, 1991a; and Chapter three). An analysis of PM data of captive swans within all the collections of The Wildfowl and Wetlands Trust Centres from 1951-1989 revealed no cases of death due to avian tuberculosis in any juvenile Whooper Swans.

As a juvenile it would never have been to Caerlaverock before and therefore either contacted the pathogenic organism in Iceland where it hatched, or in the time it was present at Caerlaverock prior to its death. Whichever the case, it is unusual in that avian tuberculosis is usually a chronic disease with a long period between primary infection and development of advanced disease. Such a rapid progression of the disease may even point to the bird being in some sort of immunocompromised state.

The long incubation usually required for establishment of *M.avium* infection could point to the bird becoming infected in Iceland. Pairs of birds often return to the same breeding site each year and the habit of building up the nest and reusing it in subsequent years (Soothill and Whitehead, 1988) may allow a focus of infection to

become established at this site if one or both of the parents are infected. However the nest site is usually in exposed marshy areas, open to the sterilising effects of ultra-violet radiation, extreme weather conditions and occasional flooding which may act to reduce the viability of any organisms present.

If the bird became directly infected from another swan in Iceland it was almost certainly from one of its parents as there is usually a great distance between pairs in the nesting areas (Kear, 1972). The cygnets also tend to remain close to one parent, usually the female (Brazil, 1981).

As seen from Chapter three, feeding habit affects the birds' risk of infection (Cromie *et al*, 1991a). Whooper Swans feed by both dabbling and grazing. The parents often paddle the water to raise edible particles to the surface which the young then take (Kear, 1972). Such a practice could also stir up *M.avium* from shallow sediments which could then be ingested with the edible particles. The parents also pull vegetation from their surrounds which they then pass to the young (Kear, 1972). A parent infected with *M.avium* has the potential for contaminating this food source.

Infected faeces is the main source of virulent *M.avium* for transmission of infection to both chickens (Thoen and Karlson, 1978) and wildfowl. Whooper Swans have been observed to indulge in coprophagy when on their wintering grounds (Black and Rees, 1984). The authors even report great competition over the faeces. This would be a possible means for infection of other individuals.

These methods of feeding may explain how infection can be spread however, direct ingestion of *M.avium* with the food tends to lead to

lesions in the gut, liver and so on. This bird died of primary pulmonary tuberculosis with no disseminated disease, a relatively unusual feature in *M.avium* infections (Cromie *et al*, 1991a). As seen in Chapter three, the only captive birds dying of primary pulmonary tuberculosis were birds that fed by dabbling. Hence dabbling may have provided the means for the transmission of the causative organism. Alternatively, very considerable aerosols must be made in the process of taking off from water. During this procedure, ventilation rate would be increased along with depth of inhalation. As cygnets take off in the spray created by their parents they may be at risk of infection from the environment in aerosol form.

Direct transmission by droplet infection from a parent bird is also a possibility. Whooper Swans are highly social and cygnets tend to remain very close to the parents (Black and Rees, 1984). As their name suggests they are very vocal. They frequently indulge in loud calling displays to maintain family bonds and organisation (Johnsgard, 1965; Craggs, 1973; Black, 1988) often when facing one another. *M.avium* can be incorporated in aerosols (George and Falkingham, 1989) and pulmonary infection in one or other parent could no doubt transmit infection in this way.

As mentioned previously, type 2 is the most common isolate from wild Anatidae, whereas type 1 is often associated with epizootics in captive collections. The type 1 isolate from the Whooper Swan (lane 15 in figure 10.1) also had a protein pattern on SDS-PAGE (lane 3 in figure 10.4) and DNA analysis analagous to those of isolates from birds of The Wildfowl and Wetlands Trust collections. Perhaps the source of infection was tuberculous birds of these collections. The bird in question would never have come into contact with the grounds

at Slimbridge. However, a small captive collection was present at the Caerlaverock Wildfowl and Wetlands Trust Centre from 1970 to 1980. Many of the birds in this collection originated from Slimbridge and as such would have provided a possible vector for transmission of *M.avium* to this area. The case of the tuberculous Barnacle Goose found in this area in 1973 (Dr. Myrfyn Owen, personal communication) may either indicate of a level of infection at this site or reflect enzootic infection in wild populations.

Although the swan would never have been to other sites where there were collections of birds, its parents could have. There have been sitings of Whooper Swans that usually winter at Caerlaverock, instead wintering at The Wildfowl and Wetlands Trust centre at Martin Mere where there is a large collection of wildfowl with enzootic avian tuberculosis.

Schaefer *et al* (1973) showed that many captive wildfowl may be infected to some degree with *M.avium* but not develop pathological disease. If this is the case within wild migratory birds they, and others with early stages of the disease, could carry infection over great distances. Of an analysis of recoveries of wild ducks ringed at Slimbridge (Carl Mitchell, personal communication) only one Gadwall was found shot at Caerlaverock, some nine months later, which suggests that northerly movements of birds from Slimbridge are an infrequent event. Another ringing recovery reported a Tufted Duck *Aythya fuligula* ringed at Slimbridge recovered at a Whooper Swan site in Iceland. However infrequent these movements, such birds could act as vectors for transmission of *M.avium*.

The role of stress and infection is discussed in Chapter three (Gross *et al*, 1989). Gardarsson and Skarphedinsson (1984) showed that

migration does not affect juvenile Icelandic Whooper Swan mortality any more so than the adult birds, indicating that migration is no more stressful to juveniles than to adults. However, if the juvenile in question had been separated from its family it would indeed have been stressed. Black and Rees (1984) report birds at the Caerlaverock site spending a greater amount of time being vigilant and suggest this is due to the greater amount of human disturbance at this site, disturbance which may be stressful to a young bird that has not experienced this before. Cold weather is known to increase mortality in birds (Beer, 1964), However, mean monthly temperatures for South West Scotland during the winter of 1987-1988 were generally a degree or so above average and so it is unlikely that cold stress was an important factor.

Had the bird been ringed, more information could have been gleaned about nest site, parents, family status, movements and habits and so shed more light on the intriguing field of *M.avium* infections in the wild.

CHAPTER ELEVEN.

DISCUSSION.

CHAPTER ELEVEN.

DISCUSSION.

Mortalities due to avian tuberculosis within the captive collection of The Wildfowl and Wetlands Centre at Slimbridge remain a serious problem. The epizootiological study (Chapter three) has revealed a 73% increase in the incidence of the disease since the study of Beer carried out from 1958-1968. Even within the 1980's there has been a 10.3% increase between the first and last five year periods ($p < 0.02$). This suggests that although the rate of increase in incidence is slowing down, the trend is still upward.

The groups of birds most susceptible and at risk of infection have been identified. However, the future of the captive breeding programme of the White-winged Wood Duck remains the gravest concern. The particularly high incidence of the disease in this species (89% : 63/71; Chapter two) presents a dilemma. As the birds are dying as they reach sexual maturity, should other birds be brought into the grounds to maintain the size of the gene pool or should the remaining birds be sent to other centres to continue the programme there? The former option would inevitably put new birds at serious risk of infection and the latter could introduce the disease to new collections.

The implications for the wild birds that use the grounds at Slimbridge have been discussed (Chapter three; Cromie *et al*, 1991b). The Rushy Pen in particular with its high incidence (40.5%) must provide a huge reservoir of *M.avium* and so put the hundreds of wild birds that use it, at risk of infection. The most obvious cases of the disease in wild birds have been seen in the cases of Bewick's Swans unable to make the return migration in the spring, remaining at

Slimbridge, and subsequently dying of the disease. In the absence of PM details of birds such as the Bewick's Swans on their breeding grounds or migration routes, it is difficult to ascertain information about the epizootiology of the disease in the wild.

The bacteriological study (Chapter ten) has revealed one epizootic caused by *M.avium* type 1 within the collections at both Slimbridge and Arundel. This study, coupled with information on bird movements, enables us to postulate whether or not *M.avium* infections in wild birds may have been through contact with the collections of The Wildfowl and Wetlands Trust. The cases of the disease in both the juvenile Whooper Swan from Caerlaverock and the Mute Swan from the Bristol area, are cases in point. The former may have been infected indirectly from a Wildfowl and Wetlands Trust collection, the latter almost certainly not so.

Possible control measures for the disease were discussed in Chapter four. The major factors in reducing the incidence of tuberculosis in humans, without control measures, have been improvements in socioecomonic, and hence living, conditions. To remove infection from the environment at Slimbridge would be an enormous undertaking and would then require putting new young birds into the grounds. The access of the grounds to wild birds means that the disease could easily become established once more.

This thesis presents an attempt to determine and understand the relationship between wildfowl and mycobacteria. It explores the possibility of immunoprophylaxis in the prevention of the disease within the individual, leading to a reduction in the incidence within the collection as a whole.

Immunity to Mycobacteria.

Mycobacterial immunity is dependent upon activation of mononuclear phagocytes by T-lymphocytes (Mackaness, 1971). This transformation of lymphocytes and subsequent events are considered to be the expression of delayed-type hypersensitivity (Pearmain *et al*, 1963; Rosenstreich and Rosenthal, 1974). As discussed in Chapter four, the actual identification of T-lymphocytes in wildfowl has yet to be demonstrated, so it has to be assumed that similar processes are responsible for their resistance to mycobacterial infection.

The first step in this project has been the development of immunological tests, namely LTT, skin test and ELISA, to assess the efficacy of the potential vaccines. Chapter five described the first successful wildfowl lymphocyte transformation in the presence of mycobacterial antigens, and the first use of skin testing wildfowl in the foot web.

Increased lymphocyte transformation *in vivo* is taken as a mechanism for protective responses and this is exploited in the *in vitro* LTT. This test has had limited use in diagnosis (Smith and Reichman, 1972) but is considered to be of use in assessing vaccine efficacy in many species including chickens (Timms, 1979) where protection may not be afforded by humoral responses.

The optimal conditions for the LTT have revealed the necessity to maintain wildfowl mononuclear cells close to avian body temperature, both in the transport of blood and when washing purified cell suspensions. For responses to mitogens, duck cells are cultured in a pool of heat inactivated duck sera (Higgins and Teoh, 1988; Higgins, 1990a; Higgins, 1990b). Although a pool of sera did support lymphocyte

transformation to mycobacterial antigens in most cases, autologous sera gave higher SI's. This is also preferable from a practical point of view, as obtaining a pool of sera from healthy White-winged Wood Ducks, in particular, would be difficult.

The main similarity between the conditions required for transformation responses to mitogens and those for antigens is the need for high cell concentrations. Higgins and Teoh (1988) suggested that close cell to cell contacts were necessary for cell communication. From their work on chicken lymphocytes, Kirchner and Oppenheim (1972) also suggested that increased cell density facilitated greater lymphocyte and macrophage cooperation and that actively proliferating cells may produce some essential product only if in close cell contact. The soluble factors involved in cellular communication in wildfowl have yet to be demonstrated but some information has been gleaned during work carried out by Dr.D.A. Higgins, of Hong Kong University, and Prof. H.-D. Flad of Forschungsintitut Borstel, Germany, and the author at The First International Duck Cytokine Workshop (Higgins *et al*, 1991).

A possible criticism of the LTT is that specific antigen driven lymphocyte proliferation can indicate not only stimulation of T cell mediated hypersensitivity but also B cell antibody production, or both. The inclusion of the non-specific T cell mitogen phytohaemagglutinin, could have differentiated between stimulation of antibody producing cells and T cell stimulation. Although phytohaemagglutinin was included in the LTT's in the early stages of the studies, during which time responses to the mitogen corresponded to responses to the mycobacterial antigens, it was not used subsequently. However, increased LTT responses were not accompanied by

increases in antibody levels, hence it can be assumed the results from LTT's were a measure of cell mediated immune responses.

Skin testing has also offered a means for assessing the immunological responses within the vaccine groups. Although results from both this test and the LTT may need careful interpretation with respect to the immunological status of the individual, the results as a whole have offered an overall evaluation of the efficacy of the study vaccines.

Timms (1979) reported the use of leucocyte migration inhibition (LMI) assays in chickens sensitised to *M.tuberculosis* and suggested that this test detected cell mediated responses for longer periods of time and on more occasions than LTT's. She also described a lack of correlation between magnitude of skin test responses and both *in vitro* tests, although the capillary LMI assay correlated better to the LTT and skin test than did the agarose LMI assay. The inclusion of an LMI assay in the present studies may have been useful, however as was pointed out by Timms, this assay produced widely different results between individuals with similar skin test responses.

Where LTT and skin test responses of the study birds did not correspond, this may be due to involvement of different cytokines or classes of lymphocytes. Chaparas *et al* (1971) also showed some carbohydrates in tuberculin capable of eliciting responses in LMI assays and skin tests, were not able to do so in LTT's. This is unlike proteins which are capable of stimulation of responses in all three tests.

The Vaccine Studies.

Mycobacterium vaccae.

The role of common mycobacterial antigens in protective immunogenicity has been mentioned in the Mallard study (Chapter six). The protection from both tuberculosis and leprosy which can be gained from BCG vaccination is evidence of this. Animal experiments using a variety of species of mycobacteria to protect from other mycobacterial infections (Lefford *et al*, 1980; Edwards *et al*, 1982; Rook and Stanford, 1981) also point to the common group i antigens being of prime importance in promotion of protective immune responses. Of the potential vaccines used in the Mallard study, killed *M.vaccae* which was subsequently used in all the trials, was shown to be an apparently highly immunogenic agent. Some of the reasons for this saprophytic environmental organism having such unusual properties and its greater implications have been discussed (Chapter six).

Whilst some species of mycobacteria promote tissue damaging Koch like responses, other species including *M.vaccae*, appear to induce protective Listeria-like responses (Stanford *et al*, 1978) i.e. responses leading to the killing of mycobacteria, rather than the killing of cells containing mycobacteria. Thus, *M.vaccae* has the potential for use as a vaccine where recognition of common antigens is required. *M.vaccae* lacks both group ii and iii antigens but has an abundance of these group i common antigens (Stanford and Grange, 1974). Swinburne *et al* (1985) showed that *M.vaccae* could enhance recognition of the group i antigens by the production of memory lymphocytes in the spleen of mice.

The presence of *M.vaccae* in the environment in Uganda, was suggested (Shield, 1983) to be responsible for the increased protective efficacy of BCG in a leprosy vaccination trial (Stanley *et al*, 1981). It was this apparent synergistic effect that led to the use of *M.vaccae* plus BCG as a potential vaccine for leprosy (Bahr *et al*, 1986; Ghazi-Saidi *et al*, 1989; Stanford *et al*, 1989). As well as a potential immunoprophylactic, *M.vaccae* has been used with some success as an immunotherapeutic agent in the treatment of both tuberculosis and leprosy in man and animals (Stanford *et al*, 1988a; Stanford *et al*, 1988c; Torres *et al*, 1988; Stanford *et al*, 1990; Bahr *et al*, 1990b). The results of the Mallard, Mandarin, Gadwall and Nene studies have shown again the efficacy of this unusual non-pathogenic organism.

The Efficacy of a Killed Vaccine.

The Mallard study (Chapter six) showed killed *M.vaccae* to possess greater immunogenicity than the comparable live vaccine and BCG. That a killed vaccine should be preferential to a live vaccine, is in contrast to a number of studies using killed mycobacterial vaccines which have reported little or no protection being afforded to experimental mice when subsequently challenged with *M.tuberculosis* (Orme, 1988; Collins, 1971). Orme (1988) suggests that although there was some non-specific resistance to challenge and delayed type hypersensitivity in these mice, only living mycobacterial vaccines generated numbers of protective T-cells. However, other workers report using killed vaccines in animal models to propagate reactive murine T-cell lines (Boom *et al*, 1987; Kaufmann and Flesch, 1986). As yet, insufficient mortality data from the vaccine studies described in this thesis are available to prove whether or not the vaccines used have afforded protection from *M.avium* infection.

There have been many reports detailing resistance to mycobacterial challenge following vaccination with killed vaccines (Weiss, 1959). Killed *M.leprae* has been shown to protect mice from both *M.leprae* and *M.tuberculosis* infection (Shepard *et al*, 1978; Patel and Lefford, 1978). It would appear that it is only a few species of mycobacteria which lose their immunogenicity once killed and then only in certain strains of mice (Rook, 1980). The prolonged antigenic stimulus provided by live vaccines, which proliferate *in vivo*, could be the reason for their apparent immunogenicity and account for why larger doses of killed vaccines tend to be required. However as Rook (1980) points out, killing mycobacteria would appear to have a qualitative effect on their immunogenicity. The work of Raffel (1948, quoted by Rook, 1980) described less necrotic skin test reactivity in those guinea pigs given killed mycobacteria. Mackaness (1967) suggests that it is the built in adjuvanticity of mycobacteria that renders them immunogenic when killed. The cell walls of *Corynebacterium parvum* have similar adjuvant properties and as such, the organism is routinely administered in a killed form.

Rook (1980) suggests that killing *M.leprae* may increase its immunogenicity by preventing it from getting into cytoplasm and nerve cells in which it effectively becomes 'hidden' from the immune system. Instead, a killed vaccine will be taken into phagosomes where it will be processed and presented to appropriate antigen recognising cells.

Both Weiss (1959) and Rook (1980) reported killed vaccines to be immunogenic regardless of the way in which they are killed. γ-irradiated vaccines were used in the Mallard, White-winged Wood Duck and Mandarin studies and autoclaved vaccines were used subsequently in the Gadwall and Nene study. Both methods used for killing the bacilli

appear to have rendered the vaccine efficient at increasing cell-mediated immune responses, although there may be qualitative differences created by the two methods. It is possible that permeability of the bacterial cell walls is altered by heating and hence internal antigens may leak out, this may not be the case in irradiated bacilli. Stainsby (1989) described autoclaved *M.vaccae* as superior to irradiated, for oral vaccination of mice. The short time span of the Gadwall and Nene study prevent us from drawing conclusions at this stage as to whether this is true in wildfowl.

An Optimum Age for Vaccination.

Some of the reasons for vaccination prior to contact with environmental mycobacteria have been discussed previously (Chapters seven and nine) and its apparent importance has been emphasised in all of the separate vaccine studies. In both the Mallard and Mandarin studies vaccination at one day old resulted in greater cell mediated immune responses than vaccination at six weeks of age. The apparent failure of vaccination of the White-winged Wood Ducks in adult life may also indicate its importance. Although there was no group vaccinated later than one day old in the Gadwall and Nene study, vaccination early in life again boosted cell mediated responses.

Schaefer *et al* (1973) carried out a limited study of environmental mycobacteria within the grounds at Slimbridge and isolated free-living mycobacteria from 22% (14/65) of samples. In those birds already sensitised to these environmental mycobacteria, vaccination may initiate inappropriate cellular responses. Such a phenomenon has been described in humans in the failure of BCG to afford protection from leprosy in Burma (Shield *et al*, 1980) and tuberculosis in South India (Tuberculosis prevention trial, Madras, 1980). In these cases the

implicated environmental organisms were *M.scrofulaceum* and *M.kansasii* in Burma (Shield *et al*, 1982; Shield and Stanford, 1982) and *M.avium* in South India (Stanford, 1983b). The latter author suggests that such contact acts in a similar way to primary tuberculosis, and the cellular immune responses following this have a necrotic component. Vaccination then enhances this Koch type response which is responsible for death of cells that express mycobacterial antigens on their surface. The vaccine therefore, does not afford protection and may even be considered detrimental to the individual where necrosis occurs around lesions in tissue. This response may however, have a beneficial role in assisting walling off lesions. In the tuberculous White-winged Wood Ducks this may have been the case although the PM reports from the birds do not describe any marked differences in the pathology of vaccinates and controls, and neither is it known whether ducks make two kinds of response to mycobacterial challenge.

Palmer and Long (1966) reported the protection from tuberculosis afforded to inhabitants of Alabama and Georgia, USA, following contact with environmental *M.scrofulaceum* and *M.avium intracellulare*. Skin testing showed Listeria-like responses i.e. responses indicative of greater bactericidal immune mechanisms. In these areas BCG failed to provide protection. Infection with any such environmental saprophytic mycobacteria in the birds prior to vaccination would result in there effectively being no unvaccinated group (Smith *et al*, 1985). Stanford (1983b) suggests that limited contact may be protective but susceptibility could be brought on by greater contact. Such effects may be responsible for the failure of the vaccine to afford protection to the White-winged Wood Ducks and the reduction of the cell mediated immune responses given by the Mallard and Mandarins vaccinated at six weeks of age. Rook *et al* (1981) suggest that even in areas where

vaccination of adults is not protective, vaccination of young children before environmental sensitisation may be so, and point out that there have never been reports of complete failures of BCG trials in children.

BCG affords protection to Europeans from *M.tuberculosis* when given after contact with environmental mycobacteria. However, the level of challenge with these organisms must be considerably lower than the challenge to *M.avium*, which the birds are subjected to on a daily basis.

An Optimum Dose of Vaccine.

Dose, as well as route, of antigen required to elicit delayed-type hypersensitivity responses has been shown to be critical in experimental situations (Lagrange *et al*, 1974; Uhr *et al*, 1957). The Nene and Gadwall study (Chapter eight) specifically investigated the effect of dose of killed *M.vaccae* on stimulating cell-mediated immune responses. It would appear from this study that high doses (10^9 bacilli) of the vaccine are required which may be intrinsically linked to the fact that the vaccine used was killed. Orme (1988) criticised the use of killed mycobacterial vaccines but did report resistance to *M.tuberculosis* in mice when given a high dose (10^8) of these killed organisms. The necessity for high doses when using killed vaccines has been discussed in Chapter eight and mentioned repeatedly in the review by Weiss (1959). It has been suggested that this greater antigenic load is required to compensate for the lack of multiplication of the vaccine *in vivo*. This may be partially true, although killing mycobacteria also has a qualitative effect.

The use of doses of 10^6, 10^7, 10^8 and 10^9 killed *M.vaccae* has been reported in this thesis and the highest of these has been chosen as optimal. The 10^7 killed *M.vaccae* used in the Mallard study (Chapter six) increased cell mediated responses more that the 10^6 dose of the same vaccine. The results of the Gadwall and Nene study indicated an optimum dose of 10^9 bacilli which was 100 fold greater than the doses used in the previous Mallard, Mandarin and White-winged Wood Duck studies. A further titration of higher doses may reveal an even higher dose to be preferable. Perhaps it should be noted that the 10^7 dose increased LTT and skin test responses in the Mallard, Mandarin and Gadwall, but not in the Nene, and did not protect White-winged Wood Ducks which may suggest a relationship between body size and optimum dose.

Results from Tuberculous Birds.

The results of LTT's, skin tests and ELISA's from tuberculous birds have revealed a number of points and suggested an immunological spectrum of disease. In the late stages of disease there were often reduced cell mediated responses and always increased humoral responses together, no doubt with a proliferation of bacilli. This situation has been recorded in a number of mammalian species, including man during chronic tuberculosis and lepromatous leprosy (Lenzini *et al*, 1977; Turk and Bryceson, 1971); and tuberculous badgers (Mahmood, 1985; Stainsby, 1989). The suppression of lymphocyte transformation may be due to a number of factors. Desensitisation of reactive lymphocytes can occur due to the release of large amounts of tuberculoproteins from tuberculous lesions. This phenomenon has been described after the administration of either intravenous or intradermal tuberculin in large amounts (Howard *et al*, 1970). Mahmood (1985) describes

increased suppression of LTT results from badgers, with increased antigen concentration. Bahr (1980) also describes this concentration dependence in man. Rook (1975) suggested the loss of skin test response in mice with experimental mycobacterial infection, could also be explained by excessive release of antigen. In guinea pigs infected with *Leishmania enriettii*, Bryceson *et al* (1974) described a similar situation of suppression, coupled with a reduction in the number of circulating sensitised lymphocytes. Although some of the results of Little *et al* (1982) have been superseded (Mahmood, 1985; Stainsby, 1989), they showed that in both naturally and experimentally infected tuberculous badgers, skin test responses were universally negative.

Cellular responses can also be suppressed by the prostaglandins that are produced during infection (Bahr, 1980). In tuberculous infection, suppression of cell mediated responses may also be due to serum containing complement dependent lymphocytotoxic activity (Herberman and Fahey, 1968).

Accompanying this hyporesponsiveness of cell mediated mechanisms to mycobacterial antigens, humoral responses increase. Increases in mycobacterial antibodies can in effect block cellular responses by suppressing transformation (Roupe and Strannegard, 1972). Rees (1976) suggests that the lysosomal enzyme activity in phagocytic cells is suppressed by high levels of mycobacterial antibodies or immune complexes. The ubiquitous rise in antibody levels to all the antigens used, is almost certainly directed against the common group i antigens and has been reported in several species during advanced tuberculous disease (Bahr *et al*, 1990a; Stainsby *et al*, 1989).

It is possible that LTT results have provided a better measure of protective cell mediated immune responses as there were no significant

differences in LTT responses in vaccinated or control tuberculous White-winged Wood Ducks. Conversely, the vaccinated tuberculous birds had significantly greater skin test responses than the control tuberculous birds.

Seasonal Variations in Results.

Wildfowl are very sensitive to seasonal change with photorefractoriness and breeding cycles being closely associated with various endocrinological changes (Bluhm, 1985; Haase *et al*, 1985; Peczely, 1985). Haematology and serum constituents also differ seasonally (Shave and Howard, 1976; Fairbrother and O'Loughlin, 1990; Fairbrother *et al*, 1990). Differing serum constituents could affect the outcome of LTT's as cells are cultured in autologous sera. Perhaps these seasonal physiological changes also affect immunological responses. Dr. D.A. Higgins, of Hong Kong University, reports varying duck LTT responses to lectins according to season (personal communication). These birds gave high transformation responses from November to June and low responses throughout July until October. These experiments were carried out on laboratory ducks in Hong Kong where breeding seasons and so on, may be somewhat different from those at Slimbridge.

Seasonal variations in antibody levels to mycobacterial antigens were found in the Mallard, Mandarins and White-winged Wood Ducks although there were too few results from the Gadwall and Nenes to elucidate any such trend. The seasonal variation in both skin tests and ELISA's may be partially explained by the ecology of *M.avium* in the environment. A study carried out by the British Tuberculosis Association (1966) on the epidemiology of skin test sensitivity of school children to avian tuberculin, revealed increased sensitivity in the summer in eastern

and southern parts of Britain and in late summer in western parts. It was suggested that contact was from localised sources of infection in the environment, more often in rural areas than urban. Environmental sensitisation to avian tuberculin is thought to account for the lack of skin test positivity in indigenous populations in Alaska, with increasing sensitisation in populations further south in temperate areas, and more so again in sub-tropical areas (Edwards *et al*, 1968). Stewart *et al* (1970) report increased tonsillar mycobacterial infections and skin test sensitivity during the spring in East Anglia possibly due to environmental sources. *M.avium* is an environmental organism and its possible proliferation during the warmer months will influence immunological responses within the birds at Slimbridge.

Stainsby (1989) suggested that the seasonal differences in LTT results from badgers may have been dependent upon the weight i.e. body condition of the animal which is intrinsically linked to seasonal effects. The avian tuberculosis mortality at Slimbridge has been shown to alter seasonally (Cromie *et al*, 1991a; Chapter three) so it must be assumed that seasonal immunological responses do exist. The increased humoral responses in the summer could therefore be associated with the high seasonal incidence of the disease during this time

Criticism of the Experimental Design.

One of the criticisms of the vaccine studies is the short time span of the Gadwall and Nene study, especially considering that their major *M.avium* challenge would be later in life and significant differences between vaccine groups were not seen in the Mandarin study until they were one year old.

Most investigations into the relationship between animals and pathogenic organisms are carried out using laboratory bred animals in very controlled conditions. The investigations in this present study have used captive wild birds, and as such may have involved problems which would otherwise have not been encountered, such as the deaths of many female Mallard in the spring and summer.

The role of stress in *M.avium* infection in birds has been shown to affect both the nature and the outcome of the disease (Gross *et al*, 1989). The epizootiological study (Chapter three) also showed stress to be an important factor in mortality in wildfowl (Cromie *et al*, 1991a). The stress experienced by the experimental birds during these investigations may therefore have had some bearing on their immunological responses. The White-winged Wood Ducks in particular may have been most at risk. These were housed in relatively large groups, partly to enable easy capture for this study. In the wild they are usually found singly or in pairs (Chapter two) and these somewhat unnatural circumstances at Slimbridge may have been stressful. The apparent territorial nature of the birds was often seen when the birds were being housed in two smaller, separated pens during the period of skin testing. If birds were accidentally replaced in the 'wrong' pen there were usually vocal displays and outright aggression shown to the new bird. Although these displays and attacks would subside with time, it indicates the heirarchy which must exist within the groups of birds. Position in such a heirarchy may influence immune responses and outcome of infection. The other species of birds used in the studies did not show such marked behaviour.

Taking biometric measurements of weight and wing length at each three monthly blood sampling, may have provided some information about body

condition index (weight/wing length) and hence how this affects susceptibility to infection. The epizootiological study (Chapter three; Cromie *et al*, 1991a) revealed a fairly close relationship between body condition index and seasonal mortality.

Perhaps histology should have been carried out on lesions from vaccinated and unvaccinated birds that died of avian tuberculosis. This may have given some information concerning both the pathogenic organism and the host response to it, in particular to elucidate whether responses were of either Listeria or Koch type. It, together with more detailed pathology, would also give some indication of the nature of the disease and the extent of dissemination of the disease in both controls and vaccinates. *M.avium* was isolated from some of the organs of tuberculous birds from the vaccine studies, but a more thorough and comprehensive study of isolates from different organs and lesions could have assessed the effects of bacterial load together with immunopathology on morbidity.

In addition to more thorough clinical and pathological examinations, a study of the haematology of both healthy and infected birds may have been useful. Haematology is known to alter during *M.avium* infection in chickens, cranes (Hawkey *et al*, 1990) and raptors (Neil Forbes, personal communication). Although differential cell counts are not specific for avian tuberculosis and may be indicative of another infection, haematological studies at every three monthly bleed may have provided more information as to when infection was at its early stages. It may have been of particular use in the White-winged Wood Ducks, some of which possibly had sub-clinical infection at the time of vaccination.

Benefits of Using a Killed Vaccine.

If killed *M.vaccae* is to be used as a potential vaccine there are a number of benefits in using a killed vaccine. Firstly, killed vaccines are easier to standardise as the bacilli cannot multiply within the vaccine buffer. For this reason storage of the vaccine is less critical, i.e. it can be supplied in a ready to use form. Many live vaccines are supplied in a lyophilised form and there can be either minor or major errors during reconstitution. The vaccine in this present study is killed in the vials from which it is administered, so there is less risk of contamination. Although unlikely, there is a possibility of live attenuated vaccine strains mutating and reverting to pathogenicity. The disaster at Lübeck in 1923 when a virulent strain of tubercle bacilli was accidentally used, illustrates the possible dangers of using a live vaccine.

Benefits of Vaccination Early in Life.

The finding that vaccination should be administered early in life is also advantageous from a practical point of view. The vaccine can be administered when the birds are contained within the duckery unit and could be carried out with the routine pinioning of young birds. The lack of requirement for repeated vaccinations later in life also reduces the complexity of the vaccination programme without the problems associated with recall of patients/birds.

It is also fortuitous that the same dose of vaccine is required for both ducks and geese as this eliminates potential areas for mistakes.

Vaccination for avian tuberculosis in wildfowl: A realistic possibility?

The development of such a vaccine presents numerous problems different from the human situation. Firstly, it involves developing a vaccine to protect about 147 different species and numerous sub-species and races, species as diverse as prehistoric-looking Magpie Geese *Anseranas semipalmata* and evolutionary more recent North American Ruddy Duck *Oxyurina jamaicensis rubida*. Racial differences in susceptibility in man are well documented (World Health Organisation, 1980), as are the responses of different mouse strains to tuberculous infection (Youmans *et al*, 1959). In other words, the genetics of a vaccinate influence response to vaccine, susceptibility to infection and outcome of infection.

Secondly, environmental factors which influence the outcome of BCG vaccination in humans (Stanford and Rook, 1983) may also affect the wildfowl at Slimbridge. Their environment may be the same but their interaction with it, i.e. their life-style, varies greatly from species to species. The reservoir of infection in man is contained within the infected individual, the birds at Slimbridge may be in contact with pathogenic *M.avium* on a daily basis.

More than a century has passed since the identification of the human tubercle bacillus, a century during which thousands of workers have carried out research into human immunity to such pathogens and the development of potential vaccines. Yet tuberculosis in man remains an enormous problem and a serious burden in The Developing World, with

three million deaths and ten million new cases each year (Grange, 1985). Humans are generally considered immunocompetent; immunologically speaking, Dr. D.A. Higgins refers to ducks as "feathered dinosaurs" (personal communication). Wildfowl immunity to mycobacteria is a relatively new field of investigation with a need for expansion.

A Potential Diagnostic Test.

The prevention and control of tuberculosis in man is two sided, firstly the identification of infected individuals and subsequent treatment; and secondly, immunoprophylaxis in the rest of the population. In this thesis work has begun to develop the latter, but in doing so has revealed information concerning the identification of the infected individual.

Whether protective or not, it is obvious that antibodies to mycobacterial antigens play a role in immunity. The work of Stainsby *et al* (1989) revealed increased antibodies to common mycobacterial antigens in tuberculous badgers. This has also been described in the human situation in patients with pulmonary tuberculosis (Bahr *et al*, 1990a). The work of Lyons and Naafs (1987) showed different patterns of antibodies to environmental mycobacteria in Zimbabwean patients with different types of leprosy.

The results from tuberculous birds, in particular the White-winged Wood Ducks, in these studies have shown a marked increase in antibody levels to mycobacterial antigens during infection. In some of the birds this rise in levels occurred one year prior to death. This indicates the problem of the infected individual within a population. If such a bird is excreting virulent bacilli during this time, the

risk of infection to other birds in contact, is enormous.

This finding of raised antibodies should be exploited. Previous serological tests have been used with some success in experimentally infected chickens (Thoen and Karlson, 1978). Sero-diagnosis in humans can to be both specific and sensitive (Nasau *et al*, 1976; Wadee *et al*, 1987). Any diagnostic test for the birds must be able to differentiate between those with mere experience of mycobacteria, whether pathogenic or not, and those with *M.avium* infection.

Although sensitivity of any diagnostic test is important, the major factor must be specificity. Unlike the human situation where the identified individual is treated with appropriate chemotherapy and/or immunotherapy, an infected bird would be destroyed. Diagnosis must be correct in the case of rare or endangered birds. A number of White-winged Wood Ducks were diagnosed incorrectly when false positive results were obtained with the previously used whole blood agglutination test (developed by the Central Veterinary Laboratory, Weybridge). The gene pool of these birds in captivity is already limited and such mistakes cannot be afforded. The inclusion of secreted mycobacterial antigens, in particular those from *M.avium*, could perhaps enhance the specificity of such a test. The lipids isolated from *M.avium* (Chapter ten) could be used in ELISA as they are specific to the serotype 1 avian tubercle bacilli prevalent within The Wildfowl and Wetlands Trust collections. Monoclonal antibodies to *M.avium* antigens may even be of use.

Rousen (1990) reported that sero-diagnosis was not practical in South African children free from tuberculosis as there was too much cross-reactivity with BCG. This however was less of a problem in older children. With respect to the birds in this study, vaccination had

little effect on antibody levels and it would be unnecessary to test the birds early in life due to infrequency of infections at this time. Therefore, a serological technique could be successful in adult birds.

If a serological test could be developed it also opens up the possibility of an assessment of the disease within wild populations of birds. A bank of sera from the population of Barnacle Geese that breed on Spitzbergen is held at Slimbridge. These birds winter on the Solway Firth centring around the Caerlaverock Wildfowl and Wetlands Trust centre. The tuberculous Barnacle Goose and Whooper Swan discussed in Chapters one and ten respectively, are cases in point of wild birds dying of the disease, in this area. Little is known about the prevalence of such infection in free flying wildfowl and screening sera could provide useful information. The feral flock of Barnacle Geese at Slimbridge are known to fly over great distances and have been recovered throughout Europe as far away as Sweden. As seen from Chapter three, the incidence of the disease in this species in captivity is high (42% of PM's) and their role in the widespread transmission of infection could be very significant. These particular birds are caught annually at Slimbridge and a diagnostic test could effectively remove any infected individuals.

The haematological technique of Hawkey *et al* (1990) could be used in conjunction. Another possibility is to develop, or amend, the previously developed agglutination test (Central Veterinary Laboratory, Weybridge). Such an agglutination test was also reported by Kwatra *et al* (1972). Although duck agglutinating antibody response is often poor, there may be means of enhancing the process. Sari and Thain (1983) enhanced this response when testing ducks for *Salmonella spp.* by the addition of an antiglobulin reagent.

Lymphocyte transformation has been used to a limited extent as a diagnostic tool in the diagnosis of tuberculosis in humans (Smith and Reichman, 1972) as has skin testing. However, the results of LTT's and skin tests from tuberculous birds in the studies reported in this thesis, were less conclusive than the antibody results. In addition, vaccination may interfere with these, or the agglutination tests, for diagnosis.

Conclusions.

The following conclusions can be drawn from this thesis:-

1. There has been a significant increase in the incidence of avian tuberculosis in the captive collection at Slimbridge since a study carried out from 1958-1968. There was a continued upward trend in incidence of the disease, shown in significantly higher incidences in the last five years of the 1980's compared to the first.

2. Since the previous study, there have been significant increases in incidence in all the tribes with the exception of the Oxyurini and Anseranatini. The Cairinini and Mergini, excluding the eiders, remain very susceptible groups. Although there was no sex predilection, there was a relationship between incidence and seasonal body condition, with significantly greater incidences of female mortality in the summer and male mortality in the winter. Feeding habit affected the incidence of the disease with those that fed by dabbling or diving for food at greatest risk of infection. There was a lower incidence in those birds that have evolved in temperate climates. No area of the grounds at Slimbridge could be considered free from infection, and areas such as The Tropical House and Rushy Pen had particularly high incidences.

3. Deaths from avian tuberculosis remain a serious problem for the captive breeding programme of the White-winged Wood Duck, with 89% of deaths being due to the disease during 1980-1989.

4. The epizootics of avian tuberculosis in the collections of birds at both Slimbridge and Arundel, were caused by *M.avium* type 1. SDS-PAGE of sonicated *M.avium* can provide a means of differentiating between strains.

5. Wildfowl lymphocytes are temperature sensitive and should be transported in whole blood diluted 1:1 in heparinised RPMI at close to avian body temperature. Similarly, cell suspensions should be washed in warmed buffers.

6. For *in vitro* wildfowl lymphocyte responses to sonicated mycobacteria, cells should be cultured at high cell concentrations (8 x 10^5 cells/well) and in 10% autologous sera. Duck mononuclear cells should be cultured for four days, and Nene cells for three. This method of lymphocyte transformation test can be used to assess the efficacy of trial vaccines.

7. Skin testing of ducks and geese should be done by administration of the reagents intradermally in the foot web. Maximum responses can be expected at 41 or 48 hours post administration.

8. There appears to be an immune spectrum in wildfowl during the development of avian tuberculosis. This is characterised by an inverse relationship between cell mediated and humoral responses.

9. The dramatic increase in humoral responses during later stages of pathological disease may provide a means to identify infected individuals by the use of a serological test.

10. γ-irradiated or autoclaved *M.vaccae* are capable of increasing cell mediated immune responses in wildfowl to a greater extent than live *M.vaccae* or BCG.

11. To increase cell mediated responses, vaccination with killed *M.vaccae* should be administered prior to environmental sensitisation with mycobacteria. Vaccination in adult life may be ineffective.

12. For maximum cell mediated responses, a high dose of killed *M.vaccae* should be used and the same dose of 10^9 bacilli appears to be optimal in both ducks and geese.

Current Vaccination Programme and Future Plans.

Since 1988 half of all the birds hatching in the Slimbridge duckery have been vaccinated within their first week of life. In 1988 the birds were given 10^7 γ-irradiated *M.vaccae* R877R intradermally in the foot web. At this time many hatchlings at The Wildfowl and Wetlands Trust centre at Arundel, Sussex, were also vaccinated. This particular centre has an enzootic avian tuberculosis problem similar, if not greater, than that at Slimbridge.

Following the results of the Gadwall and Nene study, this dose was increased and since 1989 half of all those hatching have been vaccinated with 10^9 autoclaved *M.vaccae* R877R. The half left unvaccinated were control birds. Care was taken to vaccinate half of each clutch where possible, leaving half unvaccinated. In this way the control birds were genetically similar to the vaccinated birds. All the vaccinated birds were pinioned and web tagged, each web tag (National Band and Tag Company, Kentucky, USA) is individually numbered. At several weeks old, both vaccinates and controls were ringed and released into the grounds at Slimbridge. This is a large and long term study with on average 300 birds being vaccinated in each season. It is hoped that differences in mortality rates in each group can be compared within the next few years as birds begin to develop the disease.

The Wildfowl and Wetlands Trust are opening a new centre at Llanelli in South Wales. The site has not had captive birds on it before and although no environmental isolates have been made, it is probably free from any significant level of *M.avium* infection. The centre is currently building up a captive collection prior to opening to the public in 1991. In an attempt to prevent the introduction of the

disease to this new site, the captive collection is being supplied by Slimbridge, in the form of eggs or very young birds and not as adults. In addition to this, it has been made policy to vaccinate all of the birds both sent to, as well as hatched at, Llanelli within their first one or two weeks of life. These birds receive the same vaccine as those at Slimbridge, i.e 10^9 autoclaved *M.vaccae* R877R. This vaccination programme will continue unless the results of the long term study at Slimbridge prove inconclusive.

This thesis outlines the first steps in the development of a vaccine, i.e. the optimal conditions for the immunological tests and results of a series of vaccine studies. If a vaccine is to be fully developed more time is required to assess the outcome of the vaccine studies. The ultimate proof of vaccine efficacy is a reduction in mortality of vaccinates. This will only become apparent in time. I suggest that an epizootiological survey is carried out in some years time of the vaccinated birds *versus* a similar study of the control birds. If compared to the epizootiological study reported in this thesis, it would identify which groups have been protected from infection with Grange's "ducks of the microbial world" (1987).

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APPENDICES.

Appendix 5.1 : Solutions and Buffers.

Phosphate Buffered Saline (PBS), pH 7.2, 0.15 M.

The following materials were dissolved in 1000 ml of distilled water:-

Sodium chloride (BDH 10241), 8.00 g/l

Potassium chloride (BDH 10198), 0.02 g/l

Di-sodium hydrogen phosphate (Na_2HPO_4)(BDH 30157)(0.008 M), 1.15 g/l.

Potassium di-hydrogen phosphate (Sigma P-5379), 0.20 g/l.

The solution was then autoclaved for 15 minutes at 15 lbs/in^2.

Borate Buffered Saline for Vaccines, 0.067 M pH 8.0.

The following materials were dissolved in 1000 ml of distilled water:-

Borax ($Na_2B_4O_7$ $10H_2O$)(BDH 44473) 3.63 g/l

Boric acid (H_3BO_3)(BDH 27409) 5.25 g/l

Sodium chloride (NaCl)(BDH 10241) 6.19 g/l

Tween 80 (polyoxyethylene sorbitan monoleate)(Sigma P-1754) 0.0005%

The pH was adjusted to pH 8.0 and then it was autoclaved for 15 minutes at 15 lbs/in^2

Incomplete Freund's Adjuvant.

8.5 parts light paraffin (BDH 29436)

1.5 parts Arlacel A (Sigma A-8009)

The two ingredients were mixed under pressure, by using a 1 ml syringe jammed into a 25 ml universal container, until the consistency became meringue-like.

Solutions used for Hoefer Mighty Small SDS-PAGE.

Stacking Gel Buffer: Tris-HCl buffer, 0.5 M pH 6.8.

30.3 g of Tris(hydroxymethyl)aminoethane (Sigma T-1503) was dissolved in 100 ml of distilled water. This was then titrated to pH 6.8 with 1 M HCl (BDH 1789) and the volume was adjusted to 500 ml.

Separating Gel Buffer: Tris-HCl buffer, 1.5 M pH 8.8.

45.51 g of Tris(hydroxymethyl)aminoethane (Sigma T-5753) was dissolved in 100 ml of distilled water. This was then titrated to pH 8.8 with 1 M HCl (BDH 1789) and the volume was then adjusted to 250 ml.

Electrode Buffer: Tris-glycine pH 8.3, 0.25 M tris, 1.92 M glycine.

The following materials were dissolved in 1000 ml of distilled water:-

30.3 g Tris(hydroxymethyl)aminoethane (Sigma T-5753),

134.6 g Glycine (Sigma G-7126),

10 g Sodium dodecyl sulphate (BDH 44215).

Buffers used for Horseradish Peroxidase Conjugation.

1 mM Sodium Acetate Buffer pH 4.4.

1 part of sodium acetate (BDH 30104) at 8.20 g/l was mixed with 2 parts of acetic acid (BDH 45001) at 6.005 g/l. This was diluted 1:1000 to give 1 mM.

0.2 M Sodium Carbonate Buffer pH 9.5.

Sodium carbonate (BDH 10240) was made up at 21.2 g/l and sodium bicarbonate (BDH 10247) was made up at 16.8 g/l. The first was added to the latter (approximately 6.4 ml to 18.6 ml) until the pH was 9.5.

0.1 M Borate Buffer pH 7.4.

9.54 g of disodium tetraborate decahydrate (BDH 44473) was dissolved in 250 ml of distilled water and 24.73 g of boric acid (BDH 27409) was dissolved in 4 litres of distilled water. Approximately 115 ml of borate solution was added to 4 litres of boric acid solution until the pH was 7.4.

Buffers used for the ELISA.

Coating Buffer: 0.05 M Carbonate/Bicarbonate Buffer pH 9.6.

The following were dissolved in 1000 ml of distilled water:-

1.59 g sodium carbonate (Na_2CO_3)(Sigma S-2127)

2.93 g sodium bicarbonate ($NaHCO_3$)(BDH 10247)

The pH was adjusted to 9.6

Washing/incubation Buffer pH 7.4.

The following were dissolved in 4 litres of distilled water:-

32 g sodium chloride (NaCl)(BDH 10241)

0.8 g potassium di-hydrogen phosphate (KH_2PO_4)(Sigma P-5379)

4.54 g di-sodium hydrogen phosphate (Na_2HPO_4)(BDH 30157)

0.8 g potassium chloride (KCl)(BDH 10198)

2 ml Tween 20 (polyoxyethylene sorbitan monolaurate)(Sigma P-1379)

Substrate: ABTS.

Citrate phosphate buffer 0.1 M pH 4.1 was made as follows:

Solution A: 0.1 M citric acid (Sigma C-2916), 21.01 g/l.

Solution B: 0.2 M di-sodium hydrogen phosphate ($Na_2HPO_4 2H_2O$)(BDH 30157), 35.6 g/l.

68 ml of solution A was added to 51 ml of solution B to reach pH 4.1.

The substrate: 2, 2 azino-di-(3 ethyl benthiazoline sulphonic acid)(ABTS)(Sigma A1888) was made as follows:

50 mg of ABTS was dissolved in 100 ml of the citrate phosphate buffer to which 35 ul of 20 volume hydrogen peroxide (BDH 10127) was added.

Stopping Solution

96 mg of sodium fluoride (Analar, UK) was dissolved in 50 ml of distilled water.

Buffers and Solutions for Bacteriology.

Phosphate buffer pH 6.8

The following were dissolved in 4 litres of distilled water and autoclaved for 15 minutes at 15 lbs/in^2:-

18.2 g potassium dihydrogen orthophosphate (KH_2PO_4)(BDH 29608)

23.75 g di-sodium hydrogen orthophosphate (Na_2HPO_4)(BDH 30158)

Carbol Fuchsin Stain

Solution A : 20 g of basic fuchsin (Sigma P-7632) was dissolved in 200 ml of absolute alcohol (BDH 45101).

Solution B : 125 ml of 80% phenol (Sigma P-3653) was added to 1880 ml of distilled water.

The two solutions were mixed and diluted 1/20 in distilled water for use.

Solutions for ExcelGel.

Sample Stock Buffer: 0.05 mol/l Tris-Acetic Acid pH 7.5.

3.0 g of Tris (Sigma T-5753) was dissolved in 40 ml of distilled water. The pH was adjusted to 7.5 with approximately 1.4 ml of acetic acid (BDH 45001). The volume was made up to 50 ml with distilled water.

Sample Buffer.

The sample stock buffer was diluted 1 in 10 with distilled water. To this, SDS (BDH 44215), Dithiothreitol (DTT)(Sigma D-0632) and Bromophenol Blue (Sigma B-6896) were added to obtain a final concentration of at least 10 g/l, 5 mmol/l and 0.1 g/l respectively in the sample. (NB. The sample was diluted 1 in 1 in this buffer and the buffer therefore was made at twice the final concentration).

Coomassie Staining Solutions.

Fixing Solution.

The following were mixed and made up to 1000 ml with distilled water:

500 ml Ethanol (BDH 45101)

100 ml Acetic acid (BDH 45001).

Destaining Solution.

The following were mixed and made up to 1000 ml with distilled water:

250 ml Ethanol (45101)

80 ml Acetic acid (BDH 45001).

Coomassie Solution.

To 250 ml of destaining solution, 0.29 g of Coomassie (BDH 44246) was added. This was heated to 60°C, stirring constantly, prior to use.

Appendix 6.1: LTT results from individual tuberculous Mallard.

Vaccine Group

Bird	Antigens								
Months of age	*M.vaccae* 100	50	BCG 100	50	*M.fort.* 100	50	GWT 100	50	RBP 100
Day 1/low dose/BCG									
LBA4									
21	0.2	0.5					0.5	1.0	1.3
24	1.7	5.2	2.5	6.7			1.9		
Day 1/high dose/BCG									
LBB2									
21	0.2	1.0	1.3				2.0	1.9	
24	5.1	2.2	4.6	2.7	7.4	5.4	14.2	6.7	15.2
27	6.1	2.6	4.2	12.7	9.3	4.7	19.2	4.6	23.4
31	11.4	9.5	5.1	5.0	7.6	5.2	4.9	4.0	6.6
34	0.3	0.5	1.1	1.0	0.4	0.4	0.7	1.3	2.0
6 weeks/high dose/BCG									
RBB8									
21	0.2	1.4	1.4	2.2			1.1	1.9	0.7
24	9.2	16.9	8.1				6.4	11.2	
27				dead cells					
31				"					
Control									
LYC5									
24	1.7	1.8	1.7	2.7	1.9	1.9	2.6	4.0	3.4
27	0.1	0.4	0.1	0.4	0.1	0.2	1.4	1.4	0.1
Control									
RBA9									
18	9.4	20.0	35.0	23.0			20.0	1.0	
20	0.7	2.2	2.4	0.9			4.5	0.9	

Key:

M.fort : *M.fortuitum*
GWT : *M.avium* GWT
RBP : " RBP

Appendix 6.2: Skin test results from individual tuberculous Mallard.

Vaccine group / Bird	Hours post administration				
Months of age	17	24	41	48	65
Day 1/low dose/BCG					
LBA4					
21	0.4	0.4	0.6	0.6	0.4
24	0.5	0.3	0	0	0
Day 1/high dose/BCG					
LBB2					
21	0.4	0.6	0.4	0.6	0.6
24	0.5	0.5	0.5	0.3	0
27	0.4	0.6	0.4	0.2	0.8
31	0.3	0.3	0.3	0.3	0.3
34	0.6	0.6	0.8	0	0
6 weeks/high dose/BCG					
RBB8					
21	0.8	0.6	0.6	0.8	0.6
24	0	0	0.3	0.3	0.5
27	0.4	0	0	0.4	0
31	0.5	0.5	0.3	0	0.3
34	0	0	0.6	0.8	1.0
Control					
LYC5					
24	0	0	0	0	0
27	0	0	0	0	0
Control					
RBA9					
18	0	0	0.3	Biopsy taken	

Appendix 6.3: ELISA results from individual tuberculous Mallard.

Vaccine group / Bird	Months of age	Antigen GWT	RBP	BCG	*M.gordonae*
Day 1/low dose/BCG					
LBA4					
	24	0.674	0.739	1.014	0.853
Day 1/high dose/BCG					
LBB2					
	11	0.442	0.406	0.522	0.507
	24	0.349	0.402	0.491	0.454
	27	0.677	0.606	0.437	0.685
	31	0.611	0.611	0.502	0.718
	34	0.991	0.909	0.905	0.989
6 weeks/high dose/BCG					
RBB8					
	11	0.295	0.301	0.344	0.339
	24	0.282	0.274	0.260	0.333
	27	0.404	0.368	0.267	0.406
	31	0.651	0.599	0.429	0.673
	34	0.595	0.438	0.337	0.593
Control					
LYC5					
	24	0.686	0.524	0.888	0.866
	27	0.746	0.664	1.126	0.977
Control					
RBA9					
	18	0.603	0.519	0.579	0.621

Appendix 6.4: *M.vaccae* vaccinated Mallard LTT results.

Antigen

Vaccine group		Months of age									
		15	17	20	23	26	29	32	35	38	43
***M.vaccae* 100 ug/ml**											
LGA	xSI		10.2	2.1	3.5	19.9	17.2	38.2	10.6	9.7	3.7
	SD		10.4	3.5	-	12.9	13.1	15.5	7.9	5.2	3.3
	n		4	4	1	4	4	4	4	4	3
LGB	xSI	1.2	4.2	7.4	2.7	19.7	16.9	59.5	28.2	25.5	14.4
	SD	-	2.5	4.9	0.6	9.8	21.1	50.6	17.4	10.4	7.0
	n	1	2	3	2	3	3	2	4	4	4
RGB	xSI	3.2	6.5	3.6	3.0	18.3	36.2	24.1	6.4	20.7	8.5
	SD	-	6.0	2.5	-	6.5	28.6	22.1	5.8	8.5	6.3
	n	1	4	3	1	3	3	3	3	3	3
RWB	xSI	1.0	10.2	5.4	1.2	13.7	14.1	9.1	4.4	6.6	7.1
	SD	0.3	13.5	5.3	0.5	6.2	10.7	4.8	3.0	0.6	7.3
	n	4	5	6	3	4	5	3	3	2	4
Cont	xSI	1.2	5.0	2.9	0.8	20.8	12.7	14.9	8.5	9.5	6.5
	SD	-	2.8	3.1	0.7	14.3	8.3	3.6	4.4	8.0	3.7
	n	1	2	6	3	4	6	3	3	3	4
***M.vaccae* 50 ug/ml**											
LGA	xSI		16.4	6.4	14.7	17.7	11.2	34.3	13.3	10.9	4.8
	SD		21.0	10.0	-	13.1	7.5	15.1	14.1	5.8	3.4
	n		4	4	1	4	4	4	4	4	3
LGB	xSI	5.7	11.0	26.7	5.7	11.9	10.0	55.1	14.7	19.4	12.7
	SD	-	9.3	19.0	3.7	8.0	10.9	32.3	7.2	5.4	5.7
	n	1	2	3	2	3	3	2	4	4	4
RGB	xSI	4.7	6.1	11.4	27.1	9.4	18.8	20.7	4.9	20.4	6.2
	SD	-	6.5	12.0	-	3.8	14.2	22.8	4.8	9.7	4.0
	n	1	4	3	1	3	3	3	3	3	3
RWB	xSI	1.9	6.9	8.7	15.9	9.0	9.4	8.9	4.0	5.1	5.4
	SD	0.8	5.4	10.8	9.6	6.0	5.7	4.3	1.7	0.9	4.8
	n	4	5	6	3	5	5	3	3	2	4
Cont	xSI	1.7	11.1	5.6	12.4	12.1	11.8	14.1	5.8	6.3	4.9
	SD	-	7.4	4.4	14.1	11.2	10.1	3.3	4.1	4.3	3.0
	n	1	2	6	4	5	6	3	3	3	4

Appendix 6.4 continued.

Antigen

Vaccine group		Months of age									
		15	17	20	23	26	29	32	35	38	43
BCG 100 ug/ml											
LGA	xSI		12.6	4.2	2.8	14.8	13.7	7.9	11.2	2.9	2.2
	SD		9.8	4.3	-	7.4	10.0	7.0	17.0	1.4	1.4
	n		4	3	1	4	4	4	4	4	3
LGB	xSI	9.2	1.9	15.2	4.4	27.3	15.2	17.3	4.7	11.1	9.1
	SD	-	2.4	16.0	2.1	9.7	18.6	16.1	1.9	7.1	5.7
	n	1	2	3	2	3	3	2	4	4	4
RGB	xSI	7.0	5.4	7.0	3.3	19.3	23.5	17.0	1.7	7.1	2.1
	SD	-	4.9	7.7	-	3.7	13.2	23.3	1.5	2.2	0.1
	n	1	4	3	1	3	3	3	3	3	3
RWB	xSI	2.6	5.0	9.0	1.3	14.7	10.1	2.7	3.1	3.4	2.0
	SD	1.4	2.4	13.7	0.6	9.8	6.8	0.9	2.5	0.1	1.7
	n	4	5	6	3	5	5	3	3	2	4
Cont	xSI	0.7	13.3	6.0	1.6	15.7	8.9	4.0	2.7	1.6	3.4
	SD	-	0.9	7.7	2.0	9.7	7.6	3.7	3.0	0.4	2.2
	n	1	2	6	2	5	6	4	3	3	4
BCG 50 ug/ml											
LGA	xSI		1.6	0.8	18.1	17.8	13.4	17.0	5.3	8.0	2.4
	SD		0.6	0.4	-	18.3	11.9	15.1	5.8	6.0	0.7
	n		4	3	1	3	3	4	4	4	3
LGB	xSI	0.4	0.9	1.3	-	35.0	11.5	25.3	4.7	25.6	6.1
	SD	-	0.5	1.0	-	8.8	13.0	26.2	2.7	11.9	3.6
	n	1	2	3	-	3	2	2	3	4	4
RGB	xSI	1.3	1.4	1.4	24.9	14.3	15.9	21.1	1.5	5.3	2.2
	SD	-	0.4	0.9	-	2.7	15.2	28.3	0.6	1.7	0.9
	n	1	4	3	1	3	3	3	3	3	3
RWB	xSI	1.0	1.3	2.2	16.4	8.4	7.9	3.4	3.7	2.4	2.0
	SD	0.5	6.0	1.9	11.2	7.2	3.6	2.7	2.9	1.4	1.7
	n	4	4	6	3	5	5	3	3	2	4
Cont	xSI	0.9	1.7	2.0	6.4	13.7	3.4	5.0	3.8	4.7	2.0
	SD	-	0.3	2.0	5.0	6.7	2.4	3.4	3.6	2.4	1.1
	n	1	2	5	4	5	5	3	3	3	4

Appendix 6.4 continued.

Antigen

Vaccine group		Months of age									
		15	17	20	23	26	29	32	35	38	43
***M.avium* GWT 100 ug/ml**											
LGA	xSI		2.4	5.9	1.1	16.2	4.4	38.4	6.9	9.1	3.3
	SD		1.3	7.4	-	10.2	3.6	31.9	4.5	3.5	3.2
	n		2	3	1	4	4	4	4	4	3
LGB	xSI	6.6	11.0	12.8	3.2	23.4	2.6	39.7	30.9	12.2	8.3
	SD	-	2.8	14.0	-	3.9	0.3	31.6	9.6	5.9	7.6
	n	1	2	2	1	2	3	2	4	3	4
RGB	xSI	28.0	6.4	3.5	3.8	17.7	9.7	22.2	7.8	13.9	3.6
	SD	-	8.7	2.6	-	6.4	7.2	26.0	8.4	13.5	1.6
	n	1	4	3	1	3	3	3	3	3	3
RWB	xSI	7.6	8.9	8.1	1.0	14.6	5.7	7.4	5.4	4.4	3.0
	SD	3.6	5.3	11.1	0.6	5.9	3.1	7.6	3.6	2.4	2.4
	n	4	4	5	2	5	5	3	3	2	4
Cont	xSI		10.2	4.9	0.2	8.3	1.3	9.3	11.6	5.4	4.9
	SD		5.9	3.7	-	7.7	0.4	6.2	6.0	2.3	0.8
	n		2	5	1	3	6	4	3	3	4
***M.avium* GWT 50 ug/ml**											
LGA	xSI		1.9	1.1	16.4	14.6	7.5	44.4	6.7	10.0	4.8
	SD		1.8	0.1	-	7.1	6.8	43.2	4.6	4.6	4.7
	n		3	2	1	4	4	4	4	4	3
LGB	xSI	4.6	1.6	1.1		15.9	2.6	50.6	24.0	24.1	11.2
	SD		0.6	0.1		12.8	0.4	40.0	9.0	11.2	5.8
	n	1	2	2		2	3	2	4	4	4
RGB	xSI	7.2	2.1	1.3	34.5	14.8	11.3	6.2	6.8	15.1	7.4
	SD	-	0.9	0.7	-	4.1	8.5	5.7	7.4	11.4	5.2
	n	1	4	3	1	3	3	3	3	3	3
RWB	xSI	1.0	1.9	1.4	17.8	10.6	6.5	6.6	5.0	4.3	3.7
	SD	0.2	0.5	1.0	13.2	7.4	4.3	5.7	3.0	0.1	2.4
	n	3	5	5	3	5	5	3	3	2	4
Cont	xSI		2.2	1.3	8.2	11.2	1.6	13.1	6.8	5.4	4.5
	SD		0.4	0.8	7.9	8.6	0.7	6.5	4.6	1.7	1.2
	n		2	6	3	5	5	3	3	3	4

Appendix 6.4 continued.

Antigen

Vaccine group		Months of age							
		15	17	20	23	26	29	32	35	38	43
***M.fortuitum* 100 ug/ml**											
LGA	xSI				3.8	16.7	15.9	28.8	9.6	8.0	2.2
	SD				-	10.2	6.8	23.4	7.7	6.0	1.6
	n				1	4	3	4	4	4	3
LGB	xSI				4.7	28.3	20.6	49.2	20.4	22.7	12.7
	SD				-	7.3	21.8	39.9	6.9	11.6	4.0
	n				1	3	3	2	4	4	4
RBG	xSI				10.2	15.2	23.2	25.6	4.1	13.6	4.9
	SD				-	2.3	16.2	27.7	3.5	12.3	4.6
	n				1	3	3	3	3	3	3
RWB	xSI				3.2	19.7	10.8	9.3	4.7	3.9	4.0
	SD				2.2	6.8	8.4	7.4	3.8	0.4	3.3
	n				3	5	5	3	3	2	4
Cont	xSI				0.6	13.8	9.9	9.6	5.8	4.4	4.7
	SD				0.2	6.7	5.8	2.7	4.1	1.3	2.7
	n				3	5	6	3	3	3	4
***M.fortuitum* 50 ug/ml**											
LGA	xSI				19.0	19.1	13.7	26.4	9.9	4.9	2.8
	SD				-	8.5	4.7	13.5	8.4	6.2	1.8
	n				1	3	3	4	4	4	3
LGB	xSI					34.0	20.8	67.2	15.9	23.2	12.1
	SD					7.6	26.1	-	3.9	9.2	4.7
	n					3	2	1	3	4	4
RGB	xSI				22.3	16.2	22.6	20.4	3.4	15.2	6.5
	SD				-	5.0	16.1	21.9	3.5	11.4	5.9
	n				1	3	3	3	3	3	3
RWB	xSI				12.9	19.4	10.9	5.2	7.3	1.8	6.4
	SD				9.7	12.0	6.4	1.0	5.7	0.8	8.0
	n				3	5	5	3	3	2	4
Cont	xSI				4.8	15.8	5.9	8.6	2.7	2.4	4.2
	SD				1.9	8.3	2.9	4.2	2.8	0.7	1.9
	n				2	5	5	3	3	3	4

Appendix 6.4 continued.

Antigen

Vaccine group		Months of age								
		15	17	20	23	26	29	32	35	38	43
***M.avium* RBP 100 ug/ml**											
LGA	xSI				8.9	28.4	2.0	34.4	6.7	14.8	4.5
	SD				-	13.1	0.4	18.4	3.8	7.0	3.6
	n				1	4	4	4	4	4	3
LGB	xSI	9.7			20.8	24.3	1.7	57.8	22.6	31.6	12.6
	SD	-			-	9.5	0.4	56.6	12.6	23.4	7.9
	n	1			1	3	3	2	4	4	4
RGB	xSI	21.6			46.5	20.5	1.5	19.8	5.7	22.1	8.8
	SD	-			-	5.7	0.7	18.7	4.2	20.7	5.7
	n	1			1	3	3	3	3	3	3
RWB	xSI	2.2			10.4	35.0	2.3	8.3	5.9	5.7	7.7
	SD	0.9			3.3	12.0	0.9	9.2	3.3	2.2	8.4
	n	4			3	5	5	3	3	2	4
Cont	xSI	1.2			3.2	18.3	1.2	10.9	14.2	9.2	4.6
	SD	-			1.9	11.8	0.3	7.1	13.9	8.3	2.1
	n	1			3	5	6	4	3	3	4

Vaccine group key:

LGA : Day 1/ killed/ low dose
LGB : Day 1/ killed/ high dose
RGB : 6 weeks/ killed/ high dose
RWB : 6 weeks/ live/ high dose
Cont : Control

Appendix 6.5: *M.vaccae* Vaccinated Mallard Skin Test Results.

Hours post administration

Vaccine group	Months of age								
	17	20	23	26	29	32	35	38	43
17 Hours:-									
LGA	0.80	0.60	0.50	0.32	0.70	0.24	0.38	0.35	0.50
n	4	5	5	5	5	5	4	4	4
LGB	0.70	0.75	0.17	0.40	0.58	0.45	0.44	0.55	0.67
n	2	3	3	2	3	4	4	4	4
RGB	0.87	0.50	0.58	0	0.58	0.47	0	0.33	0.58
n	3	4	3	3	3	3	3	3	3
RWB	0.37	0.45	0.50	0.12	0.56	0.56	0.06	0.45	0.56
n	6	5	4	4	4	5	4	4	4
Control	0.15	0.50	0.17	0.20	0.60	0.50	0.50	0	0.50
n	4	4	6	4	5	4	3	3	4
24 Hours:-									
LGA	0.60	0.60	0.40	0.36	0.65	0.36	0.56	0.35	0.63
n	4	5	5	5	5	5	4	4	4
LGB	0.60	0.75	0.17	0.50	0.58	0.50	0.56	0.55	0.75
n	2	3	3	2	3	4	4	4	4
RGB	0.67	0.38	0.17	0.20	0.50	0.47	0.50	0.20	0.58
n	3	4	3	3	3	3	3	3	3
RWB	0.33	0.45	0.25	0.20	0.56	0.52	0.13	0.49	0.63
n	6	5	4	4	4	5	4	4	4
Control	0.15	0.50	0.13	0.30	0.65	0.50	0.42	0.07	0.50
n	4	4	6	4	5	4	3	3	4

Appendix 6.5 continued.

Hours post administration

Vaccine group	Months of age								
	17	20	23	26	29	32	35	38	43
41 Hours:-									
LGA	0.55	0.60	0.25	0.20	0.85	0.52	0.31	0.50	0.63
n	4	5	5	5	5	5	4	4	4
LGB	0.60	0.92	0.17	0.20	0.75	0.50	0.50	0.50	0.81
n	2	3	3	2	3	4	4	4	4
RGB	0.73	0.31	0.08	0	0.58	0.47	0.50	0.27	0.58
n	3	4	3	3	3	3	3	3	3
RWB	0.33	0.45	0.06	0.12	0.63	0.56	0	0.25	0.69
n	6	5	4	4	4	5	4	4	4
Control	0.15	0.44	0.13	0	0.80	0.55	0.08	0.07	0.38
n	4	4	6	4	5	4	3	3	4
	17	20	23	26	29	32	35	38	43
48 Hours:-									
LGA	0.65	0.55	0.15	0.28	0.65	0.36	0.19	0.30	0.75
n	4	5	5	5	5	5	4	4	4
LGB	0.60	0.83	0.17	0	0.67	0.50	0.25	0.65	0.81
n	2	3	3	2	3	4	4	4	4
RGB	0.67	0.31	0.08	0	0.42	0.47	0.25	0.20	0.42
n	3	4	3	3	3	3	3	3	3
RWB	0.43	0.50	0.06	0.12	0.50	0.52	0	0.30	0.75
n	6	5	4	4	4	5	4	4	4
Control	0.15	0.31	0.13	0.12	0.70	0.55	0	0	0.44
n	4	4	6	4	5	4	3	3	4

Appendix 6.5 continued.

Hours post administration

Vaccine group	Months of age								
	17	20	23	26	29	32	35	38	43
65 Hours:-									
LGA	0.70	0.60	0	0.08	0.75	0.40	0	0.30	0.63
n	4	5	5	5	5	5	4	4	4
LGB	0.40	0.75	0.25	0.20	0.67	0.50	0	0.55	0.75
n	2	3	3	2	3	4	4	4	4
RGB	0.67	0.38	0	0	0.50	0.33	0	0.27	0.58
n	3	4	3	3	3	3	3	3	3
RWB	0.27	0.45	0.06	0.10	0.63	0.56	0	0.35	0.69
n	6	5	4	4	4	5	4	4	4
Control	0.10	0.38	0.13	0	0.65	0.40	0	0	0.44
n	4	4	6	4	5	4	3	3	4

72 Hours:-

LGA	0.70	0.05
n	4	5
LGB	0.50	0.08
n	2	3
RGB	0.67	0.33
n	3	3
RWB	0.27	0.06
n	6	4
Control	0.10	0.13
n	4	6

Appendix 6.6: *M.vaccae* Vaccinated Mallard ELISA results.

Antigen

Vaccine group.	Months of age.												
	10	11	12	13	17	20	23	26	29	32	35	38	43
BCG													
LGA	0.127	0.116	0.136		0.174	0.086	0.381	0.459	0.360	0.259	0.179	0.468	0.382
n	2	3	4		3	3	5	4	5	5	4	4	4
LGB	0.090	0.114		0.179	0.071	0.134	0.185	0.311	0.202	0.132	0.113	0.555	0.286
n	1	2		1	2	2	3	3	3	4	4	4	4
RGB		0.068	0.127	0.138	0.136		0.322	0.280	0.224	0.143	0.217	0.306	0.213
n		3	1	1	4		3	3	3	5	3	3	3
RWB	0.137	0.101		0.141	0.121	0.132	0.268	0.360	0.250	0.192	0.164	0.409	0.315
n	4	3		1	5	3	4	5	5	3	4	4	4
Control		0.152			0.097		0.287	0.423	0.279	0.177	0.187	0.421	0.334
n		5			2		5	5	6	4	3	3	4
M.fortuitum													
LGA	0.211	0.234	0.233		0.262	0.134	0.384	0.488	0.429	0.343	0.260	0.547	0.468
n	2	3	4		3	3	5	4	5	5	4	4	4
LGB	0.146	0.211		0.274	0.114	0.213	0.208	0.364	0.247	0.195	0.176	0.571	0.366
n	1	2		1	2	2	3	3	3	4	4	4	4
RGB		0.129	0.224	0.201	0.217		0.365	0.358	0.254	0.210	0.314	0.393	0.276
n		3	1	1	4		3	3	3	5	3	3	3
RWB	0.178	0.186		0.186	0.188	0.207	0.337	0.406	0.331	0.279	0.234	0.512	0.416
n	4	3		1	5	3	4	5	5	3	4	4	4
Control		0.244			0.148		0.339	0.498	0.334	0.259	0.287	0.501	0.418
n		5			2		5	5	6	4	3	3	4

Appendix 6.6 continued.

Antigen

Vaccine group.	Months of age.												
	10	11	12	13	17	20	23	26	29	32	35	38	43
M.gordonae													
LGA	0.222	0.251	0.263		0.278	0.143	0.424	0.508	0.432	0.346	0.264	0.511	0.427
n	2	3	4		3	3	5	4	5	5	4	4	4
LGB	0.151	0.223		0.293	0.117	0.223	0.228	0.388	0.254	0.214	0.181	0.474	0.310
n	1	2		1	2	2	3	3	3	4	4	4	4
RGB		0.139	0.236	0.209	0.218		0.383	0.361	0.244	0.262	0.313	0.367	0.257
n		3	1	1	4		3	3	3	5	3	3	3
RWB	0.182	0.187		0.219	0.193	0.201	0.356	0.413	0.318	0.274	0.239	0.459	0.344
n	4	3		1	5	3	4	5	5	3	4	4	4
Control		0.265			0.147		0.364	0.507	0.368	0.294	0.303	0.519	0.406
n		5			2		5	5	6	4	3	3	4
M.avium GWT													
LGA	0.100	0.142	0.129		0.124	0.064	0.287	0.291	0.282	0.337	0.206	0.339	0.303
n	2	3	4		3	3	5	4	5	5	4	4	4
LGB	0.068	0.095		0.155	0.059	0.122	0.152	0.224	0.147	0.183	0.147	0.361	0.204
n	1	2		1	2	2	3	3	3	4	4	4	4
RGB		0.059	0.110	0.104	0.090		0.250	0.227	0.164	0.184	0.261	0.248	0.182
n		3	1	1	4		3	3	3	5	3	3	3
RWB	0.082	0.086		0.093	0.097	0.103	0.228	0.267	0.217	0.251	0.168	0.322	0.254
n	4	3		1	5	3	4	5	5	3	4	4	4
Control		0.123			0.072		0.247	0.286	0.229	0.234	0.225	0.338	0.298
n		5			2		5	5	6	4	3	3	4

Appendix 6.6 continued.

Antigen

Vaccine group.	Months of age.												
	10	11	12	13	17	20	23	26	29	32	35	38	43
M.vaccae													
LGA	0.152	0.177	0.180		0.212	0.138	0.320	0.386	0.302	0.314	0.229	0.372	0.330
n	2	3	4		3	3	5	4	5	5	4	4	4
LGB	0.107	0.157		0.206	0.081	0.117	0.155	0.282	0.180	0.164	0.147	0.356	0.223
n	1	2		1	2	2	3	3	3	4	4	4	4
RGB	0.094	0.112	0.171	0.150	0.159		0.254	0250	0.174	0.178	0.276	0.259	0.185
n	1	3	1	1	4		3	3	3	5	3	3	3
RWB	0.130	0.133		0.142	0.142	0.161	0.253	0.320	0.242	0.242	0.200	0.304	0.241
n	4	3		1	5	3	4	5	5	3	4	4	4
Control		0.189			0.107		0.246	0.352	0.247	0.213	0.219	0.298	0.254
n		5			2		5	5	6	4	3	3	4
M.avium RBP													
LGA	0.174	0.203	0.219	0.340	0.209	0.126	0.463	0.512	0.421	0.338	0.248	0.610	0.503
n	2	3	4	1	3	3	5	4	5	5	4	4	4
LGB	0.122	0.189		0.234	0.086	0	0.250	0.434	0.220	0.204	0.174	0.606	0.343
n	1	2		1	2	2	3	3	3	4	4	4	4
RGB		0.121	0.196	0.172	0.183	0.134	0.398	0.370	0.246	0.245	0.293	0.387	0.312
n		3	1	1	4	3	3	3	3	5	3	3	3
RWB	0.170	0.150		0.162	0.163	0.184	0.338	0.435	0.338	0.279	0.223	0.449	0.391
n	4	3		1	5	3	4	5	5	3	4	4	4
Control		0.210			0.124		0.334	0.512	0.339	0.257	0.281	0.572	0.425
n		5			2		5	5	6	4	3	3	4

Appendix 6.7 BCG vaccinated Mallard LTT results.

Antigen

Vaccine group		Months of age							
		18	21	24	27	31	34	37	44
***M.vaccae* 100 ug/ml**									
LBA	xSI	2.2	0.3	10.4	14.0	23.6	4.5	8.1	7.8
	SD	1.8	0.2	13.4	7.6	11.2	4.5	9.5	7.8
	n	3	5	3	3	3	2	2	2
LBB	xSI	4.2	0.3	13.2	20.5	36.3	11.2	9.8	3.5
	SD	1.1	0.1	14.6	15.0	38.6	10.8	3.0	-
	n	4	3	3	3	3	3	2	1
RRB	xSI	2.7	0.3	9.5	14.4	23.9	12.7	11.6	11.3
	SD	1.6	0.2	6.4	12.0	10.6	5.8	7.4	16.4
	n	3	5	4	4	4	3	2	5
Control	xSI	2.2	0.6	9.8	11.1	16.1	7.3	8.6	3.9
	SD	1.2	0.8	11.0	8.8	8.8	5.7	4.7	3.7
	n	2	3	7	4	6	6	3	5
***M.vaccae* 50 ug/ml**									
LBA	xSI	3.9	0.8	9.7	10.7	22.9	3.5	6.8	4.7
	SD	2.0	0.3	9.3	6.8	9.0	1.9	5.1	3.7
	n	3	5	3	3	3	2	2	2
LBB	xSI	6.4	0.8	10.1	13.7	33.4	8.0	6.1	2.7
	SD	2.2	0.3	12.3	12.7	37.8	6.7	2.5	-
	n	4	4	3	3	3	3	2	1
RBB	xSI	3.6	2.1	9.9	13.4	19.4	12.9	8.9	7.2
	SD	1.8	1.6	6.8	13.9	10.6	8.8	0.1	9.8
	n	3	6	4	4	4	3	2	5
Control	xSI	1.6	3.6	6.3	16.6	16.2	6.4	9.6	3.9
	SD	0.3	4.2	5.5	12.5	9.4	5.9	5.2	3.1
	n	2	3	7	6	6	6	3	5

Appendix 6.7 continued.

Antigen

Vaccine group		Months of age							
		18	21	24	27	31	34	37	44
BCG 100 ug/ml									
LBA	xSI	4.0	1.0	8.1	9.8	28.1	2.6	4.0	3.0
	SD	2.0	0.5	10.4	4.9	12.7	2.1	2.1	3.3
	n	3	5	3	3	3	2	2	2
LBB	xSI	5.8	1.1	10.0	13.3	33.8	8.4	7.2	2.4
	SD	1.9	0.1	11.1	13.0	38.4	7.8	6.0	-
	n	4	4	3	3	3	3	2	1
RBB	xSI	2.0	2.0	8.1	13.1	17.2	3.7	5.7	3.6
	SD	0.4	1.3	0.5	14.4	10.8	2.7	0.6	3.9
	n	3	6	4	4	4	3	2	5
Control	xSI	1.0	2.9	6.8	13.4	13.5	3.0	6.3	2.5
	SD	0.1	2.2	6.1	11.6	8.7	1.7	7.7	1.8
	n	2	3	7	5	6	6	3	5
BCG 50 ug/ml									
LBA	xSI	1.1	1.1	10.0	9.7	20.2	1.7	1.7	2.5
	SD	0.7	0.2	9.7	3.5	14.9	0.6	0.5	1.3
	n	3	4	3	3	2	2	2	2
LBB	xSI	1.6	2.1	11.2	15.3	27.5	6.8	2.9	3.0
	SD	1.1	0.8	15.1	6.1	32.2	6.1	1.4	-
	n	4	3	3	3	3	3	2	1
RBB	xSI	0.9	1.3	4.8	10.6	23.8	3.3	5.6	2.0
	SD	0.6	0.5	1.2	11.5	21.4	4.0	4.5	1.2
	n	3	6	3	4	4	3	2	5
Cont	xSI	0.8	1.0	5.6	11.9	11.9	2.9	5.0	1.8
		0.1	0.2	5.9	8.3	7.2	1.7	6.9	1.0
		2	3	7	6	6	6	3	5

Appendix 6.7 continued.

Vaccine group		Months of age 18	21	24	27	31	34	37	44
***M.fortuitum* 100 ug/ml**									
LBA	xSi			12.4	12.2	23.7	3.6	7.7	2.5
	SD			10.4	4.7	13.9	3.8	8.2	1.3
	n			3	3	3	2	2	2
LBB	xSI			10.8	18.4	35.6	8.9	5.2	4.2
	SD			4.7	15.6	40.2	9.0	-	-
	n			2	3	3	3	1	1
RBB	xSI			9.9	13.6	22.4	8.9	12.0	8.3
	SD			2.9	16.8	13.4	6.5	4.1	11.7
	n			3	3	4	3	2	5
Control	xSI			7.9	14.9	11.1	8.5	4.9	3.7
	SD			4.1	11.4	5.7	8.0	2.1	3.4
	n			6	6	5	6	3	5
***M.fortuitum* 50 ug/ml**									
LBA	xSI			10.9	13.1	27.6	3.2	6.0	2.2
	SD			11.2	7.7	15.8	3.1	5.5	0.1
	n			2	3	3	2	2	2
LBB	xSI			14.5	13.5	34.5	9.9	14.3	3.8
	SD			12.8	11.0	41.4	13.4	-	-
	n			2	3	2	2	1	1
RBB	xSI			8.3	10.9	14.8	9.7	12.0	5.2
	SD			1.9	15.7	17.8	5.2	4.5	3.9
	n			3	3	2	2	2	5
Control	xSI			7.4	13.8	14.7	7.1	4.4	2.7
	SD			6.6	11.1	8.8	5.9	2.8	6.0
	n			7	6	3	6	3	5
***M.avium* GWT 100 ug/ml**									
LBA	xSI	1.7	1.1	5.4	11.3	19.7	2.9	6.6	3.4
	SD	0.1	0.7	6.4	4.1	8.2	3.4	6.8	0.4
	n	2	5	3	3	3	2	2	2
LBB	xSI	2.4	1.2	15.5	12.4	23.5	9.7	12.6	3.8
	SD	1.4	0.6	13.4	5.9	25.9	9.4	0.2	-
	n	3	4	3	3	3	3	2	1
RBB	xSI	1.3	2.4	9.2	12.4	20.3	7.9	11.9	6.8
	SD	0.4	2.0	4.7	13.8	17.8	6.7	2.8	6.4
	n	3	6	4	4	4	3	2	5
Control	xSI	1.3	3.4	9.2	9.8	13.6	9.8	6.0	2.6
	SD	0.1	3.5	8.3	5.0	6.8	9.8	4.4	1.8
	n	2	3	7	6	6	6	3	5

Appendix 6.7 continued.

Antigen

Vaccine group		Months of age							
		18	21	24	27	31	34	37	44
***M.avium* GWT 50 ug/ml**									
LBA	xSI	0.4	0.9	8.5	8.1	19.1	4.2	4.1	4.6
	SD	0.3	0.2	7.8	5.2	6.7	4.3	2.5	0.4
	n	2	5	2	3	3	2	2	2
LBB	xSI	0.9	1.4	14.1	5.2	14.0	10.1	9.1	4.8
	SD	0.6	0.5	16.2	1.0	11.6	10.1	6.3	-
	n	3	3	3	3	3	3	2	1
RBB	xSI	0.8	1.3	10.6	2.6	16.6	7.1	11.4	7.7
	SD	0.4	0.6	7.9	2.1	17.9	7.2	3.3	7.9
	n	3	6	3	4	4	3	2	5
Control	xSI	0.8	1.2	7.6	5.7	8.0	8.2	6.9	3.5
	SD	0.6	0.2	6.8	3.9	6.7	6.0	8.9	2.4
	n	2	3	7	6	6	6	3	5
***M.avium* RBP 100 ug/ml**									
LBA	xSI		1.0	11.8	24.9	29.3	9.4	3.2	5.2
	SD		0.3	10.0	18.8	18.3	10.9	1.1	1.3
	n		4	2	2	3	2	2	2
LBB	xSI		0.8	8.8	16.4	37.7	9.8	4.0	3.9
	SD		0.2	5.7	9.9	35.9	7.0	3.0	-
	n		2	3	3	3	3	2	1
RBB	xSI		2.8	16.6	23.7	30.4	18.9	8.1	10.2
	SD		2.9	2.7	19.7	20.8	4.9	3.2	7.8
	n		6	2	4	4	3	2	5
Control	xSI		6.9	7.6	26.4	16.2	13.9	4.5	3.3
	SD		6.7	5.2	18.4	6.3	9.3	5.8	1.3
	n		2	6	6	6	6	3	5

Vaccine group key:

LBA : Day 1/ low dose
LBB : Day 1/ high dose
RBB : 6 weeks/ high dose

Appendix 6.8: BCG Vaccinated Mallard skin test results.

Hours post administration

Vaccine group	Months of age							
	18	21	24	27	31	34	37	44
17 Hours:-								
LBA	0.75	0.60	0.44	0.67	0.61	0.60	0.21	0.70
n	3	7	4	3	3	2	2	2
LBB	0.50	0.65	0.67	0.48	0.44	0.73	0.50	0.50
n	5	4	3	3	3	3	2	2
RBB	0.75	0.72	0.38	0.55	0.63	0.35	0.36	0.55
n	3	5	4	4	4	4	2	4
Control	0.50	0.40	0.28	0.23	0.54	0.36	0.14	0.64
n	4	4	8	6	4	5	4	5
24 Hours:-								
LBA	0.75	0.49	0.44	0.60	0.78	0.80	0.21	0.60
n	3	7	4	3	3	2	2	2
LBB	0.45	0.60	0.33	0.53	0.39	0.67	0.50	0.60
n	5	4	3	3	3	3	2	2
RBB	0.75	0.60	0.13	0.40	0.71	0.55	0.14	0.55
n	3	5	4	4	4	4	2	4
Control	0.56	0.30	0.19	0.20	0.50	0.32	0.18	0.52
n	4	4	8	6	4	5	4	5
41 Hours:-								
LBA	0.60	0.66	0.13	0.60	0.61	0.70	0.14	0.70
n	3	7	4	3	3	2	2	2
LBB	0.55	0.45	0.25	0.47	0.50	0.60	0.64	0.70
n	5	4	3	3	3	3	2	2
RBB	0.75	0.56	0.13	0.30	0.54	0.35	0	0.55
n	3	5	4	4	4	4	2	4
Control	0.50	0.50	0.13	0.17	0.38	0.24	0.11	0.52
n	4	4	8	6	4	5	4	5

Appendix 6.8 continued.

Hours post administration

Vaccine group	Months of age							
	18	21	24	27	31	34	37	44
48 Hours:-								
LBA	0.75	0.60	0	0.40	0.61	0.30	0.29	0.50
n	3	7	4	3	3	2	2	2
LBB	0.55	0.60	0.17	0.47	0.44	0.20	0.50	0.70
n	5	4	3	3	3	3	2	2
RBB	0.63	0.60	0.19	0.40	0.46	0.35	0	0.65
n	3	5	4	4	4	4	2	4
Control	0.75	0.45	0.09	0.07	0.50	0	0.14	0.64
n	4	4	8	6	4	5	4	5
65 Hours:-								
LBA	0.75	0.49	0	0.53	0.67	0.10	0.14	0.70
n	3	7	4	3	3	2	2	2
LBB	0.60	0.65	0.17	0.73	0.39	0.2	0.29	0.70
n	5	4	3	3	3	3	2	2
RBB	0.75	0.60	0.13	0.30	0.46	0.36	0	0.45
n	3	5	4	4	4	4	2	4
Control	0.75	0.45	0.13	0.13	0.42	0	0	0.68
n	4	4	8	6	4	5	4	5
72 Hours:-								
LBA	0.75	0.57						
n	3	7						
LBB	0.50	0.55						
n	5	4						
RBB	0.75	0.52						
n	3	5						
Controls	0.75	0.45						
n	4	4						

Appendix 6.9: BCG Vaccinated Mallard ELISA results.

Antigen

Vaccine group		**Months of age**							
		11	18	24	27	31	34	37	44
BCG									
LBA		0.226	0.191	0.271	0.165	0.115	0.115	0.142	0.114
	n	3	2	2	2	3	2	2	2
LBB		0.148	0.188	0.270	0.289	0.126	0.134	0.184	0.099
	n	4	3	1	3	2	2	2	2
RBB		0.242	0.207	0.240	0.111	0.099	0.083	0.155	0.095
	n	4	3	3	2	4	3	2	4
Control		0.214	0.203	0.252	0.273	0.125	0.117	0.151	0.133
	n	7	3	6	4	8	5	4	5
M.fortuitum									
LBA		0.288	0.177	0.256	0.176	0.171	0.166	0.224	0.167
	n	3	2	1	2	3	2	2	2
LBB		0.130	0.168	0.217	0.356	0.151	0.167	0.231	0.121
	n	4	3	1	3	2	2	2	2
RBB		0.209	0.181	0.216	0.153	0.138	0.118	0.218	0.134
	n	4	3	3	2	4	3	2	4
Control		0.199	0.173	0.223	0.233	0.171	0.153	0.207	0.177
	n	7	3	6	4	8	5	4	5
M.gordonae									
LBA		0.298	0.227	0.269	0.239	0.248	0.220	0.302	0.237
	n	3	2	1	2	3	2	2	2
LBB		0.139	0.218	0.199	0.277	0.190	0.219	0.304	0.152
	n	4	3	1	2	2	2	2	2
RBB		0.238	0.184	0.244	0.207	0.192	0.156	0.320	0.187
	n	4	3	3	2	4	3	2	4
Control		0.230	0.209	0.257	0.257	0.225	0.199	0.299	0.229
	n	7	3	6	4	8	5	4	5

Appendix 6.9 continued.

Antigen

Vaccine group	**Months of age**							
	11	18	24	27	31	34	37	44
***M.avium* GWT**								
LBA	0.198	0.162	0.198	0.219	0.219	0.198	0.280	0.231
n	3	2	1	2	3	2	2	2
LBB	0.107	0.134	0.199	0.301	0.175	0.206	0.261	0.134
n	4	3	1	2	2	2	2	2
RBB	0.179	0.131	0.168	0.175	0.159	0.132	0.239	0.139
n	4	3	3	2	4	3	2	4
Control	0.180	0.152	0.179	0.187	0.194	0.162	0.227	0.181
n	7	3	6	4	8	5	4	5
M.vaccae								
LBA	0.194	0.168	0.243	0.164	0.171	0.179	0.220	0.183
n	3	2	1	2	3	2	2	2
LBB	0.112	0.142	0.183	0.218	0.149	0.167	0.227	0.119
n	4	3	1	2	2	2	2	2
RBB	0.184	0.152	0.183	0.159	0.141	0.188	0.234	0.133
n	4	3	3	2	4	3	2	4
Control	0.168	0.156	0.193	0.172	0.162	0.144	0.206	0.165
n	7	3	6	4	8	5	4	5
***M.avium* RBP**								
LBA	0.188	0.169	0.234	0.187	0.179	0.158	0.228	0.179
n	3	2	1	2	3	2	2	2
LBB	0.120	0.153	0.252	0.347	0.168	0.191	0.250	0.130
n	4	3	1	3	2	2	2	2
RBB	0.200	0.163	0.203	0.162	0.140	0.117	0.229	0.129
n	4	3	3	2	4	3	2	4
Control	0.182	0.166	0.207	0.226	0.176	0.151	0.219	0.172
n	7	3	6	4	8	5	4	5

Appendix 7.1 Mandarin LTT results.

Antigen

Vaccine group		Months of age								
		3	6	9	12	15	18	21	24	30
***M.vaccae* 100 ug/ml**										
Day 1	xSI	1.7	3.4	0.3	3.4	7.1	10.4	4.7	6.6	2.1
	SD	0.9	3.3	0.3	0.9	6.4	3.7	1.2	2.9	0.7
	n	6	3	3	5	4	4	4	4	4
6 weeks	xSI	4.0	2.7	0.3	3.9	7.2	3.4	7.4	3.9	3.1
	SD	4.1	1.5	0.2	2.2	1.4	2.1	5.9	1.6	1.6
	n	6	3	3	6	3	4	4	4	4
Control	xSI	3.3	4.2	0.2	1.8	4.2	3.2	6.1	2.9	2.1
	SD	1.7	3.4	0	1.2	2.9	1.6	3.6	1.4	0.9
	n	6	4	4	6	3	3	4	4	4
***M.vaccae* 50 ug/ml**										
Day 1	xSI	2.6	5.2	0.7	10.4	4.3	10.4	4.0	4.8	3.1
	SD	2.6	3.4	0.5	5.0	3.3	6.1	1.6	2.7	1.9
	n	6	3	3	5	4	4	4	4	4
6 weeks	xSI	4.1	2.3	0.6	7.8	4.3	2.9	6.3	3.5	3.4
	SD	3.6	0.7	0.4	7.3	1.2	1.8	5.5	1.9	2.4
	n	6	3	3	6	3	4	4	4	4
Control	xSI	3.6	5.8	0.7	5.6	3.6	2.8	4.6	2.6	2.2
	SD	2.9	4.1	0.1	2.0	2.8	1.6	0.9	1.5	0.3
	n	6	4	4	6	3	3	4	4	4
BCG 100 ug/ml										
Day 1	xSI	3.0	5.1	0.7	4.2	5.4	11.6	3.4	2.3	1.5
	SD	4.7	1.9	0.1	2.6	5.0	8.0	1.9	0.6	0.7
	n	6	3	3	5	4	4	4	4	4
6 weeks	xSI	2.3	2.0	0.6	2.9	5.3	2.6	4.1	1.8	1.3
	SD	1.2	0.6	0.3	1.2	1.0	1.4	2.7	1.2	0.7
	n	6	3	3	6	3	4	4	4	4
Control	xSI	1.7	4.2	1.0	2.3	3.4	2.9	3.0	1.6	1.5
	SD	0.8	2.1	0.5	1.6	2.3	1.4	1.2	0.3	0.6
	n	5	4	4	6	3	3	4	4	4

Appendix 7.1 continued.

Antigen

Vaccine group		Months of age								
		3	6	9	12	15	18	21	24	30
BCG 50 ug/ml										
Day 1	xSI	1.8	2.6	1.1	12.9	4.0	9.2	2.0	2.3	1.8
	SD	1.3	0.3	0.4	6.4	2.4	4.1	0.9	0.7	0.8
	n	6	3	3	5	4	4	3	4	4
6 weeks	xSI	1.3	1.5	0.9	7.4	5.4	2.1	2.7	1.4	1.3
	SD	0.5	0.9	0.2	5.9	3.2	1.1	2.0	0.7	0.4
	n	6	3	3	6	3	4	3	4	4
Control	xSI	1.1	2.2	0.9	6.4	3.1	1.5	2.9	1.6	1.2
	SD	0.3	1.3	0.2	2.0	2.1	0.6	1.5	0.3	0.6
	n	5	4	4	6	3	3	3	4	4
***M.fortuitum* 100 ug/ml**										
Day 1	xSI				1.6	5.9	9.2	3.8	6.4	4.3
	SD				0.4	5.5	4.6	1.2	2.3	2.7
	n				5	4	4	4	4	4
6 week	xSI				2.4	5.8	2.2	3.9	3.4	5.5
	SD				1.0	1.7	1.3	2.7	1.8	3.2
	n				5	3	4	3	4	4
Control	xSI				1.7	2.8	2.4	7.8	2.8	3.0
	SD				1.1	1.7	1.1	0.7	1.3	0.1
	n				5	3	3	2	4	3
***M.fortuitum* 50 ug/ml**										
Day 1	xSI				7.8	3.8	9.6	3.1	6.2	1.9
	SD				4.5	2.5	4.2	1.8	3.9	0.6
	n				5	4	4	3	4	4
6 weeks	xSI				6.4	3.8	1.7	4.1	2.5	2.7
	SD				5.6	0.4	0.9	3.4	1.4	1.4
	n				5	3	4	3	4	4
Control	xSI				3.5	3.5	1.7	4.7	2.1	2.4
	SD				1.1	1.6	0.6	2.0	1.3	1.4
	n				5	2	3	4	4	4

Appendix 7.1 continued.

Antigen

Vaccine group		Months of age								
		3	6	9	12	15	18	21	24	30
***M.avium* GWT 100 ug/ml**										
Day 1	xSI	1.4	2.8	0.8	1.4	5.1	9.2	4.7	2.6	1.9
	SD	0.5	0.8	0.2	0.5	3.4	4.6	2.9	0.4	0.6
	n	5	3	3	5	4	4	4	4	4
6 weekss	xSi	3.6	1.5	0.7	1.1	4.5	2.3	6.0	2.8	2.9
	SD	1.9	1.0	0.3	0.6	1.2	0.9	4.1	1.2	1.9
	n	6	3	3	6	3	4	4	4	4
Control	xSI	3.6	2.7	1.2	1.3	4.0	2.1	6.4	1.9	2.4
	SD	1.8	1.2	0.9	0.8	2.7	0.6	4.4	0.9	0.8
	n	5	4	4	5	3	3	4	4	4
***M.avium* GWT 50 ug/ml**										
Day 1	xSI	1.9	1.2	1.3	15.4	4.2	7.6	5.6	3.5	3.3
	SD	2.2	0.6	0.6	7.4	2.7	3.7	4.1	1.3	1.4
	n	5	3	3	5	4	4	4	4	4
6 weeks	xSI	1.7	1.2	0.9	7.7	3.6	2.2	4.2	2.6	2.0
	SD	0.8	0.6	0.2	5.9	0.5	0.8	2.8	1.4	1.4
	n	6	3	3	6	3	4	3	4	3
Control	xSI	1.1	1.0	0.9	6.9	3.8	1.6	5.3	2.3	2.3
	SD	0.2	0.2	0.1	2.6	2.0	0.2	3.1	1.1	0.8
	n	5	4	4	6	3	3	4	4	4
***M.avium* RBP 100 ug/ml**										
Day 1	xSI			0.7	3.3	7.8	11.5	4.4	2.5	4.1
	SD			0.4	1.7	7.7	5.9	2.4	0.9	2.6
	n			3	5	4	4	4	4	4
6 weeks	xSI			0.4	6.1	5.7	2.7	8.4	2.4	4.6
	SD			0.3	8.1	4.1	1.5	6.6	1.6	3.8
	n			3	6	3	4	4	4	4
Control	xSI			1.1	4.4	2.9	2.9	6.4	2.1	2.9
	SD			1.1	5.6	0.2	1.1	4.6	1.3	0.7
	n			4	5	3	3	4	4	4

Appendix 7.2: Mandarin skin test results.

Hours post administration

Vaccine group	Months of age								
	3	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**30**
17 Hours:-									
Day 1	0.45	0.65	0.52	0.30	0.75	0.55	0.5	0.5	0.45
n	5	4	5	5	3	4	4	4	4
6 weeks	0.31	0.15	0.36	0.30	0.50	0.30	0.56	0.44	0.60
n	4	4	5	5	4	4	4	4	4
Control	0	0.35	0.12	0.20	0.25	0.30	0.63	0.31	0.65
n	3	4	5	5	4	4	4	4	4
24 Hours:-									
Day 1	0.70	0.40	0.48	0.10	0.67	0.60	0.50	0.38	0.40
n	5	4	5	5	3	4	4	4	4
6 weeks	0.31	0.25	0.40	0.38	0.44	0.25	0.13	0.25	0.50
n	4	4	5	5	4	4	4	4	4
Control	0.08	0.40	0.28	0.20	0	0.30	0	0.25	0.6
n	3	4	5	5	4	4	4	4	4
41 Hours:-									
Day 1	0.60	0.55	0.60	0.10	0.50	0.50	0.44	0.31	0.45
n	5	4	5	5	3	4	4	4	4
6 weeks	0.25	0.25	0.4	0.25	0.44	0.30	0.38	0	0.45
n	4	4	5	5	4	4	4	4	4
Control	0.08	0.25	0.20	0	0	0.15	0	0	0.60
n	3	4	5	5	4	4	4	4	4
48 Hours:-									
Day 1	0.40	0.40	0.52	0.15	0.25	0.65	0.44	0.13	0.45
n	5	4	5	5	3	4	4	4	4
6 weeks	0.25	0.30	0.44	0.08	0.44	0	0.38	0	0.55
n	4	4	5	5	4	4	4	4	4
Control	0.08	0.60	0.20	0	0.06	0.35	0	0.06	0.60
n	3	4	5	5	4	4	4	4	4

Appendix 7.2 continued.

Hours post administration

Vaccine group	Months of age								
	3	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**30**
65 Hours:-									
Day 1	0.25	0.50	0.48	0.20	0.50	0.35	0	0.06	0.20
n	5	4	5	5	3	4	4	4	4
6 weeks	0.13	0.40	0.24	0.04	0.50	0	0.13	0	0.50
n	4	4	5	5	4	4	4	4	4
Control	0	0.60	0.12	0.05	0.06	0.20	0	0.13	0.50
n	3	4	5	5	4	4	4	4	4

Appendix 7.3: Mandarin ELISA results.

Antigen

Vaccine group	Months of age								
	3	6	9	12	15	18	21	24	30
BCG									
Day 1	0.178	0.238	0.224	0.383	0.344	0.196	0.222	0.302	0.264
n	5	5	4	7	4	4	4	3	4
6 weeks	0.276	0.207	0.265	0.361	0.381	0.259	0.177	0.434	0.164
n	6	3	3	7	4	4	4	4	4
Control	0.236	0.231	0.239	0.407	0.356	0.249	0.232	0.286	0.198
n	4	5	2	9	4	4	4	3	4
M.fortuitum									
Day 1	0.287	0.354	0.463	0.669	0.696	0.347	0.385	0.489	0.619
n	5	5	4	7	4	4	4	3	4
6 weeks	0.388	0.294	0.521	0.636	0.733	0.444	0.355	0.659	0.388
n	6	3	3	7	4	4	4	4	4
Control	0.373	0.382	0.537	0.703	0.701	0.474	0.376	0.464	0.425
n	4	5	2	9	4	4	4	3	4
M.gordonae									
Day 1	0.293	0.348	0.392	0.572	0.621	0.342	0.384	0.536	0.519
n	5	5	4	7	4	4	4	3	4
6 weeks	0.396	0.311	0.498	0.611	0.649	0.374	0.359	0.652	0.349
n	6	3	3	7	4	4	4	4	4
Control	0.383	0.394	0.524	0.619	0.613	0.478	0.406	0.473	0.403
n	4	5	2	9	4	4	4	3	4
***M.avium* GWT**									
Day 1	0.195	0.228	0.241	0.429	0.396	0.220	0.244	0.370	0.507
n	5	5	4	7	4	4	4	3	4
6 weeks	0.294	0.207	0.314	0.380	0.404	0.291	0.230	0.486	0.351
n	6	3	3	7	4	4	4	4	4
Control	0.251	0.259	0.320	0.404	0.368	0.268	0.238	0.324	0.276
n	4	5	2	9	4	4	4	3	4

Appendix 7.3 continued.

Antigen

Vaccine group	Months of age								
	3	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**30**
M.vaccae									
Day 1	0.274	0.318	0.420	0.545	0.593	0.310	0.379	0.461	0.500
n	5	5	4	7	4	4	4	3	4
6 weeks	0.395	0.294	0.452	0.552	0.626	0.392	0.342	0.608	0.328
n	6	3	3	7	4	4	4	4	4
Control	0.394	0.374	0.541	0.599	0.595	0.433	0.380	0.424	0.402
n	4	5	2	9	4	4	4	3	4
***M.avium* RBP**									
Day 1	0.236	0.308	0.212	0.498	0.428	0.246	0.276	0.431	0.427
n	5	5	4	7	4	4	4	3	4
6 weeks	0.320	0.261	0.344	0.466	0.462	0.310	0.252	0.554	0.291
n	6	3	3	7	4	4	4	4	4
Control	0.322	0.305	0.367	0.497	0.420	0.317	0.288	0.367	0.356
n	4	5	2	9	4	4	4	3	4

Appendix 8.1: Nene LTT results.

Antigen				Antigen			
Vaccine group (dose)	Months of age	3	6	Vaccine group (dose)	Months of age	3	6
***M.vaccae* 100 ug/ml**				***M.vaccae* 50 ug/ml**			
High	xSI	3.1	4.0	High	xSI	8.3	2.1
	SD	4.6	2.4		SD	9.9	1.1
	n	6	5		*n*	5	4
Middle	xSI	6.3	4.7	Middle	xSI	12.5	3.2
	SD	5.0	2.8		SD	7.3	3.2
	n	5	3		*n*	5	3
Low	xSI	2.8	3.6	Low	xSI	6.0	2.6
	SD	3.5	3.7		SD	4.5	1.7
	n	5	2		*n*	5	2
Control	xSI	0.8	1.4	Control	xSI	1.7	1.4
	SD	0.7	0.9		SD	1.6	0.5
	n	5	6		*n*	5	6
BCG 100 ug/ml				**BCG 50 ug/ml**			
High	xSI	3.4	3.3	High	xSI	8.2	3.5
	SD	6.3	1.3		SD	11.6	2.1
	n	6	5		*n*	5	3
Middle	xSI	4.7	4.1	Middle	xSI	11.0	3.0
	SD	3.6	4.3		SD	7.4	2.4
	n	6	3		*n*	5	3
Low	xSI	2.9	0.8	Low	xSI	3.7	0.6
	SD	1.2	0.4		SD	1.8	-
	n	5	2		*n*	5	1
Control	xSI	1.3	1.1	Control	xSI	2.6	1.2
	SD	1.0	0.8		SD	2.0	0.8
	n	4	6		*n*	5	6

Appendix 8.1 continued.

Vaccine group		Months of age 3	6
***M.fortuitum* 100 ug/ml**			
High	xSI	5.1	2.7
	SD	9.5	1.1
	n	5	5
Middle	xSI	5.4	4.3
	SD	6.9	2.8
	n	6	3
Low	xSI	1.4	1.4
	SD	0.5	1.1
	n	5	2
Control	xSI	1.2	0.9
	SD	1.1	0.6
	n	4	6
***M.avium* GWT 100 ug/ml**			
High	xSI	1.7	1.8
	SD	2.4	1.1
	n	6	5
Middle	xSI	3.3	2.4
	SD	2.7	2.3
	n	5	3
Low	xSI	1.3	0.5
	SD	1.1	-
	n	4	1
Control	xSI	0.7	0.7
	SD	0.7	0.3
	n	5	6
***M.avium* RBP 100 ug/ml**			
High	xSI	8.3	2.8
	SD	14.2	1.3
	n	5	5
Middle	xSI	18.4	5.4
	SD	10.1	5.6
	n	4	3
Low	xSI	9.0	0.9
	SD	9.6	0.5
	n	6	2
Control	xSI	2.4	0.8
	SD	2.3	0.3
	n	3	6

Vaccine group		Months of age 3	6
***M.fortuitum* 50 ug/ml**			
High	xSI	9.8	3.2
	SD	12.0	1.3
	n	4	3
Middle	xSI	14.7	3.4
	SD	11.6	2.4
	n	4	3
Low	xSI	5.1	0.4
	SD	3.9	-
	n	4	1
Control	xSI	2.9	1.3
	SD	3.0	0.7
	n	4	5
***M.avium* GWT 50 ug/ml**			
High	xSI	7.4	2.0
	SD	8.4	1.5
	n	5	3
Middle	xSI	13.2	1.7
	SD	7.8	1.6
	n	5	3
Low	xSI	4.6	1.4
	SD	2.4	-
	n	5	1
Control	xSI	2.7	0.9
	SD	2.7	0.3
	n	5	6

Appendix 8.2: Nene Skin test Results.

Vaccine group (dose)		Months of age 3	6	Vaccine group (dose)		Months of age 3	6
17 Hours:-				**24 Hours:-**			
High			0.34	High		0.33	0.37
	n		6		*n*	6	6
Middle			0.57	Middle		0.08	0.52
	n		6		*n*	5	6
Low			0.36	Low		0.08	0.36
	n		5		*n*	5	5
Control			0.29	Control		0.14	0.37
	n		5		*n*	6	5
41 Hours:-				**48 Hours:-**			
High		0.31	0.34	High		0.44	0.23
	n	6	6		*n*	6	6
Middle		0.03	0.1	Middle		0.1	0.48
	n	5	6		*n*	5	6
Low		0	0.1	Low		0.18	0.14
	n	5	5		*n*	5	5
Control		0.10	0.23	Control		0.08	0.37
	n	6	5		*n*	6	5
65 Hours:-							
High		0.17	0.23				
	n	6	6				
Middle		0.13	0.48				
	n	5	6				
Low		0.05	0.18				
	n	5	5				
Control		0	0.14				
	n	6	5				

Appendix 8.3: Nene ELISA results.

Antigen Vaccine group (dose)		Months of age 3	 6
BCG			
High		0.217	0.259
	n	5	5
Middle		0.244	0.239
	n	6	6
Low		0.269	0.209
	n	6	6
Control		0.226	0.179
	n	6	6
M.gordonae			
High		0.184	0.276
	n	5	5
Middle		0.194	0.234
	n	6	6
Low		0.219	0.213
	n	6	6
Control		0.191	0.173
	n	6	6
M.vaccae			
High		0.195	0.276
	n	5	5
Middle		0.208	0.244
	n	6	6
Low		0.239	0.218
	n	6	6
Control		0.199	0.178
	n	6	6

Antigen Vaccine group (dose)		Months of age 3	 6
M.fortuitum			
High		0.194	0.265
	n	5	5
Middle		0.211	0.242
	n	6	6
Low		0.234	0.224
	n	6	6
Control		0.204	0.182
	n	6	6
***M.avium* GWT**			
High		0.192	0.247
	n	5	5
Middle		0.214	0.228
	n	6	6
Low		0.229	0.201
	n	6	6
Control		0.224	0.158
	n	6	6
***M.avium* RBP**			
High		0.244	0.273
	n	5	5
Middle		0.266	0.258
	n	6	6
Low		0.280	0.228
	n	6	6
Control		0.249	0.201
	n	6	6

Appendix 8.4 Gadwall LTT results.

Antigen

Vaccine group (dose)		Months of age		
		3	6	9
***M.vaccae* 100 ug/ml**				
High	xSI	8.1	22.6	10.4
	SD	4.6	10.0	9.3
	n	5	7	6
Middle	xSI	4.6	20.0	7.4
	SD	2.6	13.3	6.5
	n	4	4	4
Low	xSI	5.2	27.7	11.9
	SD	3.2	10.7	9.6
	n	5	5	6
Control	xSI	4.7	7.1	6.0
	SD	2.9	4.7	3.6
	n	6	3	5
***M.vaccae* 50 ug/ml**				
High	xSI	4.2	12.1	8.9
	SD	2.2	4.3	8.2
	n	5	7	6
Middle	xSI	3.5	14.1	5.7
	SD	1.4	7.5	3.7
	n	4	4	4
Low	xSI	5.3	26.7	13.5
	SD	4.4	9.9	18.9
	n	5	6	6
Control	xSI	4.2	3.9	4.1
	SD	2.8	3.1	3.3
	n	5	3	3

Appendix 8.4 continued.

Vaccine group		Months of age 3	6	9
BCG 100 ug/ml				
High	xSI	5.5	16.7	2.0
	SD	2.1	6.6	1.7
	n	5	7	6
Middle	xSI	3.0	19.6	1.5
	SD	1.3	13.7	0.4
	n	4	4	4
Low	xSI	5.3	23.5	4.2
	SD	4.4	8.3	2.7
	n	5	6	6
Control	xSI	5.7	6.9	2.8
	SD	4.5	5.8	2.3
	n	6	4	5
BCG 50 ug/ml				
High	xSI	5.1	18.3	4.6
	SD	3.8	4.8	5.0
	n	5	5	6
Middle	xSI	2.4	16.5	4.5
	SD	0.9	11.5	0.7
	n	4	4	3
Low	xSI	6.5	21.4	6.2
	SD	3.9	9.1	3.9
	n	3	5	5
Control	xSI	5.1	9.9	2.9
	SD	3.8	-	1.7
	n	6	1	4
***M.fortuitum* 100 ug/ml**				
High	xSI	5.8	21.6	4.0
	SD	1.7	9.0	4.4
	n	5	7	6
Middle	xSI	4.6	18.7	4.6
	SD	1.9	12.7	3.2
	n	4	4	4
Low	xSI	5.6	21.7	6.5
	SD	3.1	9.7	6.7
	n	5	6	6
Control	xSI	4.7	4.2	4.4
	SD	2.8	3.9	2.1
	n	6	3	4

Appendix 8.4 continued.

Vaccine group		Months of age 3	6	9
***M.fortuitum* 50 ug/ml**				
High	xSI	4.1	19.6	1.7
	SD	1.8	5.1	1.0
	n	4	5	6
Middle	xSI	2.8	20.7	3.8
	SD	0.6	12.6	1.8
	n	2	3	3
Low	xSI	7.1	20.9	3.5
	SD	3.5	11.1	2.8
	n	4	4	5
Control	xSI	6.2	12.1	2.5
	SD	5.1	-	1.6
	n	5	1	4
***M.avium* GWT 100 ug/ml**				
High	xSI	5.5	30.9	10.7
	SD	5.6	14.3	8.6
	n	5	6	6
Middle	xSI	3.6	25.9	8.1
	SD	2.5	14.2	4.4
	n	3	4	4
Low	xSI	5.1	30.3	10.2
	SD	2.3	16.3	8.3
	n	4	6	5
Control	xSI	6.3	6.7	10.5
	SD	3.6	3.6	6.8
	n	6	4	5

Appendix 8.4 continued.

Vaccine group		Months of age 3	6	9
***M.avium* GWT 50 ug/ml**				
High	xSI	6.3	24.3	9.4
	SD	5.3	17.8	7.1
	n	5	6	6
Middle	xSI	4.6	19.7	8.7
	SD	3.4	11.7	4.8
	n	3	4	5
Low	xSI	9.1	22.7	7.3
	SD	3.1	10.6	4.2
	n	4	5	5
Control	xSI	7.0	4.6	7.0
	SD	6.6	4.8	3.0
	n	6	3	5
***M.avium* RBP 100 ug/ml**				
High	xSI	11.0	10.0	22.3
	SD	6.4	5.8	15.2
	n	4	6	6
Middle	xSI	25.7	3.0	28.3
	SD	15.6	3.1	19.4
	n	4	4	4
Low	xSI	14.6	15.3	19.0
	SD	5.9	14.2	16.9
	n	4	6	5
Control	xSI	6.9	3.9	10.3
	SD	6.1	4.8	3.1
	n	5	4	5

Appendix 8.5: Gadwall skin test results.

Hours post administration

Vaccine group (dose)		Months of age 3	6	9
17 Hours:-				
High		0.28	0.31	0.22
	n	5	7	6
Middle		0.44	0.24	0.27
	n	5	5	5
Low		0.07	0.40	0.19
	n	3	5	6
Control		0.10	0.20	0.08
	n	6	5	4
24 Hours:-				
High		0.20	0.43	0.39
	n	5	7	6
Middle		0.32	0.40	0.33
	n	5	5	5
Low		0.07	0.40	0.22
	n	3	5	6
Control		0.20	0.20	0
	n	6	5	4
41 Hours:-				
High		0.28	0.37	0.25
	n	5	7	6
Middle		0.52	0.24	0.17
	n	5	5	5
Low		0	0.20	0.31
	n	3	5	6
Control		0.20	0.24	0.13
	n	6	5	4

Appendix 8.5 continued.

Hours post administration

Vaccine group (dose)		Months of age 3	6	9
48 Hours:-				
High		0	0.20	0.11
	n	5	7	6
Middle		0.36	0.20	0.23
	n	5	5	5
Low		0	0.24	0.25
	n	3	5	6
Control		0.20	0.12	0
	n	6	5	4
65 Hours:-				
High		0	0.2	0.08
	n	5	7	6
Middle		0.28	0.24	0.27
	n	5	5	5
Low		0	0.12	0.11
	n	3	5	6
Control		0.2	0	0
	n	6	5	4

Appendix 8.6: Gadwall ELISA Results.

Antigen

Vaccine group (dose)		Months of age 3	6	9
BCG				
High		0.259	0.210	0.286
	n	5	7	6
Middle		0.222	0.251	0.310
	n	5	5	5
Low		0.188	0.228	0.295
	n	5	6	6
Control		0.210	0.240	0.243
	n	6	5	5
M.fortuitum				
High		0.409	0.324	0.392
	n	5	7	6
Middle		0.336	0.369	0.438
	n	5	5	5
Low		0.307	0.362	0.421
	n	5	6	6
Control		0.336	0.346	0.378
	n	6	5	5
M.gordonae				
High		0.395	0.343	0.382
	n	5	7	6
Middle		0.353	0.382	0.444
	n	5	5	5
Low		0.315	0.411	0.454
	n	5	6	6
Control		0.334	0.352	0.384
	n	6	5	5

Appendix 8.6 continued.

Antigen

Vaccine group (dose)		Months of age 3	6	9
***M.avium* GWT**				
High		0.294	0.267	0.321
	n	5	7	6
Middle		0.269	0.311	0.387
	n	5	5	5
Low		0.232	0.306	0.368
	n	5	6	6
Control		0.269	0.267	0.320
	n	6	5	5
M.vaccae				
High		0.319	0.268	0.310
	n	5	7	6
Middle		0.283	0.317	0.362
	n	5	5	5
Low		0.238	0.293	0.357
	n	5	6	6
Control		0.269	0.274	0.319
	n	6	5	5
***M.avium* RBP**				
High		0.313	0.271	0.340
	n	5	7	6
Middle		0.279	0.312	0.391
	n	5	5	5
Low		0.230	0.296	0.362
	n	5	6	6
Control		0.272	0.308	0.321
	n	6	5	5

Appendix 9.1: LTT results from individual tuberculous White-winged Wood Ducks

Bird / Months post beginning of study	Antigens								
	M.vaccae		BCG		*M.fort.*		GWT		RBP
	100	50	100	50	100	50	100	50	100
Vaccinated Birds.									
S1739									
9	1.5	1.7	1.2	1.6			1.0	1.0	1.0
12	2.5	2.4	1.8	1.6			2.4	1.4	
14	1.3	6.5	4.5	4.9	7.0	13.3	3.2	3.8	11.2
20	18.4	19.0	18.3	9.5	26.6	20.9	5.0	25.6	5.0
23	5.9	4.2	4.8	2.3	6.5	5.0	7.2	3.9	7.1
26	0.5	6.6	2.5		0.9	2.6	0.6	4.2	0.5
29	2.1	2.1	2.1	1.5	2.1	1.8	2.1	2.3	2.1
35	1.5	2.1	1.6	1.1	1.1	0.9			1.2
S1741									
12	1.6	0.9	1.8	0.4			0.6	0.4	
14	1.9	10.6	1.6	3.1	2.9	3.6	0.7	1.3	3.1
23	1.8	2.3	2.2	3.2	2.1	1.8	5.0	4.3	2.2
26	1.7	1.7	3.5	7.3	1.2	1.7	1.3	4.2	4.9
S1764									
20	4.4	4.0	8.8	6.7	6.4	10.1	1.5	5.7	1.5
23	8.3	5.9	7.5	9.3	11.2	6.9	4.0	3.7	8.8
S1763									
9	4.2	2.3	1.4	1.3			1.1	0.7	1.4
17	2.6	2.4	3.6	2.5	5.6	2.7	1.3	1.0	5.6
20	7.7	11.5	8.2	26.6	8.4	30.4	3.8	9.0	3.8
23	1.8	2.0	3.2	1.5	2.0	1.6	8.4	1.1	3.9
29	2.3	2.3	2.6	4.1	1.7	2.2	1.9	2.5	1.8
S1746									
9	1.3	1.4	1.6	0.7			1.0	0.5	1.0
12	1.0	1.6	2.4	1.0			2.3	0.5	
14		8.2	1.8	9.1			1.3	5.1	5.2
17	6.2	2.3	2.7	2.0	2.8	2.5	2.4	1.6	6.1
26	1.7	2.0	1.3	1.0	1.2	1.4	1.5	2.2	1.1
S1765									
9	2.0	1.0	1.2	0.9			1.1	0.7	1.0
17	1.3	1.1	1.1	0.6	0.8	1.1	1.0	3.3	0.7

Appendix 9.1 continued.

Months post beginning of study

Bird	Antigens								
	M.vaccae		BCG		*M.fort.*		GWT		RBP
	100	50	100	50	100	50	100	50	100
S1748									
14	5.4	1.4	2.2	0.9		0.8	5.7		1.2
17	0.8	0.6	1.1	0.8	0.7	0.7	0.6	0.6	0.4
S1743									
12	1.4	1.4	1.6	1.2			1.0	1.1	
17	1.6	1.4	2.0	1.5	1.4	0.7	2.7	0.8	0.7
Control Birds.									
S1761									
9	1.8	3.2	2.0	1.1			2.4	0.5	1.7
12	1.8	3.0	4.7	1.8			5.6	1.3	
17	17.7	11.0	13.7	12.0	12.6	15.2	8.6	4.1	14.4
23	10.8	10.9	9.2	9.8	9.0	7.4	11.1	5.9	19.3
26	1.2	3.7	6.3	18.3	1.9	1.9	1.4	1.9	3.8
29	1.4	2.7	2.0	2.1	1.8	4.4	2.7	2.6	2.4
S1757									
9	2.7	2.8	2.7	1.4			1.5	1.4	1.9
12	1.0	1.8	2.2	1.3			1.7	0.8	
20	5.6	7.4	14.8	9.5	11.9	9.2	2.5	5.4	1.3
23	2.7	2.3	2.8	2.7	3.0	1.5	2.1	1.5	2.2
26	3.9	10.8	1.0	8.6	10.8	6.4	5.6	4.8	3.0
29	1.0	1.1	0.9	1.1	1.1	0.9	1.4	1.3	1.1
S1749									
9	1.2	1.4	1.0	0.8			1.5	1.0	1.4
12	1.4	1.8	2.2	0.7			1.4	1.6	
17	2.3	1.5	1.7	0.7	1.4	1.4	3.3	0.7	3.6
23	0.7	1.4	3.3	2.6	0.9	2.3	3.3	1.1	2.5
S1758									
12	2.9	4.0	3.8	1.1			3.2	0.9	
23		1.0	0.7	1.4	1.6	0.8			
S1755									
20	1.0	1.2	1.1	1.4	1.8	1.3	1.0	2.4	1.1
S1754									
9	3.1	1.5	1.8	0.8			1.6	0.7	1.5
20	1.2	2.0	3.7	1.0	1.0		1.1	1.1	

Antigen key:

M.fort. : *M.fortuitum*
GWT : *M.avium* GWT
RBP : *M.avium* RBP

Appendix 9.2 Skin test results from individual tuberculous White-winged Wood Ducks

Months post beginning of study / Bird	Hours post administration				
	17	24	41	48	65
Vaccinated Birds.					
S1739					
14	0.8	0.6	0.6	0.2	0
20	1.0	0.8	0.8	0.8	0.8
23	0.9	0.9	0.9	1.0	0.7
26	0.8	1.0	1.0	0.6	0
29	0	0	0	0	0
33	0	0.7	0.7	0.7	0.5
S1741					
14	0.2	0.6	0.8	0.6	0.2
20	1.0	1.0	0.8	0.5	0.8
23	0.7	0.7	0.9	0.7	0.9
S1764					
9	0.2	0.2	0.2	0	0.4
20	1.0	0.8	0.7	0.7	0.5
23	1.0	1.0	1.0	1.0	1.0
S1763					
9	1.0	1.0	1.0	1.0	1.0
17	1.0	1.0	1.0	1.0	1.0
20	0.3	0.5	0.7	0.5	0.5
23	0.9	0.9	0.9	0.7	0.9
26	0.8	0.9	0.9	0.8	0
29	0.9	1.0	1.0	0.9	0.9
S1746					
9	0.2	0.6	0.8	0.8	0
17	0.3	0.3	0.3	0.3	0
26	0.6	0.9	0.9	0.6	0
S1765					
9	0.6	0.6	0.6	0.4	0.6
17	0.4	0.4	0.4	0.4	0
S1748					
14	0.8	0.8	1.0	0.8	0.6
17	0.7	0.7	0.7	0.7	0
S1743					
17	0.7	0.7	0.7	0.7	1.0
S1742					
9	0.4	0.6	0.4	0	0

Appendix 9.2 continued.

Months post beginning of study	Hours post administration				
Bird	17	24	41	48	65
Control Birds.					
S1761					
17	0.4	0.4	0.1	0.3	0
26	0	0	0	0	0
29	0	0.1	0	0	0
S1757					
9	0.6	0.6	0.6	0.6	0.6
23	0.7	0.7	0.7	0.7	0.7
26	0	0	0	0	0
S1749					
9	0.8	0.8	0.8	0.8	0.8
17	0.3	0.1	0	0.1	0.1
S1758					
9	0.4	0.6	0.4	0.6	0.4
S1755					
9	0	0	0	0	0
20	0.8	0.7	0.8	0.7	0.7

Appendix 9.3: ELISA results from individual tuberculous White-winged Wood Ducks

Bird	Months post beginning of study	Antigen					
		GWT	RBP	BCG	*M.fort.*	*M.gord.*	*M.vaccae*
Vaccinated Birds.							
S1739							
	5	0.126	0.146	0.063	0.166	0.171	0.124
	6	0.117	0.139	0.067	0.148	0.139	0.109
	11	0.118	0.298	0.101	0.176	0.174	0.145
	14	0.112	0.125	0.102	0.142	0.150	0.113
	20	0.166	0.184	0.136	0.204	0.208	0.188
	23	0.099	0.165	0.124	0.186	0.194	0.145
	26	0.108	0.119	0.076	0.120	0.122	0.101
	29	0.288	0.301	0.155	0.320	0.299	0.224
	33	1.083	1.311	0.807	1.336	1.096	0.956
S1741							
	5	0.099	0.108	0.056	0.165	0.147	0.130
	11	0.125	0.154	0.103	0.161	0.169	0.145
	14	0.144	0.163	0.124	0.183	0.179	0.146
	20	0.178	0.190	0.143	0.213	0.227	0.189
	23	0.132	0.208	0.162	0.232	0.245	0.207
	26	0.108	0.119	0.076	0.120	0.122	0.101
S1764							
	5	0.071	0.092	0.042	0.100	0.110	0.089
	20	0.123	0.141	0.119	0.156	0.177	0.136
	23	0.103	0.131	0.077	0.138	0.152	0.120
S1763							
	17	0.161	0.198	0.152	0.214	0.202	0.170
	20	0.161	0.181	0.152	0.204	0.209	0.193
	23	0.219	0.213	0.131	0.228	0.227	0.223
	26	0.251	0.271	0.198	0.276	0.295	0.257
	29	0.800	0.845	0.497	0.905	0.765	0.636
S1746							
	5	0.087	0.102	0.045	0.122	0.126	0.102
	11	0.113	0.136	0.091	0.141	0.154	0.129
	14	0.135	0.161	0.125	0.172	0.174	0.148
	17	0.142	0.186	0.133	0.185	0.188	0.170
	26	0.413	0.405	0.274	0.416	0.426	0.439
S1765							
	6	0.130	0.178	0.090	0.181	0.177	0.157
	17	0.584	0.584	0.486	0.649	0.646	0.637
S1748							
	14	0.115	0.141	0.108	0.143	0.175	0.134
	17	0.469	0.464	0.392	0.526	0.545	0.486

Appendix 9.3 continued.

Bird	Months post beginning of study	Antigen					
		GWT	RBP	BCG	*M.fort.*	*M.gord.*	*M.vaccae*
S1743							
	11	0.083	0.102	0.064	0.110	0.115	0.094
	17	0.577	0.601	0.526	0.641	0.603	0.604
Control Birds.							
S1761							
	5	0.116	0.164	0.093	0.186	0.244	0.152
	6	0.084	0.085	0.053	0.122	0.153	0.096
	11	0.075	0.097	0.070	0.115	0.122	0.088
	17	0.146	0.155	0.084	0.202	0.193	0.153
	23	0.125	0.156	0.137	0.164	0.177	0.139
	26	0.097	0.121	0.065	0.117	0.127	0.105
	29	0.335	0.336	0.171	0.382	0.517	0.257
S1757							
	6	0.143	0.180	0.101	0.194	0.249	0.133
	11	0.064	0.078	0.056	0.086	0.088	0.070
	20	0.128	0.174	0.131	0.182	0.194	0.156
	23	0.158	0.168	0.125	0.188	0.201	0.155
	26	0.394	0.374	0.342	0.399	0.413	0.383
	29	0.662	0.744	0.451	0.827	1.024	0.441
S1749							
	6	0.051	0.123	0.073	0.101	0.103	0.111
	11	0.121	0.138	0.091	0.145	0.167	0.126
	17	0.126	0.148	0.117	0.183	0.182	0.138
	23	0.446	0.407	0.342	0.449	0.475	0.452
S1758							
	6	0.117	0.124	0.067	0.159	0.156	0.122
	11	0.171	0.191	0.104	0.212	0.225	0.160
	23	0.500	0.468	0.350	0.468	0.506	0.440
S1755							
	20	0.486	0.460	0.328	0.402	0.437	0.406
S1754							
	6	0.142	0.141	0.081	0.178	0.221	0.121
	20	0.512	0.546	0.488	0.588	0.629	0.598

Antigen key:

GWT : *M.avium* GWT
RBP : *M.avium* RBP
M.fort. : *M.fortuitum*
M.gord. : *M.gordonae*

Appendix 9.4 White-winged Wood Duck LTT results.

Antigen

Vaccine group		Months post beginning of study								
		9	**12**	**14**	**17**	**20**	**23**	**26**	**29**	**35**
***M.vaccae* 100 ug/ml**										
Vaccinates	xSI	2.1	2.2	2.9	2.5	9.0	4.9	2.1	2.5	4.8
	SD	1.2	1.9	2.2	2.2	6.4	3.0	1.6	0.6	4.7
	n	5	5	3	5	4	5	4	3	2
Control	xSI	1.8	1.6	0.9	5.6	5.1	4.5	2.2	1.4	2.3
	SD	0.6	0.8	0.7	6.8	5.9	4.4	1.3	0.9	1.6
	n	4	5	2	5	5	4	4	4	3
***M.vaccae* 50 ug/ml**										
Vaccinates	xSI	1.6	1.9	6.7	1.6	9.5	4.4	4.0	2.2	2.7
	SD	0.4	0.9	3.9	0.8	7.3	2.3	2.5	0.1	0.8
	n	5	5	4	5	4	5	4	3	2
Control	xSI	2.2	2.3	1.8	4.2	5.4	3.6	5.9	1.8	3.6
	SD	0.9	1.2	1.1	3.9	5.5	4.1	3.8	1.0	1.7
	n	4	5	3	5	5	5	4	4	3
BCG 100 ug/ml										
Vaccinates	xSI	1.5	2.5	2.5	2.1	9.8	5.3	2.4	2.0	2.0
	SD	0.4	1.3	1.3	1.1	6.0	2.7	0.9	0.7	0.4
	n	5	5	4	5	4	5	4	3	2
Control	xSI	1.9	2.9	0.9	4.7	7.7	3.7	3.0	1.6	1.9
	SD	0.7	1.3	0.3	5.2	6.4	3.2	2.4	0.7	0.4
	n	4	5	2	5	5	5	4	4	3
BCG 50 ug/ml										
Vaccinates	xSI	1.1	1.2	4.5	1.5	11.7	4.0	3.9	2.6	1.3
	SD	0.3	0.6	3.5	0.8	10.2	3.1	3.2	1.3	0.2
	n	5	5	4	5	4	5	3	3	2
Control	xSI	1.0	1.1	3.8	4.1	7.4	3.6	9.6	1.7	2.2
	SD	0.3	0.4	0	4.5	8.9	3.5	6.3	0.9	0.4
	n	4	5	2	5	5	5	4	4	4

Appendix 9.4 continued.

Antigen

Vaccine group		Months post beginning of study								
		9	12	14	17	20	23	26	29	35
***M.fortuitum* 100 ug/ml**										
Vaccinates	xSI			5.0	2.3	11.9	6.1	1.8	1.8	2.9
	SD			2.9	2.0	9.8	4.0	1.4	0.2	2.4
	n			2	5	4	5	4	3	2
Control	xSI			1.3	4.2	4.8	3.5	4.8	1.4	5.2
	SD			0.7	4.8	5.0	3.2	4.1	0.7	4.5
	n			2	5	4	5	4	4	4
***M.fortuitum* 50 ug/ml**										
Vaccinates	xSI			5.9	1.3	16.5	4.2	2.1	1.9	2.2
	SD			6.6	0.7	11.4	2.4	0.7	0.2	1.8
	n			3	5	4	5	4	3	2
Control	xSI			1.0	5.0	7.9	2.8	4.4	2.2	3.9
	SD			-	5.8	8.0	2.6	2.2	1.7	1.6
	n			1	5	4	5	4	4	4
***M.avium* GWT 100 ug/ml**										
Vaccinates	xSI	1.7	2.7	2.7	1.6	3.2	5.8	1.7	2.4	9.7
	SD	1.2	2.7	2.3	0.9	1.6	1.9	1.1	0.8	-
	n	5	5	4	5	4	5	4	3	1
Control	xSI	2.7	2.6	0.5	4.1	2.6	4.4	2.9	1.7	6.3
	SD	2.3	1.9	0.1	2.6	2.2	3.8	2.0	1.0	3.1
	n	4	5	2	5	5	5	4	4	4
***M.avium* GWT 50 ug/ml**										
Vaccinates	xSI	1.0	0.9	3.4	1.5	12.2	3.4	3.5	2.6	4.2
	SD	0.2	0.4	1.9	1.1	9.1	1.3	0.9	0.3	-
	n	5	5	3	5	4	5	4	3	1
Control	xSI	1.8	1.1	3.6	2.0	6.0	3.4	4.1	1.7	7.3
	SD	0.4	0.4	0.2	1.4	6.8	2.8	1.6	0.9	4.2
	n	4	5	2	5	5	5	4	4	4
***M.avium* RBP 100 ug/ml**										
Vaccinates	xSI	1.1	-	5.2	2.7	2.9	6.9	3.9	2.3	3.4
	SD	0.2	-	4.3	2.9	1.8	4.0	3.9	0.7	3.1
	n	5	-	4	5	4	5	4	3	2
Control	xSI	1.6	-	2.6	5.1	1.6	6.8	3.6	1.7	7.4
	SD	0.2	-	1.6	5.3	0.5	8.3	2.0	0.9	3.9
	n	4	-	3	5	4	4	4	4	4

Appendix 9.5: White-winged Wood Duck Skin test results.

Hours post administration

Vaccine group	**Months post beginning of study**							
	9	**14**	**17**	**20**	**23**	**26**	**29**	**33**
17 Hours:-								
Vaccinates	0.50	0.50	0.46	0.83	0.83	0.67	0.58	0.25
n	8	4	5	4	5	3	3	2
Control	0.46	0	0.23	0.78	0.43	0	0	0.70
n	7	4	5	3	4	3	3	5
24 Hours:-								
Vaccinates	0.50	0.50	0.63	0.79	0.83	0.88	0.46	0.75
n	8	4	5	4	5	3	3	2
Control	0.43	0.1	0.14	0.60	0.43	0	0.04	0.80
n	7	4	5	3	4	3	3	5
41 Hours:-								
Vaccinates	0.53	0.60	0.60	0.75	0.86	0.84	0.42	0.67
n	8	4	5	4	5	3	3	2
Control	0.54	0	0.09	0.72	0.43	0	0	0.73
n	7	4	5	3	4	3	3	5
48 Hours:-								
Vaccinates	0.38	0.40	0.63	0.63	0.80	0.66	0.38	0.58
n	8	4	5	4	5	3	3	2
Control	0.46	0	0.11	0.50	0.43	0	0	0.77
n	7	4	5	3	4	3	3	5
65 Hours:-								
Vaccinates	0.35	0.20	0.29	0.67	0.77	0	0.29	0.67
n	8	4	5	4	5	3	3	2
Control	0.43	0	0.03	0.33	0.43	0	0	0.87
n	7	4	5	3	4	3	3	5
72 Hours:-								
Vaccinates	0.30	0.35						
n	8	4						
Control	0.37	0.1						
n	7	4						

Appendix 9.6: White-winged Wood Duck ELISA results.

Antigen

Vaccine group	Months post beginning of study									
	5	6	11	14	17	20	23	26	29	33
BCG										
Vaccinated	0.064	0.078	0.089	0.114	0.338	0.134	0.122	0.139	0.281	0.465
n	5	5	5	4	5	5	5	5	3	2
Controls	0.087	0.073	0.081	0.099	0.112	0.239	0.214	0.144	0.212	0.122
n	2	6	5	6	5	5	5	4	4	3
M.fortuitum										
Vaccinated	0.161	0.173	0.144	0.159	0.443	0.197	0.191	0.211	0.535	0.792
n	5	5	5	4	5	5	5	5	3	2
Controls	0.184	0.153	0.140	0.155	0.191	0.304	0.288	0.201	0.416	0.271
n	2	6	5	6	5	5	5	4	4	3
M.gordonae										
Vaccinated	0.163	0.150	0.149	0.169	0.437	0.206	0.203	0.220	0.460	0.654
n	5	5	5	4	5	5	5	5	3	2
Controls	0.194	0.134	0.149	0.157	0.203	0.327	0.305	0.207	0.377	0.266
n	2	6	5	6	5	5	5	4	4	3
***M.avium* GWT**										
Vaccinated	0.113	0.119	0.110	0.126	0.365	0.154	0.142	0.180	0.458	0.635
n	5	5	5	4	5	5	5	5	3	2
Controls	0.130	0.110	0.107	0.113	0.137	0.268	0.263	0.155	0.333	0.189
n	2	6	5	6	5	5	5	4	4	3
M.vaccae										
Vaccinated	0.131	0.134	0.127	0.135	0.413	0.181	0.171	0.200	0.376	0.566
n	5	5	5	4	5	5	5	5	3	2
Controls	0.137	0.121	0.114	0.123	0.149	0.294	0.264	0.181	0.254	0.185
n	2	6	5	6	5	5	5	4	4	3
***M.avium* RBP**										
Vaccinated	0.134	0.148	0.138	0.147	0.406	0.174	0.176	0.211	0.484	0.766
n	5	5	5	4	5	5	5	5	3	2
Controls	0.164	0.133	0.128	0.139	0.163	0.303	0.269	0.191	0.373	0.230
n	2	6	5	6	5	5	5	4	4	3

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Susceptibility of captive wildfowl to avian tuberculosis: the importance of genetic and environmental factors

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Summary—This study reports the findings of an epidemiological survey of death due to avian tuberculosis in the captive collection of wildfowl at The Wildfowl and Wetlands Trust Centre, Slimbridge, Gloucestershire. Both genetic and environmental factors have been shown to affect the incidence of, and the birds' susceptibility to, the disease.

Seasonal body condition was related to the occurrence of death due to the disease in both males and females. Birds from either hot or cold climates appeared to have a higher incidence than those from temperate climates. What the birds ate did not affect incidence but the method they used for obtaining their food did. Higher susceptibility was found in those species evolved for marine or arboreal habitats. Anomalies in susceptibility which suggest a higher level of genetic immunity in some groups have also been found. Reasons are put forward to explain these findings.

Introduction

The Wildfowl and Wetlands Trust, at Slimbridge in Gloucestershire, maintains the most comprehensive collection of wildfowl in the world. However, endemic tuberculosis due to *Mycobacterium avium* infection now accounts for at least a third of adult mortalities. This study assesses the disease within a heterogenous group of ducks, geese and swans. Attempts are made to elucidate the reasons for susceptibility to this chronic and debilitating disease.

Methods and Materials

Adult *post mortem* (PM) data from 1980–1989 were analysed. An adult bird was classed as one surviving to its first January or the equivalent for those southern hemisphere birds that breed earlier. During this study period there were only 3/531 (0.6%) deaths of juvenile birds caused by avian tuberculosis.

Diagnosis was made on macroscopic appearance of tuberculous lesions in the dissected bird, supported by the presence of acid-fast bacilli in Ziehl-Neelsen stained smears. All PMs used for this study were carried out by the same pathologist (MJB).

Incidences shown indicate the number of cases of tuberculosis found at PM as a percentage of the total number of PMs for that group.

M. avium was the only mycobacterial species isolated from lesions of tuberculous birds. 42 of these isolates were subjected to lipid chromatography by the method of Jenkins [1] and were all found to be type 1, in agreement with the study of Schaefer *et al* [2].

The birds were classified according to a

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taxonomy based on evolutionary and behavioural relationships, which divides the 148 species of wildfowl into 13 tribes [3]. A classification has also been made by dividing the birds into different feeding groups. Firstly, dividing the birds by what they ate i.e. animal, vegetable or mixed foodstuffs and, secondly, by the way in which they obtained their food i.e.

1. Grazers: those that fed on vegetable matter by grazing on land.
2. Divers: those that fed on either vegetable or animal matter from the pelagic zone or benthos.
3. Dabblers: those that fed on vegetable or mixed foodstuffs by sifting mud and silt on the water surface or just beneath it, generally in shallow water.

Statistical analyses have been by Fisher's Exact test and Student's t-test.

Results

The incidence of avian tuberculosis according to taxonomic tribe varied enormously, ranging from 0% (0/13) in the *Tachyerini,* steamer ducks, to 52% (133/256) in the *Cairinini,* perching ducks ($p<0.001$). The *Mergini,* the sea ducks, had the second highest incidence of tuberculosis (48.7%, 113/232). This group can be divided into the eider ducks as one sub-group, and the mergansers, scoters and goldeneyes as the other [4, 5]. These two sub-groups had very different incidences of avian tuberculosis: eiders, 23.2% (23/99); mergansers, scoters and goldeneyes, 67.7% (90/133) ($p<0.00001$).

The greatest numbers of total deaths occurred in winter and spring, but the greatest number of deaths from tuberculosis, occurred in winter and summer (Table 1). These levels differ significantly from that of spring ($p<0.0001$ and $p<0.00001$ respectively) but not of autumn.

Table 2 shows there was no overall sex predilection to *M. avium* infection, but there was a difference in when the sexes died (Table 1). Female mortality was significantly greater than male mortality in the summer ($p<0.005$).

The increase in tuberculosis mortality between the first and second 5-year-periods of the study ($p<0.02$) was exclusively amongst females ($p<0.003$).

The manner in which food and feeding habit affected the incidence of the disease is shown in

Table 1 Incidence of death according to sex and season*

Season	*TB deaths Male (%)*	*Female (%)*	*TB deaths*	*Other deaths*	*Total deaths*
Winter	124 (55.1)	101 (44.9)	225	423	648
Spring	87 (49.4)	89 (50.6)	176	531	707
Summer	90 (43.5)	117 (56.5)	207	302	509
Autumn	84 (49.4)	86 (50.6)	170	300	470
Total	385	393	778	1556	2334

*The table shows the incidence of death due to avian tuberculosis divided into male and female mortality as well as seasonal mortality.

Table 2 Incidence of death according to sex*

	1980–84 TB (%)	*Total*	*1985–89 TB (%)*	*Total*	*1980–89 TB (%)*	*Total*
Male	200 (32.9)	608	188 (32.8)	573	388 (32.9)	1181
Female	178 (29.2)	610 (i) ($p<0.003$)	217 (37.0)	586	395 (33.0)	1196
Total	378 (31.0%)	1218 (ii) ($p<0.02$)	405 (34.9%)	1159	783	2377

*The table shows the incidence of death due to avian tuberculosis according to sex. (The p values indicate the level of significance of the difference in (i) female tuberculosis mortalities and (ii) total tuberculosis mortalities between the two five year periods).

Table 3 Incidence of death according to food and feeding habit*

	Food			*Feeding habit*		
	Vegetable	*Animal*	*Mixed*	*Diver*	*Grazer*	*Dabbler*
Total PMs	1682	377	321	499	803	1079
TB cases	539	132	114	191	223	372
% TB	32.0	35.0	35.5	38.3 p<0.002	27.8	34.4 p<0.001

*The table shows how food and feeding habit affects the incidence of death due to avian tuberculosis. The data was divided into (i) what the bird ate, and (ii) how the bird obtained its food, by either diving, grazing or dabbling. (p values show the significance levels of the differences between the diving and dabbling feeding groups related to the grazers).

Table 3. It can be seen that what the birds fed on did not affect their incidence of tuberculosis, but how they obtained their food did. There were significant differences between the grazers and divers; and the grazers and dabblers ($p<0.002$ and $p<0.001$ respectively).

If the diving ducks are split into two groups: sea duck divers and fresh water divers, there is a significantly greater incidence in the former group ($p<0.001$).

The few birds with primary pulmonary tuberculosis and no disseminated disease (8 out of 787 PMs of tuberculous birds) were ducks that fed by dabbling.

There was a trend in incidence according to climatic origin, with the lowest incidence in those birds from temperate climates (mean temperatures of 10°C–21°C). Higher incidences were found in those birds from cold (below 10°C) and hot (21°C and over) climates.

An analysis of the incidence within the different pens at Slimbridge showed that no area containing wildfowl was free from infection. However, shaded pens had a particularly high incidence. The Tropical House, an enclosed unit with its own water supply, had the highest incidence of any of the pens with 55.0% (22/40) of mortality due to tuberculosis.

Discussion

Slimbridge has held a large captive wildfowl collection at relatively high density since 1946, and there is evidence of an increasing incidence of avian tuberculosis. J. V. Beer carried out an unpublished study on PM data from 1958–1968 and found a mean annual incidence of 19.4% (±8.1%) in contrast to 33.2% (±5.1%) in the current study, a statistically significant rise ($p<0.001$). Although methodology could explain the difference, the current pathologist (M.J.B.) has witnessed an increase in annual incidence since coming to Slimbridge in 1971.

Schaefer *et al* [2] isolated *M. avium* from almost 50% (10/21) of Slimbridge birds considered non-tuberculous at PM, indicating that birds infected with *M. avium* may not necessarily develop pathological disease. Thus, changes in mortality can be explained in two ways. First, if the percentage of infected birds that develop disease remains constant, then increased infection will result in increased mortality. This might be expected as the environment becomes progressively contaminated with wildfowl pathogenic strains, concentrated and excreted by successive generations of tuberculous birds. Secondly, increased susceptibility to disease could result in increased mortality without an increase in infection. The data in this study are considered in both these ways.

The high incidence of the disease in the *Cairinini,* perching ducks, could be explained by the fact that in the wild they spend a great deal of time in arboreal habitats where mycobacterial immunity may be of less importance than in ground living species [6]. Additionally, in captivity, they are pinioned and therefore brought into far more frequent contact with the ground. At ground and water level they encounter environmental mycobacteria and both their own and other birds' potentially infected droppings and so are at an increased risk of infection.

The results from the *Mergini,* sea ducks, show that even within tribes, anomalies in incidence can be found. These birds share similar life-styles and environments in both the wild and captivity,

yet the eider ducks appear to have a high level of genetic immunity to avian tuberculosis, whilst the mergansers, scoters and goldeneyes are exceptionally susceptible to disease.

The role of stress in tuberculosis disease is difficult to quantify. Gross *et al* [7] showed that the nature and number of tuberculous lesions in chickens was affected by not only their genetic make-up, but also by the level of 'social stress' the birds experienced. The birds at Slimbridge must experience a number of potential stress factors. They are pinioned, which must be stressful to those groups of wildfowl that would naturally take to the air when in danger. Other groups such as the *Oxyurini,* the stiff-tailed ducks, with their particularly short wings, would use their ability to fly far less and might be less stressed. The relatively low incidence (24.8%, 27/109) found in this group of birds may be a reflection of this.

Cold weather is known to increase mortality in birds [8, 9] and the study of seasonal mortality confirms this. The birds at Slimbridge may be less susceptible to avian tuberculosis in the spring because this is a time when both sexes are in good condition in preparation for breeding.

The lack of sex predilection to infection confirms the finding made in other collections [10]. However, the high female mortality due to avian tuberculosis in the summer, may be due to the loss of body condition during this time. Females, in particular, lose body condition throughout the summer due to the stress and energy expenditure of both the summer moult and breeding. Besides laying the eggs, the incubating and brooding is often carried out entirely by the females. Summer weights of female Lesser Snow Geese, *Anser coerulescens coerulescens,* are markedly lower than those of males [11]. A similar pattern is also shown in Mallard, *Anas platyrhynchos platyrhynchos,* condition indices (weight/wing length) [12]. During this summer breeding period, female Mallard lose about 25% of their body weight [13] and female Eiders, *Somateria mollissima,* may lose 50% of their prelaying body weight [14]. Conversely, the lowest weight of male Lesser Snow Geese is in the winter [11] and male Mallard experience a progressive decline in body condition throughout the winter [12] which may increase their susceptibility to tuberculosis during this season.

The increase in female mortality in the latter 5-year-period suggests that summertime stress has increased more than wintertime stress. There are two possible explanations for this. First, there was a 28% increase in the number of visitors to Slimbridge in the second 5-year-period, particularly in 1988 and 1989, compared to the first (from 0.91 million to 1.17 million per 5 years). The increase in summer visitors may prevent the females from leaving the nest as frequently to feed and drink. The long hot and dry summers of 1988 and 1989 would have increased the dehydration of incubating birds and exacerbated the problem. Secondly, there has been a slight shift in avicultural practices in the second 5-year-period. To ensure maximum survival of offspring, the eggs are removed from the females and are hatched, and the young reared, in an hygienic duckery unit where there is less risk of predation and infection. In the latter years of this study, clutches were removed at a later stage of incubation. This may be more stressful in terms of anxiety to the bird and, as she has begun incubating, her body condition will be reduced from the longer period spent sitting on eggs.

As the pathology of avian tuberculosis suggests an oral route of infection, feeding habit could be an important factor if the risk of infection is related to the bacterial load encountered by the birds. This is supported by the data if we assume that infection and mortality have a constant relationship.

The grazers may be able selectively to avoid potentially contaminated vegetation, i.e. that which is covered in faeces, and, in any case, much of their food is exposed to the sterilising effect of ultraviolet irradiation.

Grange [15] referred to mycobacteria as 'ducks of the microbial world' as they are found at air/water interfaces. The divers and dabblers, in particular, must encounter a higher level of mycobacteria, both pathogenic and saprophytic, and this could increase their risk of infection. The constant use of the same grounds must steadily increase the pathogenic proportion of environmental mycobacteria.

The fresh water divers and sea duck divers feed on vegetable and animal foodstuffs respectively. Since food seems not to affect incidence, and they are at equal environmental risk of infection, it may be concluded that a genetic factor is responsible for the increased susceptibility of the sea ducks.

M. avium is capable of inclusion in aerosols [16] and dabbling may be a good means of producing

them. This could lead to the primary pulmonary tuberculosis found in some of the ducks that feed in this way.

Birds housed in shaded pens or the Tropical House may experience an increased risk of infection due to a lack of the sterilising effects of ultraviolet irradiation. Those birds that actively seek the shelter of vegetation may also be particularly at risk.

Although few of the birds within the collection are wild caught, the different species originate from throughout the world and have therefore evolved to suit a specific climate. It is therefore not surprising that there was a low incidence in those birds which are adapted to the temperatures found in Great Britain. The birds from both hot and cold climates experience adverse temperature conditions and this may affect their susceptibility to pathological disease due to *M. avium*.

Birds infrequently subjected to challenge with wildfowl pathogenic serotype 1 *M. avium* in their natural habitat may be more at risk under the conditions of captivity at Slimbridge both in terms of exposure to mycobacterial infection and susceptibility to develop disease. Despite being bred at Slimbridge, many exotic species would have the evolutionary experience of their forebears reflected in their genes. In contrast, species from temperate zones and of freshwater habitats may have been selected for resistance to avian tuberculosis, and continue to exhibit this at Slimbridge. Thus, some of the wildfowl of the arctic regions and of the sea, have the increased incidence that might be expected of them.

This study has pinpointed the groups of wildfowl most at risk of dying of avian tuberculosis and the factors which increase this risk. Hopefully it gives some indication of how and where control measures should be implemented to improve matters in the future.

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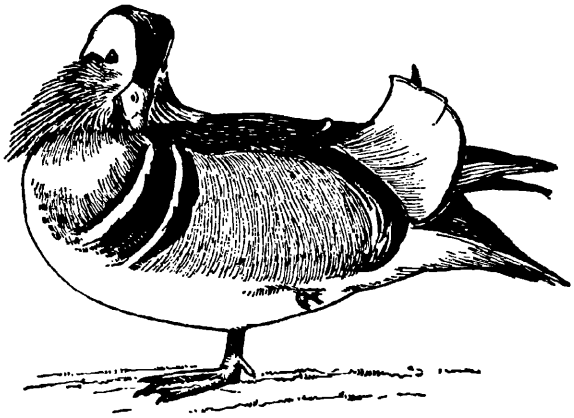
A Progress Report of the project to develop a vaccine against avian tuberculosis in wildfowl



RUTH L. CROMIE, JOHN L. STANFORD, MARTIN J. BROWN and DAVID J. PRICE

Techniques for monitoring the efficiency of an anti-tuberculosis vaccine for wildfowl have been developed. These include web testing, lymphocyte transformation test (LTT) and an enzyme linked immunosorbance assay (ELISA).

Using these methods a number of trial vaccines have been assessed with a fair degree of success. A vaccine has been found that gives increased LTT and web test responses and will, we hope, therefore increase protection from infection. An optimum dose of this potential vaccine has been found and this should be administered to day-old birds.

Avian tuberculosis caused by *Mycobacterium avium* has been endemic at The Wildfowl Trust at Slimbridge since 1948 and is now at epidemic proportions accounting for, on average, a third of post-fledgling mortalities. This is a serious problem in itself, but there are also the problems of spreading the disease when birds are translocated to other collections and spreading infection to the wild populations that use the refuges in great numbers. Adult mortality rates as high as 49% in the perching ducks, a particularly susceptible group, have been recorded (Hillgarth & Kear 1981). Indeed, the captive breeding programme of the White-winged Wood Duck *Cairina scutulata*, a species classified as 'vulnerable' in the Red Data Book (King 1978–79), is still seriously affected by deaths from tuberculosis.

During the 2.5 years that this project has been running, considerable progress has been made in developing immunological techniques for assessing a series of potential vaccines. (Precise details of the vaccine are omitted due to possible patent pending.)

Materials and Methods

Techniques for assessing vaccine efficacy

(i) The *in vivo* method of web testing in which tiny doses of reagents prepared from different mycobacteria (the group of bacteria including those that cause tuberculosis) are injected into the foot web and the size of local responses are measured over a 2–3 day period. Responses indicate immune recognition of the reagents injected.

(ii) The *in vitro* technique of Lymphocyte Transformation Test (LTT) in which lymphocytes purified from small blood samples are cultured with soluble extracts of mycobacteria. Proliferation of lymphocytes recognising these reagents is measured by the test.

(iii) An enzyme linked immunosorbance assay (ELISA) which measures the levels of antibodies in the serum to different mycobacteria.

The vaccine studies

A series of tests have been set up in which groups of ducklings and goslings were vaccinated and control groups were left unvaccinated. The trial vaccines have been given in different forms and doses, and at different ages. After vaccination the test birds and controls were released into the grounds at Slimbridge where infection rates are high.

1986

One hundred and fifty Mallard *Anas platyrhynchos* were vaccinated with one of six potential vaccines at either one day or six weeks old. (Mallard were used due to the comparative ease of raising sufficient num-

bers and their relatively high susceptibility to infection as dabbling ducks.)

1986–87

Half of all White-winged Wood Ducks both present and brought into Slimbridge were vaccinated as adults.

1987

Sixty Mandarins *Aix galericulata* were vaccinated at either one day or six weeks old to find an optimum age for vaccination. (Mandarins were used because of their particular susceptibility as perching ducks).

1988

To find an optimum dose of vaccine in both ducks and geese, 40 Gadwall *Anas strepera* and 40 Ne-ne *Branta sandvicensis* were given one of three doses of vaccine as day-old birds.

1988–89

Half of all birds bred at Slimbridge (and a small percentage of those bred at The Wildfowl Trust Centre at Arundel) were vaccinated.

Every three months, birds from the various vaccine groups are caught, web tested and small blood samples are taken for laboratory analysis.

Results

Techniques for assessing vaccine efficacy

(i) Web testing.
This method has been developed and has shown that local responses vary depending upon vaccine received, vaccinated birds responding more than control birds.

(ii) LTT.
Using the culture conditions for duck lymphocytes developed by Higgins & Teoh (1988) and modified by us (Cromie *et al.* 1989), lymphocyte transformation in the presence of mycobacterial antigen has been made to work for the first time. This has proved valuable as a way of showing that immune cells of vaccinated birds recognise mycobacteria better than do those of control birds.

(iii) ELISA.
This method has been optimalised from the method described by Hudson & Hay (1980). The reagents needed are commercially unavailable and suitable ones have only been produced recently. A detailed study of the birds' antibody levels is now being carried out.

The vaccine studies

So far too few of the birds involved in the studies have died to assess directly vaccine efficacy; thus we have discriminated between vaccines on the basis of greater responses in web tests and lymphocyte transformation tests.

In the Mallard study the vaccine giving the best LTT and web test responses was determined and then used in the Mandarin study. This study confirmed the optimum age for vaccination to be one day old.

The results from the Gadwall and Ne-ne study have now helped to determine an optimum dose of vaccine for both ducks and geese.

The results of the study in adult White-winged Wood Ducks have been disappointing with apparently no differences between vaccine and control groups. To date three vaccinated and four control birds have died of tuberculosis.

Discussion

The project is now at the stage where the vaccine giving the best results has been identified and its optimum dose and age for vaccination have been found. It is hoped that results obtained from web tests, LTT and ELISA are a true reflection of the protection afforded by the vaccine, but this will only be proved in time.

The disappointing results from the White-winged Wood Duck study may be due to the vaccine being ineffective when administered in adult life, as previous exposure to environmental myobacteria would already have been great, or may be chance as numbers are so small.

Monitoring continues as the project reaches the critical stage where many of the birds approach the age when *M. avium*

infection would be expected. It is the policy to vaccinate half of all birds bred at Slimbridge leaving half as controls. Comparative mortalities due to tuberculosis within the two groups can be assessed over the next few years when birds may begin to develop the disease. There is also a policy to vaccinate all wildfowl sent to and bred at the new Wildfowl and Wetlands Trust Centre in Llanelli, South Wales.

Avian tuberculosis remains the most devastating and costly bacterial infection of captive birds and, should a successful vaccine be developed, it would be invaluable not only for The Trust but for zoos and private collections around the world.

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Seeing Gel Wells Well

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Indicator dyes have been incorporated in the stacking gel monomer used in acrylamide gels to render readily visible and delineate the sample wells. Some dyes, such as bromphenol blue, migrate during the electrophoresis, whereas others, such as the ortho-unsubstituted phenol red, become chemically bound to the polymerized gel. The method can be used for agarose gels.

KEY WORDS: gel electrophoresis; indicators; gel dyeing.

Many workers find difficulty in applying sample to gel wells as both the wells and the stacker gel are of similar density and almost invisible to those without 20/20 vision. Among the apparatus manufacturers, Hoefer has recognized the problem and supply a plastic mask with their "mighty small" apparatus in which the wells are outlined.

Bromphenol blue (BPB),[2] added to the sample, has been used as a front marker since the earliest days of paper electrophoresis. In gel electrophoresis, the experiment is stopped when the small-molecular-weight dye reaches the bottom edge of the gel. Microbiologists, who incubate their samples in media containing phenol red (PR), have noted (private communications) that the red dye is an equally useful marker but migrates slightly faster than the BPB.

It occurred to us that if one or the other of these dyes was incorporated into the stacking gel buffer then the clear wells would be outlined by the dark blue or purple indicator held in the polymerized gel. We have separately incorporated either BPB or PR into the buffer at a concentration of 4 mg/ml giving a final concentration of 1 mg/ml in the stacking gel monomer mixture (see Fig. 1). The BPB clearly outlines the wells with a dark blue border. The PR at the buffer pH of 6.8 first appears as a light yellowy pink or purple color which changes rapidly to a darker purple from the interface upward during the course of the polymerization reaction. This indicates that buffer salt diffusion is occurring rapidly and that this should be taken into account by those examining the theory of discontinuous electrophoresis. Coomassie blue may also be used.

We then carried out electrophoresis in the usual way on both the Hoefer "mighty small" and standard 15-cm-long gels. In all cases the BPB rapidly closed up to a thin line at the stacker–running gel interface and then moved as a sharp band just as it normally does when added to the sample. Runs were stopped when the dye reached the far end of the gels. Protein separations obtained were identical to those where no BPB was added to the stacker but incorporated only into the sample in the usual way.

However, on electrophoresis of the PR gels, the whole stacker gel remained purple and only the "excess" PR migrated when high concentrations were used. Results were again identical with the above. Gels were also blotted but the PR remained bound to the stacking gel (see Fig. 2) although, of course, BPB does migrate from the gel. Clearly the explanation for the binding of PR to the gel

[1] To whom all correspondence should be addressed.
[2] Abbreviations used: BPB, bromphenol blue; PR, phenol red.

0003-2697/88 $3.00

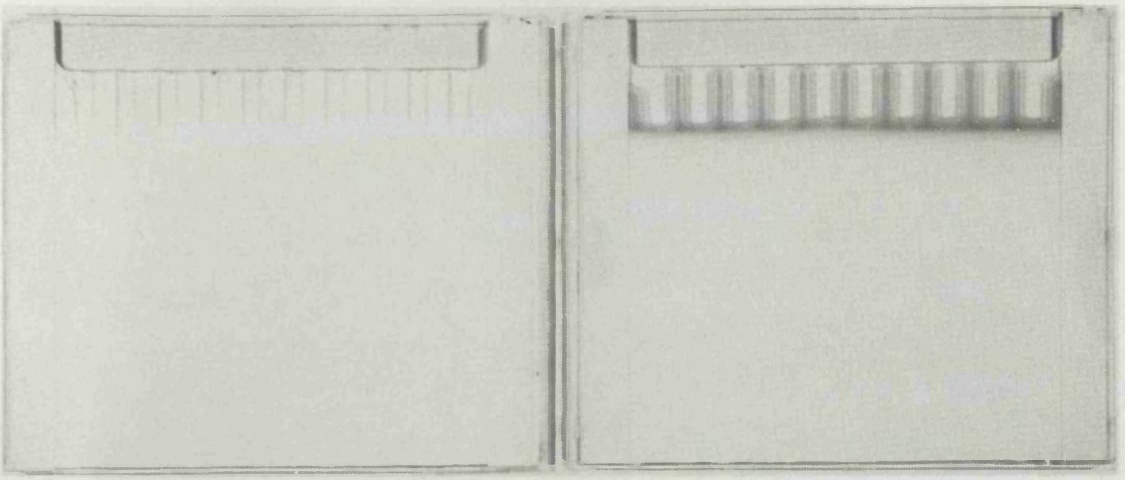

FIG. 1. Comparison of stacking gels prepared in the normal manner without (left) and with (right) phenol red indicator dye. The phenol red gel retains its appearance identically after electrophoresis showing that it is chemically bound into the gel polymer. Gels prepared with bromphenol blue look darker than the phenol red gels but, after electrophoresis, no residual dye remains, thus showing that it is not bound and so migrates right out of the gel. Note that the left gel wells appear partly visible due to shadowing during the photography.

and the nonbinding of BPB must be found in their respective chemical structures. PR is a simple, ortho-unsubstituted phenolic dye which must actively polymerize into the polyacrylamide gel structure, whereas BPB, which is an ortho-tetrabromo-substituted PR, is unable to participate in the polymerization because the large bromine atoms effectively obstruct the reaction. Similar results were obtained with a number of other ortho-unsubstituted phenolic indicators, most of which retained their indicating properties albeit, unfortunately, with a slower reaction time due to the necessary time for diffusion. Parenthetically, it might be noted that this may well prove to be a useful method for insolubilizing other ortho-unsubstituted phenols such as tyrosyl peptides, etc., while possibly retaining some of their chemical and biological properties.

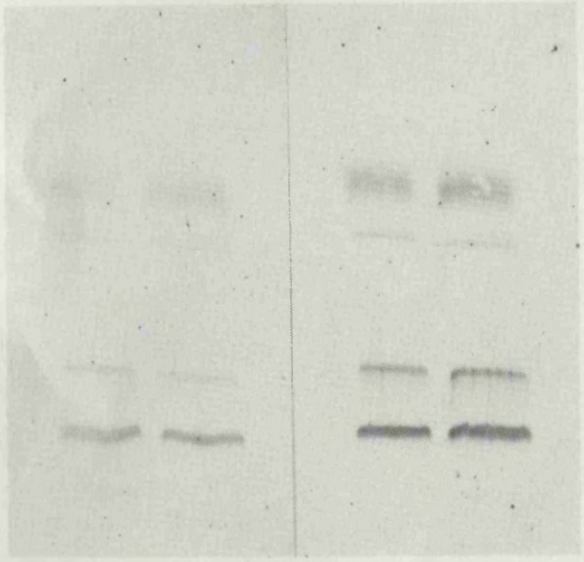

FIG. 2. Nitrocellulose blots of proteins separated by gel electrophoresis. The left pair separated on gels without PR and right pair on gels with PR, showing that the PR method yields identical separations.

Our work has been on the microbiological uptake of labeled methionine, thio-ATP, and other compounds, and we have routinely electrophoresed the resultant proteins, phosphoproteins, and macromolecules under the above dyed gel conditions. We now routinely use this method for enhancing the visualization of wells and either dye can be used equally satisfactorily. We have also used both dyes in admixture with identical results. The BPB is preferred for front marking as it travels slightly slower than PR, which can be allowed to run off the gel, but PR is valuable if one wishes to retain a visible marker for the interface or origin of the separating gel. Clearly the same general method of visualizing wells could be used for work with other gels such as agarose and nucleic acids and in other fields.

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The transport and culture conditions for optimum transformation responses of wildfowl lymphocytes to mycobacterial antigens

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A method has been developed for increasing the survival of wildfowl lymphocytes during transport over considerable distances. Blood in an equal volume of heparinised RPMI was maintained at close to avian body temperature, i.e., approximately 40 °C. Using this system lymphocyte transformation in the presence of antigen (mycobacterial) has been successfully performed with wildfowl mononuclear cells for the first time. Duck cells were cultured in 10% autologous sera with 8×10^5 cells/well for 4 days. Cells from Hawaiian geese (*Branta sandvicensis*) required similar culture conditions but were incubated for 3 days.

Key words: Lymphocyte transformation test; Mycobacterial antigens; (Wildfowl)

Introduction

Avian tuberculosis is a significant cause of death amongst captive wildfowl (*Anatidae*: ducks, geese and swans) in major collections the world over. In our attempt to develop a vaccine against this disease the in vitro assay of lymphocyte transformation test (LTT), has been employed. The test has been used to monitor the response of wildfowl peripheral blood lymphocytes to soluble mycobacterial antigens.

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Abbreviations: LTT, lymphocyte tranformation test; PHA, phytohaemagglutinin; CVPDS, Cherry Valley-pooled duck sera; WwWDPDS, white-winged wood duck-pooled duck sera; PBS, phosphate-buffered saline pH 7.2; RPMI, Roswell Park Memorial Institute 1640 medium; BCG, Bacille Calmette-Guérin; SI, stimulation index; cpm, counts per minute; SD, standard deviation; *n*, number of samples.

There is a tendency to assume that all avian immunology is represented by that of the chicken which has been much studied. This is mainly due to the bursa of Fabricius providing such an ideal model for studying early B cell development. However, wildfowl immunology would appear to be somewhat different from that of chickens resembling instead that of reptiles and even amphibians. Wildfowl are a particularly ancient evolutionary group and unlike chickens, no distinct populations of T 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). Even wildfowl immunoglobulins share similarities with those of more primitive groups. Ducks possess two antigenically similar and independently produced IgG molecules with different sedimentation coefficients of 7.8 S and 5.7 S (Zimmerman et al., 1971). Whilst chickens lack this smaller immunoglobulin, it has been found in the sera of some turtles, the lung-

fish and groupers (Kubo et al., 1973). We have therefore based our study on work already performed with duck lymphocytes rather than that done with chicken cells.

The culture conditions for the optimum transformation of duck lymphocytes by the plant lectin phytohaemagglutinin (PHA) have been documented previously (Higgins and Teoh, 1988) but these were not ideal for our purposes. Since we have not found any account in the literature of antigen driven LTT in wildfowl, we have investigated a variety of lymphocyte culture methods and conditions. One of the major problems to overcome was the distance between the site of the experimental ducks and geese and the laboratory. This could result in a 5 h delay before cell separation. A number of different systems were evaluated in an attempt to overcome the problem (Cromie et al., 1988) and methods have been developed producing satisfactory results in several species of duck and in the Hawaiian goose or nene.

Materials and methods

Wildfowl used

Adult mallards *Anas platyrhynchos*, adult white-winged wood ducks *Cairinini scutulata* and 3-month-old nenes *Branta sandvicensis* were used.

Pooled duck serum, CVPDS from clinically healthy 7-week-old white Peking ducklings *Anas platyrhynchos* bred at Cherry Valley Farms Ltd., was used, as well as autologous sera. Pooled serum was inactivated by heating for 1 h at 56 °C.

Blood collection and transport

Blood was collected from the medial metatarsal vein since this site is usually surrounded by supporting muscles and tendons that assist in haemostasis (Murdock and Lewis, 1964; Dein, 1982).

On average there was a 5 h delay between taking the blood and its arrival in the laboratory. Transport of whole heparinised blood over this distance resulted in lymphocyte death within 24 h; therefore transport of whole blood in different media at different temperatures and the transport of purified lymphocytes were investigated.

Blood was taken into an equal volume of either 0.1 M phosphate-buffered saline (PBS) pH 7.2 (Higgins and Chung, 1986), containing 10 $U \cdot ml^{-1}$ of sodium heparin (Monoparin, Heparin Injection B.P., Weddel Pharmaceuticals) or heparinised Roswell Park Memorial Institute 1640 medium (RPMI 1640 medium with 25 mM Hepes buffer with L-glutamine, Gibco) Samples were then transported in siliconised Vacutainers (no additive, Silicon coated Vacutainer, Becton Dickinson.)

TABLE I

LTT RESPONSES OF MALLARD LYMPHOCYTES COLLECTED FROM BLOOD TRANSPORTED IN RPMI OR PBS PRIOR TO SEPARATION

Final antigen concentration $\mu g \cdot ml^{-1}$	Transported in RPMI			PBS		
	Mean cpm (Mean SI)	SD	*n*	Mean cpm (Mean SI)	SD	*n*
PHA						
50	1186 (6.8)	174 (1.2)	3	704 (4.0)	378 (1.3)	3
10	1042 (5.8)	304 (0.9)	3	500 (3.1)	294 (1.8)	3
1	433 (2.5)	206 (1.2)	3	319 (1.6)	88 (0.6)	3
M. vaccae						
100	450 (2.6)	261 (1.5)	3	281 (1.6)	149 (0.5)	3
10	448 (2.6)	298 (1.7)	3	230 (1.4)	118 (0.6)	3
1	193 (1.1)	39 (0.2)	4	226 (1.4)	132 (0.8)	3
M. avium						
100	394 (2.2)	95 (0.6)	4	228 (1.4)	124 (0.7)	3
10	254 (1.4)	80 (0.4)	4	183 (1.1)	85 (0.3)	3
1	285 (1.6)	69 (0.5)	4	185 (1.1)	82 (0.2)	3
Control unstimulated wells	180	21	4	171	76	3

Key: Blood travelled at 40 °C. Cells cultured in 10% CVPDS. Cell concentration 10×10^6 ml^{-1}. 4 day cell incubation. Mean cpm of control unstimulated wells shown represent cpm of the maximum number of birds in each group. Mean SIs are calculated from individual SIs not mean cpm divided by mean control unstimulated cpm.

Lymphocyte separation

2.5 ml of the blood/buffer mixture were layered over an equal volume of lymphocyte separation medium, specific gravity 1.077 (Flow Laboratories) (Higgins and Chung, 1986) in Vacutainers. These were centrifuged at $200 \times g$ for 25 min (Higgins and Chung, 1986). Cells collected from both the lymphocyte separation medium and the interface were washed three times in RPMI.

Lymphocyte transformation test

The species of mycobacteria used for the preparation of antigens were *Mycobacterium avium, M. fortuitum, M. vaccae* and Glaxo BCG (Bacille Calmette-Guérin). Antigens were prepared from fresh cultures on Sauton's agar medium. Bacilli were suspended in 0.15 M borate-buffered saline pH 8.0, and treated for 15 min in an MSE 100 W ultrasonic disintegrator with an amplitude of 6–8 μm (Stanford et al., 1975). The resultant mixture of residual whole organisms, broken cell walls and cytoplasm was then centrifuged at 3000 rpm for 20 min. The supernatant was filtered serially through a 0.45 μm and two 0.2 μm membrane filters (Schleicher and Schuell). Protein concentration was measured spectrophotometrically by the method of Warburg and Christian (1941), using UV absorbance values at 260 and 280 nm. These preparations were used in different final concentrations (1, 5, 10, 20, 40, 50, 100 $\mu g \cdot ml^{-1}$).

20 μl of antigen in RPMI (or 20 μl of RPMI alone in control wells) were incubated at 41.6°C with 100 μl of 20% serum supplemented RPMI in flat-bottomed microtitre trays (Microwell, Nunc) (Higgins and Teoh, 1988). To this mixture were added 80 μl of cells suspended in RPMI at 2.5 × final concentration. All RPMI used contained streptomycin sulphate BP (Evans) at 100 $\mu g \cdot ml^{-1}$ and sodium benzylpenicillin BP (Crystapen, Glaxo) at 100 $U \cdot ml^{-1}$. After culture for 4 days at 41.6°C (Higgins and Teoh, 1988) in a humid atmosphere with 5% CO_2, to maintain pH, newly synthesised DNA in proliferating lymphocytes was radiolabelled for 17 h with 0.6 μCi/well of [^{3}H]thymidine.

Cells were washed onto cell harvester paper (Skatron) with distilled water using a semi-automatic cell harvester (Skatron). Discs were placed in 4 ml of scintillation fluid (Ecoscint A, National Diagnostics). Counts per minute were measured over a 1 min interval using a liquid scintillation counter (1211 Rackbeta, LKB).

Stimulation indices (SIs) were calculated from the mean counts per minute (cpm) of triplicate antigen stimulated cultures divided by the mean cpm of triplicate unstimulated control cultures (Timms, 1979).

Experimental design

Experiment 1. Blood samples were taken into equal volumes of either heparinised PBS or heparinised RPMI. Using a half of each sample the lymphocytes were separated at Slimbridge immediately after the blood was collected and the

TABLE II

LTT RESPONSES OF MALLARD LYMPHOCYTES AT DIFFERENT CELL CONCENTRATIONS

Final antigen concentration $\mu g \cdot ml^{-1}$	10×10^6 cells·ml^{-1}			5×10^6 cells·ml^{-1}		
	Mean cpm (Mean SI)	SD	*n*	Mean SI (Mean SI)	SD	*n*
PHA						
50	1726 (3.3)	1230 (1.4)	3	667 (1.1)	345 (0.7)	3
10	674 (1.5)	252 (0.1)	3	824 (1.2)	637 (0.5)	3
1	585 (1.4)	161 (0.3)	3	1099 (1.4)	770 (0)	2
BCG						
100	1088 (1.6)	1084 (0.2)	4	240 (0.6)	0 (0)	1
50	935 (1.5)	554 (0.4)	4	679 (1.0)	542 (0.2)	3
20	794 (1.5)	378 (0.3)	4	747 (1.0)	666 (0.3)	3
10	914 (1.4)	550 (0.4)	4	642 (0.9)	537 (0.2)	3
5	892 (1.5)	456 (0.5)	4	605 (0.9)	394 (0.1)	3
1	1041 (1.3)	1056 (0.4)	4	614 (0.9)	443 (0.2)	3
Control unstimulated wells	701	505	4	651	435	3

Key: Blood travelled at 40°C in RPMI. Cells cultured in 10% CVPDS. 4 day cell incubation. See also key to Table I.

TABLE III

LTT RESPONSES OF WHITE-WINGED WOOD DUCK LYMPHOCYTES AT DIFFERENT CELL CONCENTRATIONS

Final antigen concentration $\mu g \cdot ml^{-1}$	10×10^6 cells $\cdot ml^{-1}$			5×10^6 cells $\cdot ml^{-1}$		
	Mean cpm (Mean SI)	SD	*n*	Mean cpm (Mean SI)	SD	*n*
PHA						
50	11370 (62.8)	5625 (29.4)	4	956 (5.2)	1069 (5.7)	4
10	61222 (300.0)	43924 (179.0)	3	17378 (111.4)	25576 (178.4)	4
1	5399 (27.0)	5228 (22.2)	4	4217 (25.3)	5625 (40.7)	4
M. vaccae						
100	875 (3.6)	903 (3.5)	5	898 (3.5)	717 (3.0)	4
10	698 (2.4)	657 (1.2)	3	536 (2.2)	353 (1.2)	4
1	524 (3.3)	217 (2.4)	3	598 (2.3)	450 (1.2)	4
Control unstimulated wells	218	91	5	223	256	4

Key: Blood travelled at 40 °C in RPMI. Cells cultured in 10% autologous sera. 4 day cell incubation. See also key to Table I.

separated cells transported to London in RPMI. The remaining half samples were transported to London prior to separation of the lymphocytes. In both cases the samples were transported on ice, at ambient temperature, or at 40 °C. Cells were cultured in 10% CVPDS.

Experiment 2. Blood taken into heparinised RPMI at Slimbridge was transported to London at 40 °C. Lymphocytes were then cultured in 10% CVPDS or 10% autologous sera at two cell concentrations, 5 and 10×10^6 cells $\cdot ml^{-1}$. White-winged wood duck lymphocytes were also cultured in a pool of sera from this species, WwWDPDS.

Results

Experiment 1

The transport of purified lymphocytes at various temperatures suspended in RPMI resulted in cell death within 24–48 h and a reduced yield of lymphocytes and macrophages. There was also a higher percentage of contaminating cells (erythrocytes, thrombocytes and granulocytes) probably due to the imperfect speed control of the centrifuge used (Zinkl, 1986).

Lymphocytes purified in London from the blood transported on ice or at ambient temperature appeared unhealthy and died within 24 h. Whole blood transported in heparinised RPMI at 40 °C, gave the best transformation results.

Experiment 2

The lymphocyte transformation results are presented in Tables I–VI. Lymphocytes from all three species gave their highest SIs when cultured at 10×10^6 ml^{-1} (Tables II and III) and in autologous sera (Tables IV, V and VI). The nene lymphocytes died at the lower cell concentration and in CVPDS (Table VI) (the low *n* value in the table indicating many wells containing dead cells).

The SIs from the white-winged wood duck lymphocytes were higher when cultured in WwWDPDS than when in CVPDS (Table V). The optimum incubation times were found to be 4 days for the ducks and 3 days for the nenes.

Discussion

Our study clearly shows that wildfowl lymphocytes transform in the presence of mycobacterial

TABLE IV

LTT RESPONSES OF MALLARD LYMPHOCYTES IN 10% CVPDS AND AUTOLOGOUS SERA

Final antigen concentration $\mu g \cdot ml^{-1}$	10% autologous sera			10% CVPDS		
	Mean cpm (Mean SI)	SD	*n*	Mean cpm (Mean SI)	SD	*n*
PHA						
50	908 (4.8)	453 (2.7)	5	793 (2.8)	663 (2.3)	4
10	2629 (13.3)	1574 (10.5)	4	1222 (4.2)	365 (2.7)	5
1	837 (4.1)	490 (2.4)	5	922 (4.1)	1039 (2.9)	4
M. vaccae						
100	518 (2.6)	213 (1.2)	5	557 (1.4)	378 (0.6)	4
10	435 (2.1)	290 (0.9)	5	590 (1.5)	169 (1.0)	3
1	355 (1.8)	35 (0.5)	4	399 (1.1)	163 (0.1)	4
Control unstimulated wells	204	56	5	365	181	5

Key: Blood travelled at 40 °C in RPMI. Cell concentration 10×10^6 ml^{-1}. 4 day cell incubation. See also key to Table I.

antigens, providing the transport and culture conditions are optimized. The culture conditions differ from those previously recommended for mitogen driven transformation with the most obvious distinctive feature being the use of autologous sera at high cell concentrations. The probable explanation of RPMI increasing the longevity of the cells is that the sodium bicarbonate buffer ($2 mg \cdot ml^{-1}$) temporarily controlled the CO_2 levels. Moreover, the slightly acidic conditions in the RPMI would reduce loss of macrophages by adherence to the Vacutainer.

The effect of temperature on the longevity of lymphocytes was found to be considerable. Those transported on ice or at ambient temperature appeared dead after 24 h with no thymidine uptake after 2, 3 or 4 days' incubation. This would indicate dead or non-metabolising cells rather than cooled cells simply being more sluggish in their responses. Washing solutions, stored at 4 °C, are now prewarmed to avoid unnecessary temperature shock.

The superior results for the higher cell concentrations suggest that the close proximity of cells improves survival and permits better functional responses to antigens. In addition the close cell-to-cell contact probably facilitates antigen presentation. Higgins and Teoh (1988) have previously suggested the use of U or V shaped culture wells to increase cell contact. However, we did not take up this suggestion.

Higgins and Teoh (1988) also found that 20% foetal calf serum supported duck LTT to PHA at lower cell concentrations ad did 5, 10 and 20% chicken sera. Although we did not try these sera our results for both mallard and white-winged wood ducks in CVPDS showed that thymidine incorporation in antigen-stimulated cells may be similar, but the SIs tended to be lower due to higher values for unstimulated cells. Microscopic observations of lymphocyte morphology in control wells showed some degree of clumping and clustering of lymphocytes around macrophages suggesting a degree of antigen presentation. The CVPDS was heat-inactivated for 1 h at 56 °C, to destroy complement activity, but this does not destroy immunoglobulins and it is possible that these non-self molecules have alloantigenic properties which would result in stimulation of the cells. The mallard lymphocytes cultured in autologous sera gave higher SIs than those cultured in CVPDS (derived from white Peking ducks *Anas platyrhynchos* which would therefore be considered allogeneic). Although these two breeds of duck share the same species name it is highly likely that they have genetically diverged since white Peking ducks were domesticated from wild mallards and appeared in Chinese literature as early as 1597 AD (Shaw, 1940). In evolutionary terms this is relatively recent. However the processes used in domestication are likely to induce genetic divergence in a relatively short time. As the physiology, anatomy and behaviour of the two breeds of duck are also distinct (Delacour, 1964; Farner and Sossinka, 1982), it is probable that mallard lymphocytes recognise some aspects of the CVPDS as foreign.

The white-winged wood duck lymphocytes cultured in WwWDPDS appeared to be healthy and

TABLE V

LTT RESPONSES OF WHITE-WINGED WOOD DUCK LYMPHOCYTES IN 10% CVPDS, WwWDPDS AND AUTOLOGOUS SERA

Final antigen concentration $\mu g \cdot ml^{-1}$	10% autologous sera			10% WwWDPDS			10% CVPDS		
	Mean cpm (Mean SI)	SD	*n*	Mean cpm (Mean SI)	SD	*n*	Mean cpm (Mean SI)	SD	*n*
PHA									
50	11370 (62.8)	5625 (29.4)	4	19824 (115.4)	19620 (113.4)	4	1842 (8.5)	2348 (9.5)	3
10	61222 (300.0)	43924 (179.0)	3	63040 (395.5)	46406 (317.8)	4	18990 (133.5)	12418 (177.1)	3
1	5399 (27.0)	5228 (22.0)	4	5925 (40.1)	5631 (46.7)	4	15648 (69.3)	8169 (65.6)	3
M. vaccae									
100	875 (3.6)	903 (3.5)	5	204 (1.2)	105 (0.3)	3	793 (3.3)	572 (3.5)	3
10	698 (2.4)	657 (1.2)	3	417 (2.2)	324 (0.8)	3	791 (1.3)	809 (0.5)	2
1	524 (3.3)	217 (2.4)	3	252 (1.4)	140 (0.4)	3	487 (0.9)	376 (0.1)	2
M. avium									
100	578 (2.9)	175 (0.6)	3	197 (1.7)	47 (0.4)	2	115 (0.3)	9 (0.2)	2
10	1304 (6.3)	1118 (4.7)	3	227 (1.9)	102 (0.8)	2	444 (0.8)	349 (0)	2
1	1379 (6.5)	1858 (8.3)	3	148 (1.2)	4 (0)	2	457 (1.0)	282 (0.2)	2
Control unstimulated wells	218	91	5	168	71	4	400	414	3

Key: Blood travelled at 40 °C in RPMI. Cell concentration $10 \times 10^6 ml^{-1}$, 4 day cell incubation. See also key to Table I.

gave reasonable SIs although these were not as high as those cultured in autologous sera. Those cultured in CVPDS gave the lowest SIs, although the cpm were generally higher than those in WwWDPDS, backgrounds were higher. There were also many dead cell cultures. This was almost certainly due to the ducks not belonging to the same species.

The nene lymphocytes appeared 'crinkled' and unhealthy and either died or responded very weakly at all cell concentrations in CVPDS with many cell cultures dying and the remaining cultures giving low cpm and SIs. When they were cultured in autologous sera the culture medium became very yellow after 3 days' incubation indicating acidic conditions produced by very actively metabolising cells. Radiolabelling of these cells after 4 days' incubation in both sera gave poor results. Radiolabelling at 3 days' incubation gave higher cpm in antigen stimulated cultures and therefore higher SIs.

The optimal culture conditions for mallards and white-winged wood ducks also proved satisfactory for mandarin ducks *Aix galericulata* and gadwall *Anas strepera.*

Our conclusions are that wildfowl mononuclear cells are temperature sensitive and should be kept as near to the bird's body temperature as possible. Antigen driven lymphocyte transformation proceeds most effectively at higher cell concentrations

TABLE VI

LTT OF NENE LYMPHOCYTES IN 10% CVPDS AND AUTOLOGOUS SERA

Final antigen concentration $\mu g \cdot ml^{-1}$	10% autologous sera			10% CVPDS		
	Mean cpm (Mean SI)	SD	*n*	Mean cpm (Mean SI)	SD	*n*
M. Vaccae						
40	676 (1.8)	346 (0.4)	5	149 (0.9)	26 (0.1)	2
10	796 (1.7)	799 (1.6)	5	172 (0.9)	47 (0.4)	3
BCG						
40	540 (1.3)	494 (1.0)	4	170 (1.0)	58 (0.2)	2
10	1088 (2.6)	950 (2.0)	5	121 (1.1)	104 (0.4)	3
M. fortuitum						
40	585 (1.2)	583 (1.1)	4	155 (0.9)	49 (0.4)	3
10	1557 (2.9)	1521 (3.0)	4	211 (1.2)	37 (0.4)	3
M. avium						
40	222 (0.7)	113 (0.7)	5	126 (0.7)	19 (0)	3
10	1248 (2.7)	1437 (2.7)	5	250 (1.4)	42 (0.4)	3
Control unstimulated wells	528	235	5	189	28	3

Key: Blood travelled at 40 °C in RPMI. Cell concentration 10×10^6 ml^{-1}. 3 day cell incubation. See also key to Table I.

and in the presence of autologous sera rather than pooled sera, even when the pool is from birds of the same species.

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Letter to the editors

Duck lymphocyte transformation

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Dear Editors,

We read with interest the recent paper of D.A. Higgins and C.S.H. Teoh (1988). Whilst following these and earlier methods (Higgins and Chung, 1986), we would like to forward some additional information and point out some ammendments made to enable successful duck lymphocyte transformation (LT), to be carried out under different circumstances.

Due to the distance between the ducks and our laboratory, travelling time for the blood is considerable, at least 5 h, prior to cell separation. We found that transport at about 40°C led to successful LT whilst transport at room temperature gave inferior results. Transport on ice resulted in cell death within 24 h. Since temperature seems to be important in the longevity of the lymphocytes, the warming of washing solutions, stored at 4°C, to room temperature prior to cell contact, was also tried and found to be an improvement. Rather than the blood being taken into an equal volume of heparinised phosphate-buffered saline (PBS) (Higgins and Chung, 1986), it was taken into, and transported in, an equal volume of heparinised RPMI 1640 (Gibco with 25 mM Hepes buffer with L-glutamine). Transport of purified lymphocytes over this 5 h period in either PBS or RPMI was unsatisfactory as the number of macrophages was significantly reduced even though the containers (Becton Dickinson Vacutainers) were siliconised.

Whilst these conditions were optimised for Mallard (*Anas platyrhincos*), they also prove very satisfactory for Mandarin ducks (*Aix galericulata*), and White Winged Wood ducks (*Cairina scutulata*).

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