The epidemiology of ulcerative mycosis of the platypus (*Ornithorhynchus anatinus*)

by

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ABSTRACT

Platypuses (*Ornithorhynchus anatinus*) in the north of the island state of Tasmania suffer from a serious disease called ulcerative mycosis, which is responsible for high morbidity and, presumably, mortality rates in areas where the disease occurs. The disease is caused by the fungus *Mucor amphibiorum*, which is also found in Queensland and New South. However, it does not cause disease in platypuses in those states. This thesis reports on the results of an integrated study of the epidemiology of the disease. aimed at determining why only northern Tasmanian platypuses are affected.

Pathogenicity trials involving various cane toads (*Bufo marinus*) as the experimental model indicated that Tasmanian, platypus-derived isolates of *M. amphibiorum* were more likely to cause a serious, long-term infection than Queensland or West Australian toad-derived isolates. Also, analysis of DNA sequences from four Tasmanian, platypus-derived isolates of *M. amphibiorum*, two Queensland toad-derived isolates and one West Australian slender tree frog (*Litoria adelensis*)-derived isolate suggest that the area sequenced is not highly variable between isolates from different areas. Based on the DNA analysis and pathogenicity trials, the isolates could be split into three groups; firstly, Tasmanian, platypus-derived isolates, secondly, West Australian slender tree frog-derived isolates and Queensland cane-toad (+) mating type isolates, and thirdly the Queensland cane toad-derived (-) mating type. A closely related fungus, *Mucor circinelloides*, was also sequenced and tested in pathogenicity trials to determine if it was implicated in the disease. The results suggest that its isolation from diseased animals represents accidental environmental contamination.

Sampling of platypus tail fat for the immunomodulating xenobiotics PCB, DDT and Lindane from animals within and outside the endemic area for ulcerative mycosis showed equal levels in all areas. Ulcerated animals did not have elevated levels in comparison to healthy animals. However, some animals from both enedemic and non-endemic areas had greatly elevated levels of PCBs, which may impact on other areas of platypus health.

Lymphocyte proliferation assays developed for the platypus showed a low response to mitogens in comparison to most other mammals, but there was no difference between responses for animals from endemic and non-endemic areas. A lowered response to some mitogens was noted in ulcerated animals. A Western blot was developed that could detect antibodies to *M. amphibiorum* in animals with both active and healed ulcers, but not in healthy platypuses. The possibility that Tasmania possesses a sub-

population of atopic platypuses was investigated with an ELISA for the detection of *M. amphibiorum* specific IgE.

There are a number of hypotheses why ulcerative mycosis occurs in Tasmanian, but not mainland, platypuses. The fungus *M. amphibiorum* may have been introduced into the state, exposing a previously naive population. Tasmanian platypuses, having been isolated from mainland Australia for at least 12,000 years, may be genetically distinct and inherently susceptible to the disease. It is possible that the Tasmanian platypuses' immune system is suppressed, either genetically or by some environmental contaminant. Alternatively, the fungus *M. amphibiorum* in Tasmania represents a more pathogenic strain than that found in mainland Australia.

This thesis will argue that the fungus *M. amphibiorum* has been introduced into the state on numerous occasions, but in most instances these introductions did not result in infections of platypuses. However, prior to 1982, an isolate of *M. amphibiorum* successfully infected a platypus, probably because of its ability to produce infective bodies at a much faster rate than other isolates, and the inherent susceptibility of Tasmanian platypuses. Also, some Tasmanian platypuses have suppressed T cell function, which ultimately results in the death of such ulcerated animals.

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General Introduction

General Introduction

The platypus, *Ornithorhynchus anatinus*, is one of only three extant monotremes, and the only one to inhabit an aquatic environment. Its habitat is restricted to the eastern states of Australia; Queensland, New South Wales, Victoria and the island state of Tasmania, although it has been introduced to Kangaroo Island in South Australia. Although not officially endangered, it faces threats from land degradation, habitat destruction and predation by domestic and wild dogs. Many platypuses become entangled in fishing nets and drown, whilst others suffer serious injury through wounds caused by plastic beer wrap, fishing line and other discarded material (Munday *et al.* 1998). Motor vehicles also run over many animals each year. In the north of Tasmania, a new threat has appeared which may cause significantly more mortalites than all of the other threats combined, and which could threaten the entire Tasmanian platypus population.

The threat is a mycotic infection which was first observed in 1982, when three moribund and one debilitated platypus were found on the banks of the Elizabeth River at Campbell Town in the north of Tasmania (Munday and Peel 1983) (Figure 1). The debilitated platypus was returned to the river, and two of the moribund platypuses, which died, were submitted to the Mt. Pleasant Laboratories for necropsy. Both of the animals were suffering from extensive ulceration, but were otherwise in reasonable condition. Lesions were found on the bill, the back, the tail, manus and pes. The ulcers were rough and greyish in appearance, with a granulating surface. In places, they had invaded the musculature to a depth of 10 mm below the skin (Figures 2, 3). One of the animals also had miliary lesions approximately 1 mm in diameter in the lungs. Scrapings and needle biopsies from the ulcers were inoculated onto Sabouraud's agar, algae culture agar and algae culture broth. Histological sections revealed the presence of large, round organisms approximately 18 µm in diameter in affected tisssues (Figure 4). Some of these organisms appeared to be unicellular, whilst others contained elements which did not occupy the entire cell. The only organism consistently isolated from the ulcers was a *Mucor* sp which grew on the Sabouraud's agar. At the time, this was considered likely to be an environmental contaminant. Up to that time, with the exception of Frank *et* al. (1974), all cases of mucormycosis mentioned in the literature were distinguished by the presence of hyphae in affected tissue. Despite extensive investigation, no hyphae were found in any of the sections. The tentative initial diagnosis was infection with an achloric alga. This diagnosis was supported by the presence of abundant chloric algal growth in the river at the time, due to recent drought conditions. However, the authors were intrigued by the consistent isolation of a *Mucor* sp.

These were the first reported cases of this intriguing condition. The disease is not found in platypus in other parts of Australia, nor does it affect other aquatic mammals such as the water rat, although these animals live in close proximity to platypuses. Animals that are otherwise healthy, with good body weight and high tail fat levels, may be affected. Animals in poor condition also contract the disease, but whether the disease causes the poor body condition, or vice versa, is not known. The ulcers can cause severe discomfort for platypus, with affected animals often being observed scratching at the ulcers with their claws, or rubbing ulcers against rocks and pieces of wood. In summer, it is not unusual for the ulcers to become fly-blown. It is likely that the disease itself does not cause death directly in platypuses. The ulcers leave large areas of flesh and skin exposed to the elements. For an animal that spends extended periods of time in water of very low temperature, any constant heat loss reduces the ability of the animal to successfully thermoregulate, and it is likely that in winter badly ulcerated animals suffer from hypothermia. The large areas of exposed flesh also leave the animals open to secondary infections.

The disease is not mentioned in the literature again until 1993, when nine animals suffering from similar ulcerative mycosis were examined (Obendorf *et al.* 1993). All of these animals were found either dead or moribund on the banks of three natural waterways in Tasmania's north. The organisms seen in histological sections were similar to those reported in the original cases, and once more the only consistent isolate was a *Mucor* sp. This time, isolates were sent to the National Reference Laboratory for Medical Mycology, Royal North Shore Hospital, New South Wales, and identified as *Mucor amphibiorum.*

In 1994, with knowledge of the putative causative agent of the disease, a 12 month investigation commenced, with extensive trapping at Brumbys Creek (Connolly *et al.* 1998). Thirty six animals were trapped, with 13 being ulcerated. Lesions were found on the hindlimbs, forelimbs, tail, trunk, head, webbing of the forelimbs and the bill. Thirteen isolates of the fungus were isolated from 9 ulcerated animals. The study also examined water quality parameters, both at Brumbys Creek, and in areas where the disease had not been found. The results were similar, regardless of the presence or absence of diseased animals. The fungus was not isolated from soil or water samples, or from platypus or amphibian faeces, despite extensive sampling.

The initial rate of infection in affected areas is between 30% and 35%, with more males than females affected (Connolly *et al.* 1998, Stewart 1998). It is difficult to

estimate how many of these animals die as a result of the disease. Most mortalities in platypuses are not reported, with animals dying in burrows, or in undergrowth lining the waterways. The dead, ulcerated animal found by a passer-by is the exception, not the rule.
Although the rate of infection remains high in some areas, other areas have high initial rates followed by complete disappearance of affected animals. This latter situation is probably due to a combination of some affected animals dying and others recovering. The reason (s) for the disease persisting in some areas is not clear.

It is still not known the disease is transmitted from animal to animal. Some suggest that the fungus infects previous wounds, perhaps caused by eel or water rat bites, or by abrasions caused by snags in the rivers (Obendorf *et al.* 1993). Intraspecific fighting, resulting in spur wounds, has also been suggested (Obendorf *et al.* 1993). Ticks may act as a vector, transmitting spores to the epidermis of the platypus, although to date the fungus has not been isolated from a tick. Inhalation of spores is another posible portal of entry for the fungus. Spores carried to the lungs may be taken up by phagocytic cells, and carried to other parts of the body, where they develop into sphaerule-like bodies, which are the infectious stage of the fungus (Munday *et al.* 1998). Platypuses regularly share burrows, which provide an ideal environment for the fungus. The temperature is constant, the air humid, and the fungus is protected from direct sunlight. An ulcerated platypus that slept in a burrow would leave behind sphaerule-like bodies on the floor and walls of the burrow. These would then develop into hyphae and mycelia, produce sporangia, and then release spores into the burrow. Other animals entering the burrow would then be exposed to the spores. Of course, it is possible that there are several routes of infection, although the inhalation of spores is partly supported by the isolation of *M. circinelloides*, a closely related species, from the nares of several platypuses (Stewart 1996), and the frequent occurrence of mycotic pneumonitis in infected animals.

The true extent of the endemic area in Tasmania is still unknown. Until recently, it was thought that the disease was restricted to several streams that drain the northern Midlands district (Connolly *et al.* 1995). However, an ulcerated animal was caught at Lake Lea in the Cradle Mountain National Park in early 2000 (pers. com. Bethge). This animal had been previously trapped in 1998, and at that time was perfectly healthy. There have also been reliable reports of ulcerated animals at Derwent Bridge and Dee Lagoon, and in the upper reaches of the Mersey River. There has also been a less reliable report of an ulcerated animal at New Norfolk, and in July 2000 an ulcerated platypus was captured at Springfield, near Scottsdale in the north east of the state (Figure 1).

The fungus *Mucor amphibiorum*

This fungus was first isolated in 1972 from an Australian green tree frog (*Hyla* syn. *Litoria caerulea*), recently imported by a collector into West Germany (Frank *et al.* 1974). The green tree frog became sick, and shortly thereafter the infection spread to other amphibians in the collection. The authors suggested that the frog may have been infected in Australia. At the time, the fungus isolated from diseased amphibians could not be identified. Experimentally infected European frogs (*Rana temporaria, R. esculenta*) and toads (*Bufo bufo spinosa*) quickly died. On necropsy, numerous lesions were found in the internal organs, predominantly affecting the liver and spleen. Experimentally infected reptiles remained healthy, with only small lesions being found in the internal organs. Mice, rats and guinea pigs were unaffected by the fungus.

This species of *Mucor* differed from other *Mucor* sp in its ability to form bodies termed sphaerules by Frank *et al.* (1974). The sphaerules were tissue forms of sporangia/sporangiospores, and contained daughter cells which were capable of forming further sphaerules *in vivo*. If the sphaerules were grown *in vitro*, they grew in the normal manner associated with *Mucor* sp. Throughout this thesis, these bodies are referred to as sphaerule-like bodies (slb), to avoid confusion with the sphaerules that are associated with infection by *Coccidioides immitis*. It was not until 1978 that the fungus was described as a new taxon, *Mucor amphibiorum* (Schipper 1978). The fungus exists as two mating types, known as (+) and (-) mating strains. The fungus is capable of asexual reproduction, but if a (+) and a (-) mating strain come into contact, they reproduce sexually by producing zygospores (Figure 5). By definition, (+) mating strains of *M. amphibiorum* produce zygospores only with (-) mating strains of the same species. If a known (+) and (-) mating strain are available, this provides an excellent method for determining whether a fungal isolate is *M. amphibiorum.*

Other diseases associated with the fungus *Mucor amphibiorum*

As the name of the fungus suggests, it is commonly associated with mycoses in amphibians. Other than the outbreak described by Frank *et al.* (1974), the fungus has been isolated from cane toads (*Bufo marinus*) in Queensland, the Northern Territory and northern New South Wales (Speare *et al.* 1994), and from slender tree frogs (*Litoria adelensis)* and white-lipped tree frogs (*Litoria infrafrenata*) held at the Perth

Zoological gardens (Creeper *et al.* 1998). In 1994, a mass mortality of amphibians occurred at the Melbourne Zoo. Although the agent responsible was not definitely identified, a *Mucor* sp was isolated, and the symptoms were consistent with infection by *M. amphibiorum*. The disease outbreak followed the introduction of an amphibian from the north of Australia to the collection (Slocombe *et al.* 1995).

Unlike the platypus, in which the disease affects predominantly the extremities, and occasionally the lungs, in amphibians the fungus can infect the liver, spleen, kidneys, urinary bladder, heart, lungs, subcutaneous lymph spaces, skin, gastro-intestinal tract, voluntary muscle, bone, cranial cavity and the oral cavity (Creeper *et al.* 1998, Speare *et al.* 1997).

It is not clear why the fungus affects the platypus, and not other aquatic mammals. It may in part be due to the body temperature of the platypus (32°C), which is lower than the maximum permissive temperature for *M. amphibiorum*. The disease occurs predominantly on the extremities of the platypus, which are at a lower temperature than the internal organs, so it is likely that the maximum temperature for growth of the fungus *in vivo* is lower than 32°C. Temperature is unlikely to be the sole determinant however. The water rat, *Hydromys chrysogaster*, has a low body temperature when swimming in water at temperatures below 25C°, and presumably its skin temperature is lower even than its core body temperature (Dawson and Fanning 1981), but the disease has not been found in this species, which frequently occurs in association with ulcerated platypuses.

Although the suspicion is that the fungus is native to Australia, as suggested by Frank *et al.* (1974), it should be noted that although the first report of the disease followed the importation of an Australian frog into a German collection, the collection also contained frogs imported from South America. Also, the (-) mating strain examined by Schipper was isolated from a diseased poison arrow frog (*Dendrobates* sp.) shortly after its importation into West Germany from South America. It is possible that the fungus occurs naturally in both Australia and South America, as both of these countries were once part of the super continent Gondwanaland.

As stated earlier, body temperature is obviously not the only factor involved in susceptibility to the disease. There are several hypotheses that would explain the presence of the disease in defined areas of Tasmania, but not in mainland populations, even though the fungus and platypuses are known to occur together in Tasmania, NSW and Queensland. This thesis investigates those hypotheses, which will be espoused below.

Description of chapter contents

In Chapter one, the possibility that *M. amphibiorum* has been introduced into the state was investigated. This hypothesis is strongly supported by the evidence. The disease appeared suddenly in a localised area. It seems unlikely that the disease had been in Tasmania prior to the reported outbreak. The waterways of Tasmania are much frequented by anglers and bushwalkers. Ulcerated animals would have been seen and reported if present. The most likely source of the introduction would be via green tree frogs in bunches of bananas imported from Queensland. These frogs are known to be carriers of the disease, and are regularly found by both supermarket employees and customers. There are several methods available that could be used for determining whether the fungus has recently been introduced. The most basic method involves a comparison of the morphology of different isolates, to determine whether there is some feature that is common to both Tasmanian isolates and a discrete mainland population. That is, whether the Tasmanian isolates are morphologically readily identifiable as having originally come from a mainland population.This method is imprecise, as fungal morphologies can differ depending upon culture conditions. A more powerful method involves DNA sequencing. There are many methods avaliable for such sequencing, and many areas of the genome that can be investigated. In fungus, the rDNA sequence is often used for determining phylogenetic relationships. The sequence comprises the genes that code for ribosomal DNA, and is highly conserved, as would be expected for genes that are coding for such an important product. It is comprised of the 18S, 5.8S and 26S regions. Between the 18S and 5.8S genes is an area known as the internal transcribed spacer region 1 (ITS1), and between the 5.8S gene and the 26S gene is the area known as ITS2. The ITS regions are non-coding and not highly conserved between species, and thus tend to be highly variable between species. Within species, some geographical areas have strains with ITS sequences unique to that area (Bryan *et al.* 1994). Other species appear to have sequences associated with pathogenicity and virulence (Jackson *et al.* 1999). Sequencing of the ITS1 and ITS2 regions was used to determine the relationship between Queensland, West Australian and Tasmanian isolates of *M. amphibiorum.* Sequencing of *Mucor circinelloides* isolated from an ulcerated platypus, and from diseased frogs in West Australia was also performed.

Chapter two investigates the possibility that the Tasmanian strain of *M. amphibiorum* is more pathogenic than its mainland counterparts. Pathogenicity is known to vary between strains in a species. It is not uncommon for species to contain both pathogenic and non pathogenic strains. For example, Ellis *et al.* (1991) found there

was significant variation in both virulence and antigenicity between different isolates of *Dermatophilus congolensis*. In Tasmanian ulcerated platypuses, only (+) mating types of *M. amphibiorum* have been isolated (Stewart 1996, Connolly *et al.* 1998, Stewart 1998).The presence of only (+) mating strains supports not only the introduction of the fungus, but also the increased pathogenicity of the isolates. In other *Mucor* pathogens of plants, such as *Mucor piriformis*, it is accepted that the (+) mating strains are more pathogenic than their (-) counterparts (Michailides and Spotts 1986). The pathogenicity of the fungus may also have increased through passaging through the platypus. Often, passaging of a pathogen through a highly susceptible host can result in increased pathogenicity and/or virulence. Designing pathogenicity trials involving *M. amphibiorum* was complicated by the low maximum temperature for growth of the species (35°C). Because of this, normal experimental species such as mice, rats and guinea pigs were unsuitable. It was not possible to use platypuses for experimental infection, due to both ethical and practical considerations. For the trials, cane toads were considered a suitable model. The trial also involved the experimental infection of cane toads with *Mucor circinelloides*, a species closely related to *M. amphibiorum* (Frank *et al.* 1974). This species has been isolated from an ulcerated platypus before (Stewart *et al.* 1999), and can form sphaerule-like bodies when grown *in vitro* (Stewart 1998).

Chapter three reports on an extensive survey of platypuses from throughout Tasmania, involving the analysis of tailfat samples for the xenobiotics polychlorinated biphenyls (PCBs), 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane (DDT) and the gamma isomer of 1,2,3,4,5,6 hexachlorocyclohexane (Lindane). With many species, data on levels of xenobiotics in tissues can only be gathered by euthanasing the animal. The method described in this chapter is simple, quick, non lethal and causes no long term problems for the platypus. Although the dangers of xenobiotics are well recognised in other countries, there is little data available on Australian terrestrial or aquatic species. The literature mentions only three investigations of PCB levels in native Australian animals, one concerning kangaroos (National Residue Surveys 1989-1997), one relating to brushtail possums (Bolton and Ahokas 1997) and a preliminary study on levels in Tasmanian platypuses (Munday *et al.* 1998). Given the potential risks associated with xenobiotics, the lack of information on levels in native wildlife in general, and platypuses in particular, is surprising. In Tasmanian, PCBs were commonly used in electrical equipment, and in a variety of industrial processes. Lindane is an insecticide that is still widely used today, and DDT use was widespread in Tasmania prior to its banning in 1987. Xenobiotics can directly affect the immune system, leaving an animal susceptible to a range of diseases (Luster and Rosenthal 1993). Many also affect the endocrine system, resulting in inappropriate levels of

hormones (Brouwer *et al.* 1989). The effect of xenobiotics on particular species is complicated by differences in susceptibility between species, and by the interrelationship between different xenobiotics (Ross *et al.* 1996). Some can act synergistically with others, resulting in increased risk, whilst others, when combined, result in a reduced risk. This study was aimed at determining whether the levels in Tasmanian platypuses were as high as those recorded for a variety of animals in other countries, and which were considered to be a risk to the animals, especially in relation to the immune system.

Chapter four investigates some questions concerning the immune status of Tasmanian platypuses. In humans, a mycosis is often associated with an underlying immune deficiency. Truly pathogenic fungi are rare, and do not normally cause severe infections in healthy individuals . Conditions such as HIV/AIDS leave the host susceptible to a range of ubiquitous fungi which pose no problem to a healthy individual (Threlkeld and Dismukes 1989). Likewise, wild animals are not normally prone to serious fungal infections. Wild animals from which fungi have been isolated are normally suffering from a range of disease conditions, and are often in poor health prior to infection (Adaska 1999). In such cases, the fungi are acting opportunistically. In this chapter, several areas of immunity were examined. Firstly, a lymphocyte proliferation assay was developed. This assay measures the ability of B and T cells of the immune system to proliferate in response to a mitogen, and is a routine test performed on many species to help determine the immune status of the individual. This assay is non-specific, and does not provide information on the specific immune reaction of an individual to infection by *M. amphibiorum*. To ascertain the response of ulcerated and non ulcerated animals, and of healthy animals from endemic and non endemic areas of Tasmania and mainland Australia to *M. amphibiorum* antigens, sera were tested in a collaborative investigation with Dr Richard.Whittington of the Elizabeth MacArthur Agricultural Institute in New South Wales. In addition, a Western blot was developed to investigate both the response of individual animals to the fungus, and the possibility that Tasmanian isolates of *M. amphibiorum* possess a unique banding signature. A Western blot was developed because a previous study had demonstrated that there was a high degree of cross-reactivity of sera from rabbits exposed to antigens from either *M. amphibiorum* or *M. circinelloides* (Stewart 1996). In a collaborative project with Dr Lars Hellman of Upsalla University, splenectomies were performed on two Tasmanian platypuses, and the immunoglobulin classes possessed by the platypus were determined. Using recombinant IgE produced at Upsalla, antibodies were raised in rabbits, and an ELISA developed for the detection of IgE in platypuses. The disease ulcerative mycosis may be an atopic dermatitis, and

there may be a unique subpopulation of atopic platypuses in Tasmania. Elevated IgE levels in some, but not all platypuses, would support that hypothesis.

In Chapter five, possible differences in weight, body condition and sex ratios were investigated between animals from endemic and non endemic areas. Weights and sex ratios between ulcerated male and female platypuses were also recorded. This chapter also investigates the possibility that Tasmanian platypuses are genetically predisposed to the disease. Such predisposition is recognised in several diseases. For example, the mycosis caused by *Coccidioides immitis* is more likely to affect people of Afro-American descent than those of Hispanic background (Fierer *et al.* 1998). Possible genetic differences between ulcerated and non ulcerated platypuses, animals from endemic and non endemic areas, and Tasmanian and mainland platypuses were investigated in a collaborative project with Dr Shiro Akiyama of LaTrobe University. Skin samples were taken from platypuses, and microsatellite analysis used to determine relationships between populations. Many areas of DNA contain highly repetitive sequences of nucleotides known as microsatellites. The more closely related populations and individuals are, the more similarity there is between microsatellite sequences.

Platypus Immunology

Little research to date has been done on the platypus immune system and function, and what is available can be contradictory. Some sexually mature platypuses possesses a thymus, but in others the thymus hasn't been found. It has been suggested that the platypus thymus, like that of reptiles and amphibians, undergoes seasonal involution and development. Peyer's patches are uncommon and in one study, the entire intestinal tracts of four animals were sectioned every 3cm, with Peyer's patches only being found in one section of jejunum from one animal and in one section of terminal ileum from another. Like the other monotreme, the echidna, the platypus does not possess lymph nodes. Rather, it has lymph nodules that are scattered throughout the loose connective tissues associated with blood vessels. The platypus possesses a relatively large spleen, which is presumably a compensation for the paucity of other tissues with immunological function.

Limitations on the study of platypus

Given the unique status of the platypus, it would at first seem surprising that very little is known about basic immune functions of the animal. However, the paucity of knowledge is easily explained by the problems inherent in studying this animal.

Platypus are secretive in nature, and feed predominantly at night. Trapping them is achieved either through the use of fyke nets or gill nets. Fyke nets, with the cod ends run up on the bank of a river, can be left unattended for several hours, but gill nets need to be continually monitored. Many man-hours are required to set and monitor nets, and there is no guarantee that animals will enter them. Although platypuses are abundant in Tasmania, many of the waterways in which they are found do not lend themselves to easy trapping. Many rivers and dams which are ideal for placing nets lack platypus, and many rivers which are almost impossible to trap have the most platypuses.

Although the platypus is a primitive animal, and is credited with having poor eyesight, it is quite capable of detecting gill nets, and is often observed climbing over the top of a net, or swimming around it. Animals that have been caught in fyke nets seem to have little trouble in remembering the event, and in future avoid the nets. Recaptures in fyke nets are rare, and many areas that have been trapped once will not produce any animals on subsequent occasions, even after a year. The inability to consistently recapture platypuses limits the range of options open to the experimenter. Measuring antibody response over time, while desirable, is not likely to be possible. Ideally, select platypus could be held in captivity, thus obviating the need for recapture. However, setting up an enclosure for platypus is prohibitively expensive. Also, platypus are particularly prone to stress and have been known to die in captivity as a result (Whittington 1991, 1993).

Because the platypus cannot easily be held in captivity or predictably recaptured, assays have to be developed accordingly. The number of assays that can be performed is limited by the amount of blood that can be taken from a platypus. Blood can only safely be taken from the sinus that runs around the perimeter of the upper bill. Depending on the size of the animal, amounts of between 0.5 and 2ml can be taken.

Aims of the thesis

The aims were to test the following hypotheses:

1: That there are differences between ulcerated and non ulcerated animals, and between animals in endemic and non-endemic areas of Tasmania. These differences can be split into the following categories.

a) Physical, gender and size

b) Genomic

c) Immunological

d) The presence of persistent anthropogenic chemical

2: That there are differences between Tasmanian and mainland platypuses based on:

a) Genomic characteristics

b) Immunological parameters

3: That there are differences between platypus-derived isolates of *M. amphibiorum*, and cane toad derived isolates based on:

a) Genomic characteristics

b) Pathogenicity and/or virulence

4: That the closely related fungus *Mucor circinelloides* is also involved in the disease, based on:

a) Genomic characteristsics

b) Pathogenicity and/or virulence

5: That *M. amphibiorum* has been introduced into Tasmania

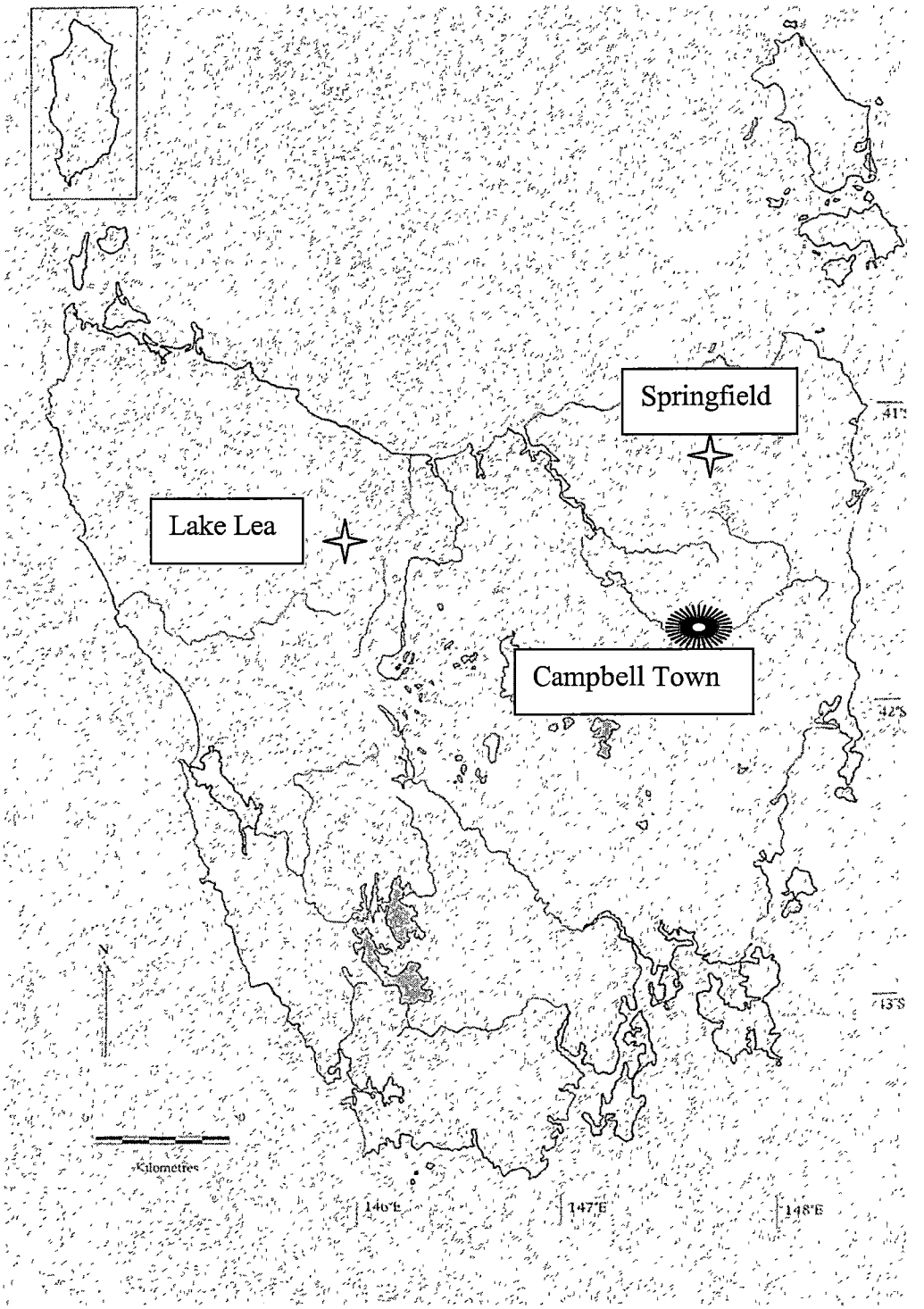


Figure 1: Map of Tasmania showing Campbell Town, the site of the first reported cases of ulcerative mycosis of platypuses in 1982. Also shown are Springfield and Lake Lea, areas in which ulcerated platypuses were captured in 2000

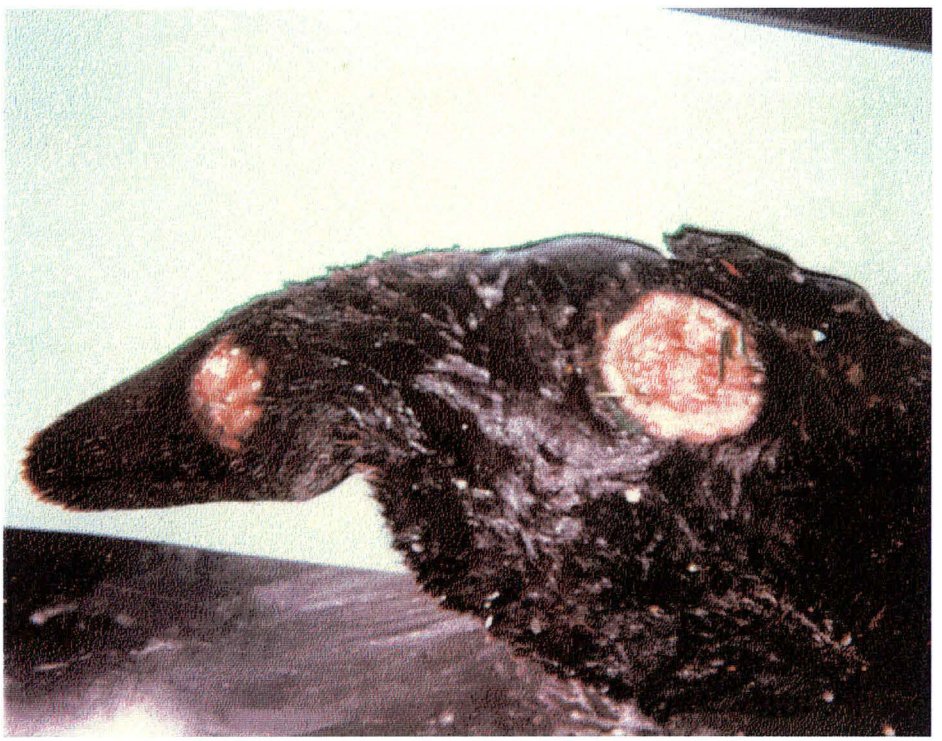


Figure 2: Ulceration on the back and tail of a male platypus captured at Campbell Town in 1982.

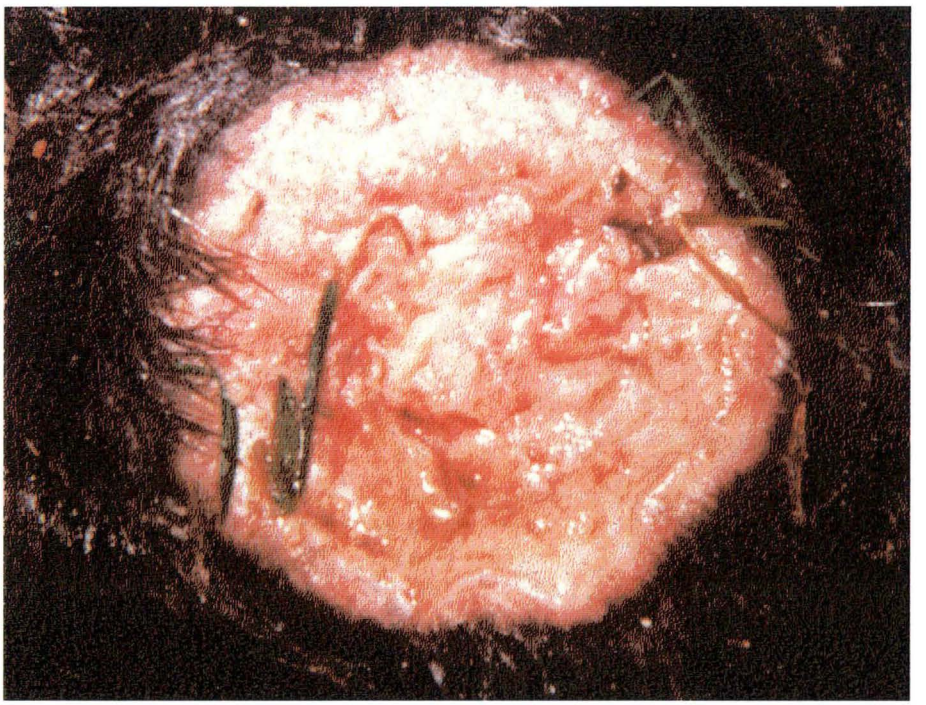
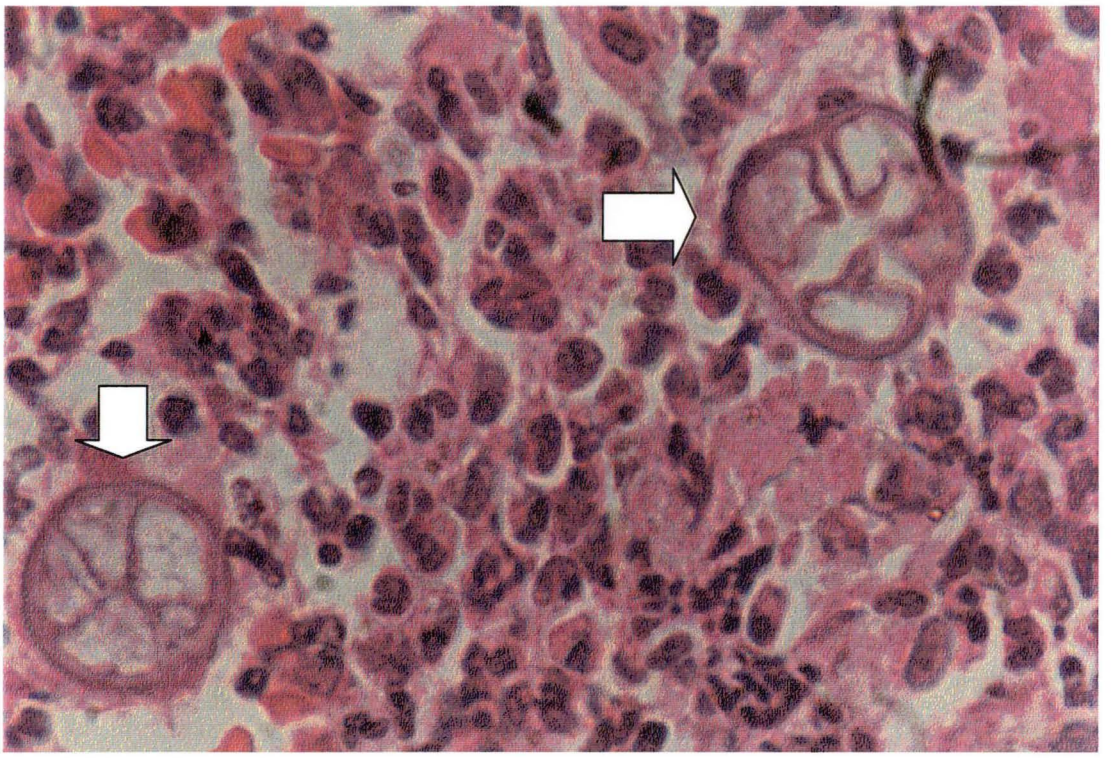
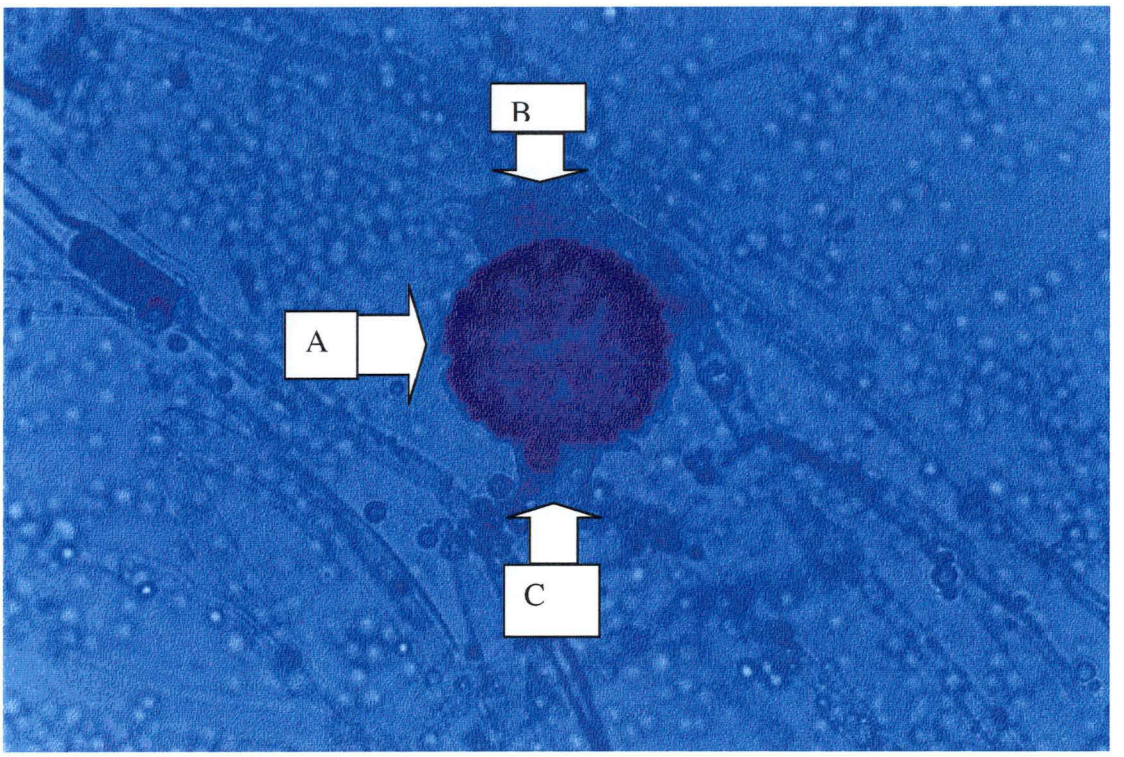


Figure 3: Close-up view of ulcer on the back of the platypus depicted in Figure 3.



Scale bar = 20um

Figure 4: Sphaerule-like bodies in H and E stained section of platypus skin



Scale bar = 50um

Figure 5: Zygospore (A) formed between *Mucor amphibiorum* (+) and (-) mating types. Letters (B) and (C) denote hyphae from each mating type which have fused and formed specialised suspensors holding the zygospore.

Chapter 1

A comparison of the internal transcribed spacer regions of seven isolates of *Mucor amphibiorum* and six isolates of *Mucor circinelloides*

1.1 Introduction

One of the hypotheses to explain the sudden appearance and apparent spread of the disease ulcerative mycosis is that the fungus responsible, *Mucor amphibiorum*, has been introduced into the island state of Tasmania, or an endemic strain of the fungus has mutated to a more pathogenic biovar. Determining whether this is the case is, however, difficult. Identification of fungi is based primarily on morphology. The fungus *M. amphibiorum* is identified initially by the width and shape of the sporangiophores, the diameter of sporangia, the shape and size of the columellae, the shape and size of the sporangiospores, and the shape and size of the zygospores (Schipper 1978). These morphological observations only allow for primary identification, as other fungal species can look similar to *M. amphibiorum*. For a positive identification, an isolate must be crossed with the two known mating types of the species. These types, designate (+) and (-), produce specialised spores known as zygospores, when crossed with their opposite mating type. The production of zygospores is species specific, thus ensuring complete accuracy in identification to species level.

Although identification to species level is relatively easy, determining the geographical origin of the isolate is not. In some species, morphology can differ slightly from isolate to isolate (Jackson *et al.* 1999), but not all isolates from a particular area will exhibit the same unique morphology. Also, the morphology and characteristics of some fungal species can be influenced by culture conditions such as incubation temperature, light regime and media used. The length of time that the isolate has been held in a collection, and the presence of bacteria in a culture, can also affect the morphology of some species (Summerbell and Kane 1997). Thus, even if some characteristics are noted which appear to be unique to isolates from a particular area, caution must be exercised in using the characteristics as markers.

One method which may be suitable for determining the geographical origin of an isolate involves the use of DNA sequencing. Of particular interest is the ribosomal DNA sequence comprising the 18S, 5.8S and 26S genes. These genes are highly conserved, and have been used for establishing coarse scale phylogeny of fungal species (Berbee *et al.* 1995). Of greater significance are the two internal transcribed spacer regions, one located between the 18S and 5.8S genes (ITS1), and the other between the 5.8S and 26S genes (ITS2). The amount of variation that occurs in gene sequences depends on the stringency of the functional constraints affecting the transcribed product. The ITS sequences are non coding, but they are not void of structural constraints. The moderate selective pressures on these sequences result in a significant level of variation between species (Goffinet and Bayer 1997). This variation has been used to establish

phylogenetic relationships between species, and to identify species (Paul *et al.* 1998, Sanders *et al.* 1995). Within species, as would be expected, there is less variation. However, the variation can be great enough for biovars of some fungal species to be classified according to their ITS sequences. For example, the fungus *Gaeumannomyces graminis*, a fungus responsible for disease in wheat, is found worldwide. Australian samples of the fungus are identical in their ITS1 and ITS2 regions, but differ by four nucleotides from United Kingdom samples. Also, the fungus is divided into two groups; one capable of infecting wheat, the other rye. It is possible to distinguish these groups based on ITS1/ITS2 sequencing (Bryan *et al.* 1994). Another phytopathogenic fungus, *Gibberella fujikuroi*, contains two distinct strains based on ITS2 sequencing, but which are identical morphologically (O'Donnell and Cigelnik 1997). Similarly, many isolates of the fungus *Erynia neoaphids* can be geographically grouped based on random amplified polymorphic DNA from the ITS sequences (Rohel *et al.* 1997). In some fungal species, virulence is also reflected in ITS sequences. The fungal plant pathogen *Leptosphaeria maculans* contains both highly and weakly virulent strains that are morphologically identical. The two strains can be separated according to ITS1 sequences (Xue *et al.* 1992).

There are different methods that can be employed to analyse the ITS sequences. The simplest method is to count the nucleotides that comprise the ITS sequences. This method has been used to identify three different strains of *Erynia neoaphidid*, a pathogen of aphids (Rohel *et al.* 1997). Two of the strains were found in several countries, but one was unique to France. Another strain of the fungus, which has a non-aphid host, had a significantly shorter ITS sequence than any of the other isolates. Although crude, this method can produce useful comparisons.

Some researchers produce restriction maps of the ITS sequences and the 5.8S gene. These maps are based on restriction fragment length polymorphisms (RFLP). Basically, the ITS1-5.8S-ITS2 region is amplified using polymerase chain reaction (PCR), then the product is digested using a restriction endonuclease such as *MVA*1. The products are then run on agarose gels. If the ITS1-5.8S-ITS2 region differs between isolates at the sites cut by the endonuclease, then the restriction endonuclease cuts the DNA in different areas, resulting in different lengths of DNA. Of course, this particular method relies on the isolates differing in the areas where the restriction endonuclease cuts. If the isolates are identical in these areas, then no difference will be observed between isolates, even if they differ in areas not recognised by the endonuclease. This method has been successfully used to differentiate between isolates of *Trichophyton rubrum*, a dermatophyte causing a variety of mycoses (Jackson *et al.* 1999). The broad grouping associated with this method does not seem to discriminate on geographical grounds,

with many isolates with the same RFLP profile coming from several different countries. Of interest in the study was the preponderance of a strain type designated A, which represented approximately one third of the isolates. This strain was found in five different countries. The high percentage of the strain suggests that virulence and/or pathogenicity may be detected in the ITS sequences, even if the sequences themselves play no part in the disease process. Although more refined than simple ITS length determination, this method suffers from the inability of restriction endonucleases to recognise all areas of nucleotide differences between samples.

The most refined method for ITS sequence analysis involves determining the entire nucleotide sequence of the ITS regions and the 5.8S gene, which normally comprises around 500 to 700 nucleotides This method has the advantage of allowing direct nucleotide comparison between samples, so that differences of only one nucleotide can be detected. The sensitivity of this technique has allowed for ITS genotypes to be described in several species, with particular genotypes being restricted to particular countries (Mathis *et al.* 1999), and it has been used to show species specificity of some isolates of the parasite *Encephalitozoon cuniculi* (Mathis *et al.* 1996).

The drawback is that the method requires expensive equipment and reagents, so that analysis of large sample numbers can be a costly business. Despite this, it is becoming increasingly popular, especially in the area of phylogeny, where it can be used to determine the exact relationships between and within fungal species (Berbee *et al.* 1995, Lloyd-Macgilp *et al.* 1996).

1.2 Materials and methods

1.2.1 Details of isolates sequenced

Seven isolates of *Mucor amphibiorum* were obtained. Four were from platypuses, two from cane toads and one from a slender tree frog from Western Australia (Table 1.1). Four isolates of *Mucor circinelloides* were also obtained, one from a platypus, and three from slender tree frogs from Western Australia (Table 1.2). Two other isolates, WA5 and TAS6, were originally identified as *M. amphibiorum* , but were later identified as *M. circinelloides* after DNA analysis. All cultures were grown for five days at 25°C on Sabouraud's agar, checked for contamination and, in the case of *M. amphibiorum* isolates, crossed with tester strains to confirm mating type. The cultures were frozen at -80°C until required.

1.2.2 Extraction of DNA

A small amount of material (the size of a match head) was teased from each culture and placed in a 1.5ml eppendorf tube with 0.5mm glass beads. The tissue was ground by vortexing the eppendorf, to which 200µl of 2xCTAB (hexadecyltrimethylammonium bromide had been added, for 10 seconds. A further 500µl of 2xCTAB was added, and grinding continued for a further 10 seconds. Then 5µl of proteinase-K (20mg/ml) was added, and the tube briefly vortexed. The tubes were mixed and incubated at 65°C for one hour before 600µl of chloroform -isoamyl was added. The tubes were shaken and centrifuged for 20 minutes at 14,000 rpm. Fresh tubes were prepared with 600µl of phenol-chloroform-isoamyl, and the supernatant added. The tubes were well mixed and then centrifuged for 10 minutes at 14,000 rpm. The supernatant was removed and added to fresh tubes with 600µl of chloroform-isoamyl, shaken well and centrifuged for 60 seconds at 14,000 rpm. The supernatant was added to fresh tubes with cold (-20°C) isopropanol and mixed gently. The tubes were placed in a -20°C freezer overnight and centrifuged at 4°C for 30 minutes at 10,000 rpm. The supernatant was decanted and discarded, and 1ml of cold (-20°C) ethanol added to the pellet. The tube was well mixed and centrifuged for 10 minutes at 10,000 rpm at 4°C. The ethanol was decanted, and the tube placed in a vacuum centrifuge for 1 hour (until dry). The pellet was rehydrated in 200µl of Milli-Q water, and stored at 4°C.

1.2.3 PCR protocol

To each Micro Amp reaction tube (Perkin Elmer Cetus Pty Ltd) was added 6µl of a $MgCl_2$ solution, 5µl 10x PCR buffer II (Perkin Elmer), 1µl dNtP's (10mM, Promega), 2µl primer (forward), 2µl primer (reverse), 0.2µl AmpliTaq Gold (5µl, Perkin Elmer), DNA from sample and Milli-Q water to give a final volume of 50µl).

All reagents were removed from the freezer immediately prior to use and placed on ice.The stock solution, comprising all reagents except the fungal DNA, was vortexed and centrifuged prior to use. The fungal DNA was flick-mixed and centrifuged before adding to the PCR tubes.

1.2.4 PCR cycling conditions

The PCR cycling was performed in in a Perkin Elmer GeneAmp PCR System 9700 Thermal Cycler.

1:	Ten minutes at 95°C	
2:	Thirty cycles of:	94°C for 1 minute
		50°C for 30 seconds
		72°C for 1 minute 30 seconds
3:	One cycle of:	94°C for 1 minute
		50°C for 30 seconds
		72°C for 7 minutes

The product was then held at 4°C

1.2.5 Agarose Gel

The agarose gel was prepared by adding 1gm agarose (ultrPURE, Life Technologies) to 100ml TBE (108g Tris, 55g boric acid, 7.44g EDTA, pH 8.2-8.5) in a conical flask. The mouth of the flask was sealed, and boiled in a microwave until the crystals dissolved. The flask was allowed to cool to 50°C before 3µl of ethidium was added and mixed.

1.2.6 Gel loading

The bp ladder was prepared by pipetting 5µl of 2x Novex (1:3 dilution of 6xTBE sample buffer, Novex, with Milli-Q H_2O), onto a piece of paraffin film and adding 0.5µl bp ladder [100bp (Base Pair) Ladder (1µg/µl, Life Technologies)] and 4.5µl Milli-Q H_2O. This was loaded into the first well of the gel. For each of the PCR products, 5µl of 2x Novex was added to paraffin film with 5µl of the PCR product, and mixed by pipetting up and down before loading into the gel. The loaded gel was run at 150V and 150A for 43 minutes.

1.2.6.1 Photographing the gel

The gel was removed from the running tank and rinsed under deionised water. It was placed on a tray and taken to a darkroom where the gel was slid onto an ultraviolet (UV) transilluminator. The UV light was turned on, the camera aligned and focused. A black and white Polaroid 57 film was loaded, and the film exposed for 2 seconds.

1.2.7 Preparation of PCR products for sequencing using the QIAquick method (Quiagen, USA)

Five volumes of Buffer PB (approximately 225µl) was added to 1 volume of the PCR product and mixed. A QIAquick spin column was placed in a 2mL collection tube and the sample added before centrifuging for 1 minute. The flow through was discarded and the QIAquick column placed back in the same tube. The product was washed by adding 0.75mL Buffer PE to the QIAquick column and centrifuging for 1 minute. The flow through was discarded and the QIAquick column placed back in the same tube, and centrifuged for a further 1 minute. The column was placed in a 1.5mL eppendorf tube. The product was eluted by adding 20µl of elution buffer, centrifuging for 1 minute, adding a further 10µl of elution buffer and spinning for a further 1 minute. The product was stored at -20°C until required.

1.2.8 ABI Prism Big Dye Terminator Cycle Sequencing

The primer was diluted by adding 6.8ul of Milli-Q H_2O to 3.2ul of primer. The cycle sequencing reaction was prepared in a new 0.2ml MicroAmp Reaction tube. The tubes were vortexed and centrifuged at 14,000rpm before being added to a Perkin Elmer GeneAmp PCR System 9700 Thermal Cycler, and processed as follows.

Thirty cycles of:	96°C for 10 seconds
	50°C for 5 seconds
	60°C for 4 minutes

1.2.9 Purifying extension products

2ul of 3M sodium acetate and 50ul of 95% ethanol were added to a 1.5ml eppendorf tube. 20ul of the sequencing reaction was added to the eppendorf and vortexed before being placed on ice for 10 minutes. The tube was centrifuged for 30 minutes at 14,000 rpm, and the supernatant was aspirated and discarded. The pellet was rinsed with 250 ul of 75% ethanol and briefly vortexed before centrifuging at 14,000 rpm for 5 minutes. The supernatant was aspirated and discarded. The pellet was dried for 15 minutes in a vacuum centrifuge. The tube was then wrapped in aluminium foil and kept in a -80°C freezer until sequencing.

1.2.10 Quantification of DNA in PCR products

The DNA content of each sample was determined fluorometrically, using calf thymus as the standard.

1.3 Results

1.3.1 DNA extraction

DNA was successfully extracted from all isolates of *M. amphibiorum* and *M. circinelloides*. The PCR amplification resulted in DNA yields of between 83 and 129 ng/ul.

1.3.2 Gel photography

To check the quality of genomic DNAs, PCR reactions were run on agarose gels and photographed. Each sample produced a single DNA band with an approximate molecular weight of 0.7kb (Figure 1.1).

1.3.3 DNA sequencing

All isolates were successfully sequenced and aligned (Figures 1.2, 1.3). Isolates of *M. circinelloides* had sequences of 615 bp, and *M. amphibiorum* sequences comprised 593 bp

1.3.3.1 *Mucor amphibiorum*

The four Tasmanian isolates of *M. amphibiorum* were identical, and differed by two nucleotides at positions 195 and 434 from the West Australian (+) mating type and the Queensland (+) mating type. The Tasmanian isolates differed by ten nucleotides from the Queensland (-) mating type at positions 195, 434, 577, 578, 580, 581, 584, 585, 586 and 587 (Figure 1.2, Table 1.3). The West Australian isolate differed from the Queensland isolate by 8 nucleotides at positions 577, 578, 580, 581, 584, 585, 586 and 587 (Figure 1.2, Table 1.3).

1.3.3.2 *Mucor circinelloides*

There were 13 positions at which isolates of *M. circinelloides* differed from each other; 90, 113, 130, 139, 154, 304, 407, 433, 498, 538, 540, 572 and 598 (Fig 1.3, table 1.4).

Mucor amphibiorum and *M. circinelloides* did not share common positions for differences between isolates. The majority of differences between *M. amphibiorum* isolates occurred between positions 577 and 587. Differences between isolates of *M. circinelloides* did not occur in one block, but were evenly spread throughout the sequence.

Isolate	Cultured from:	Location	Year	Mating type
Tas1	platypus	Tasmania	1996	+
Tas2	platypus	Tasmania	1999	+
Tas3	platypus	Tasmania	1994	+
Tas4	platypus	Tasmania	1994	+
Q1	cane toad	Queensland	1994	-
Q2	cane toad	Queensland	1994	+
WA1	tree frog	W. Australia	1998	+

Table 1.1: Details of isolates of *Mucor amphibiorum* used for ITS1 and ITS2 DNA sequencing

Isolate	Cultured from:	Location	Year
WA2	frog sp. unknown	W. Australia	1998
WA3	green tree frog	W. Australia	1998
WA4	green tree frog	W. Australia	1998
WA5	*slender tree frog*	*W. Australia*	*1998*
Tas5	platypus	Tasmania	1996
Tas6	*platypus*	*Tasmania*	*1994*

Table 1.2: Details of isolates of *Mucor circinelloides* used for ITS1 and ITS2 DNA sequencing. Italicised isolates represent the two isolates originally identified as *M. amphibiorum*

	TAS1	TAS2	TAS3	TAS4	Q1	Q2	WA!
TAS1	0	0	0	0	10	2	2
TAS2	0	0	0	0	10	2	2
TAS3	0	0	0	0	10	2	2
TAS4	0	0	0	0	10	2	2
Q1	10	10	10	10	10	8	8
Q2	2	2	2	2	8	0	0
WA1	2	2	2	2	8	0	0

Table 1.3: The number of nucleotides by which different isolates of *M. amphibiorum* differ from each other in their ITS sequences

	WA2	WA3	WA4	WA5	TAS5	TAS6
WA2	0	11	10	4	13	13
WA3	11	0	5	13	4	7
WA4	10	5	0	10	4	5
WA5	4	13	10	0	13	14
TAS5	13	4	7	14	0	3
TAS6	13	4	4	13	0	3

Table 1.4 The number of nucleotides by which different isolates of *M. circinelloides* differ from each other in their ITS sequences

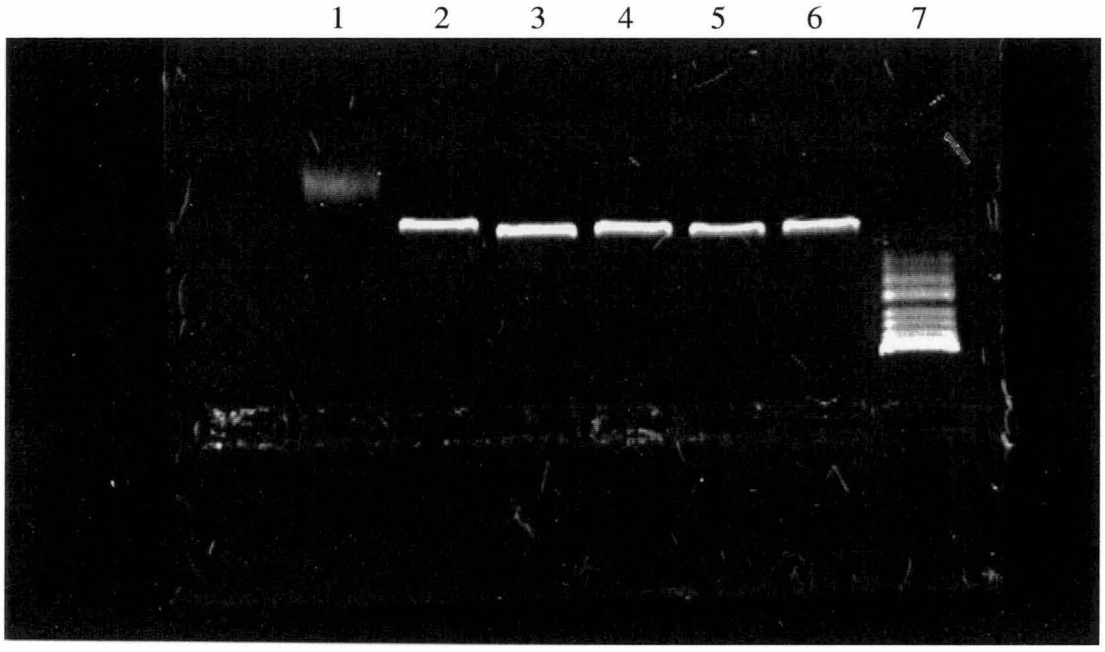


Figure 1.1: Photograph of agarose gel run with amplification products from selected isolates of *M. amphibiorum* and *M. circinelloides*. Lane 1 is a negative control, Lane 2 is Tas1, Lane 3 Q1, Lane 4 WA1 and Lane 5 Tas5. Lane 6 is a positive control and Lane 7 is a molecular weight marker. The products had a molecular weight of approximately 0.7kb.

Figure 1.2: The nucleotide sequence of the ITS1 and ITS2 regions of seven isolates of *Mucor amphibiorum*. (Sequence shown for Tas1; nucleotides for other isolates are only shown where they differ from this isolate).

```
                10          20          30          40          50
Tas1    TCCGTAGGTG AACCTGCGCA AGGATCATTA AATAATTCGA TAATTCCAAA
Tas2
Tas3
Tas4
Q1
Q2
WA1

                60          70          80          90          100
Tas1    ATTATCTTAT TTACTGTGAA CTGTTTTAAT TATGGCACAT AAGGGGATGA
Tas2
Tas3
Tas4
Q1
Q2
WA1

               110          120         130         140         150
Tas1    CTGTATACCA TAAGGGTAGG TATATAGAAT GTTAACCTAG CCATAGTCAA
Tas2
Tas3
Tas4
Q1
Q2
WA1

               160          170         180         190         200
Tas1    GCTTGATGCT TGGTACCCTA TTATTATTTA CCAAAGAAT TCAGATTAAA
Tas2
Tas3
Tas4
Q1                                                      T
Q2                                                      T
WA1                                                     T

               210          220         230         240         250
Tas1    TATTGTAACA TAGATCTAAA CAATCTATAA AACAACTTTT AACAATGGAT
Tas2
Tas3
Tas4
Q1
Q2
WA1

               260          270         280         290         300
Tas1    CTCTTGGTTC TCGCATCGAT GAAGAACGTA GCAAAGTGCG ATAACTAGTG
Tas2
Tas3
Tas4
Q1
Q2
WA1
```

```
                310         320         330         340         350
Tas1    TGAATTGCAT ATTCAGTGAA TCATCGAGTC TTTGAACGCA ACTTGCACTC
Tas2
Tas3
Tas4
Q1
Q2
WA1

                360         370         380         390         400
Tas1    ATTGGTATTC CAATGAGTAC GCCTGTTTCA GTATCAAAAA CATCCCTTAT
Tas2
Tas3
Tas4
Q1
Q2
WA1

                410         420         430         440         450
Tas1    TCAAACATTT TTGTTTGAAT AGACTTGAGT GTAGCGAGTT TCGAGACACT
Tas2
Tas3
Tas4
Q1                                          A
Q2                                          A
WA1                                         A

                460         470         480         490         500
Tas1    TTAAATATAC TAAGGCCTGA TTTGTTTCAC TGCCTATATT TTTTTTAAAT
Tas2
Tas3
Tas4
Q1
Q2
WA1

                510         520         530         540         550
Tas1    TAAAAAAGAT AGAAGCGAAT GAAGCTGTGT TAGGGCCTCC CAAAAAAACT
Tas2
Tas3
Tas4
Q1
Q2
WA1

                560         570         580         590
Tas1    TTTAAACTTG ATCTGAAATC AGGTGGGATT ACCCGCTGAA CTT
Tas2
Tas3
Tas4
Q1                                  AT A  GCTG
Q2
WA1
```

Figure 1.3: The nucleotide sequence of the ITS1 and ITS2 regions of six isolates of *Mucor circinelloides*. (Sequence shown for WA2; nucleotides for other isolates are only shown where they differ from this isolate).

```
                10          20          30          40          50
WA2   TCCGTAGGTG AACCTGCGGA AGGATCATTA AATAATCAAT AATTTTGGCT
WA3
WA4
WA5
Tas5
Tas6

                60          70          80          90          100
WA2   TGTCCATTAT TATCTATTTA CTGTGAACTG TATTTATTATT TGACGCTTGA
WA3                                   A           C
WA4                                   C           C
WA5                                   C
Tas5                                  A           C
Tas6                                  C           C

               110         120         130         140         150
WA2   GGGATGCTCC ACCGCTATAA GGATAGGCGA TGGGGATGTT AACCGAGTCA
WA3                 T                 G           C
WA4                 T                 G
WA5
Tas5                T                 G           C
Tas6                T                 G

               160         170         180         190         200
WA2   TAATCAAGCT TAGGCTTGGT ATCCTATTAT TATTTACCAA AAGAATTCAG
WA3
WA4
WA5
Tas5      A
Tas6      A

               210         220         230         240         250
WA2   AATTAATATT GTAACATAGA CCTAAAAAAT CTATAAAACA ACTTTTAACA
WA3
WA4
WA5
Tas5
Tas6

               260         270         280         290         300
WA2   ACGGATCTCT TGGTTCTCGC ATCGATGAAG AACGTAGCAA AGTGCGATAA
WA3
WA4
WA5
Tas5
Tas6

               310         320         330         340         350
WA2   CTAATGTGAA TTGCATATTC AGTGAATCAT CGAGTCTTTG AACGCAACTT
WA3
WA4       G
WA5       T
Tas5      C
```

```
Tas6        C

                360         370         380         390         400
WA2   GCGCTCATTG GTATTCCAAT GAGCACGCCT GTTTCAGTAT CAAAACAAAC
WA3
WA4
WA5
Tas5
Tas6

                410         420         430         440         450
WA2   CCTCTATCCA ACATTTTTGT TGAATAGGAA TATTGAGAGT CTCTTGATCT
WA3                                       C
WA4                                       C
WA5
Tas5                                      C
Tas6            T                         C

                460         470         480         490         500
WA2   ATTCTGATCT CGAACCTCTT GAAATGTACA AAGGCCTGAT CTTGTTTAAA
WA3                                                       G
WA4   T                                                   G
WA5   T                                                   T
Tas5  C                                                   G
Tas6  C                                                   G

                510         520         530         540         550
WA2   TGCCTGAACT TTTTTTTAAT ATAAAGAGAA GCTCTTGTGA TAAACTGTGC
WA3                                           C  G
WA4                                           C  G
WA5
Tas5                                          C  G
Tas6                                          C  G

                560         570         580         590         600
WA2   TGGGGCCTCC CAAATAATAC TCTTTTTAAA TTTGATCTGA AATCAGGTGG
WA3                       T                               C
WA4                                                       C
WA5                       A
Tas5                                                      C
Tas6                                                      C

                610
Wa2   ATTACCCGCT GAACT
WA3
WA4
WA5
Tas5
Tas6
```

1.4 Discussion

The sequencing of the ITS regions of *M. amphibiorum* and *M. circinelloides* has demonstrated that these two species vary greatly from one another, and can be identified using such sequencing. As discussed in the following paragraph, the sequencing supports the hypothesis that *M. circinelloides* represents an environmental contaminant, and plays no role in the disease ulcerative mycosis. There was little difference between (+) mating types of *M. amphibiorum*, with the Tasmanian isolates being identical, and only varying by two nucleotides from the West Australian and Queensland isolates. The greatest difference was noted between the Queensland (-) mating type and the other (+) isolates. The lack of variation in the sequences for *M. amphibiorum* supports the argument that this fungus is a true pathogen, and not an opportunistic fungal pathogen. As discussed later, opportunistic fungi tend to display great heterogenicity in ITS sequences.

The high variability in the sequences of the isolates of *M. circinelloides* isolated from the amphibians in West Australia further supports the theory that this species is a contaminant when isolated from ulcerated platypuses. It seems unlikely that, if this species was truly pathogenic, there would be so many different isolates present in the one outbreak of disease. Saprobic fungi that occasionally cause disease in a compromised host show high genetic diversity in the ITS regions. For example, the fungus *Aspergillus fumigatus* is a common saprobe that can cause disease in susceptible hosts, such as those with cystic fibrosis. In such cases, many different isolates can be isolated from the one host, and these isolates do not differ from environmental isolates (Debeaupuis *et al.* 1997). If *M. circinelloides* was acting in such a fashion, then numerous isolates would be expected to be cultured from each ulcerated animal. The isolation of different isolates from different species in West Australia suggests contamination is a more likely explanation than a compromised host.

That *M. circinelloides* is a contaminant is further supported by the results for WA5 and TAS6, both of which were originally identified as *M. amphibiorum* in mating trials. Subsequently, each isolate had been subcultured at least six times prior to DNA sequencing. Directly before the sequencing, neither had reacted positively when crossed in a further mating test. It was decided to continue with the sequencing, as the results could help to determine whether the isolates were still *M. amphibiorum*, or whether the isolates had become contaminated by another species. It was suspected that the cultures may have contained spores of *M. circinelloides* at the time of original isolation, and the sequencing data supports the premise that *M. circinelloides* was present in both samples at that time. All subculturings were carried out in a laminar flow which was regularly

cleaned, and UV light treated prior to use. It seemed unlikely that it could become contaminated by *M. circinelloides*, however, it was possible that contamination had occurred during subculturings of isolates of *M. circinelloides*. If this was the case, then the sequences of the contaminating fungi would be identical to one of the sequences of the other isolates of *M. circinelloides* subcultured in the laminar flow. This was not the case, with the two isolates WA5 and Tas6 differing by 13 nucleotides. The isolate WA5, from West Australia, only differed by four nucleotides from one other West Australian isolate, but by 14 from the other Tasmanian isolate of *M. circinelloides*. The Tasmanian isolate Tas6 only differed by three nucleotides from the other Tasmanian isolate.Thus the Tasmanian isolates were likely to have been more closely related to each other than to the West Australian isolates. This suggests that both WA5 and TAS6 were contaminated by local strains of *M. circinelloides* common to Western Australia and Tasmania respectively at the time of isolation, and do not represent cross-contamination by one of the other isolates of *M. circinelloides*.

This contamination of *M. amphibiorum* isolates by the ubiquitous environmental fungus *M. circinelloides* further supports the argument that this fungus is not implicated in the disease ulcerative mycosis. The outbreak of disease in amphibians in West Australia showed classic sphaerule-like bodies in the organs of the frogs, and *M. amphibiorum* was isolated from many of the animals. As the frogs were collected dead, from the wild, it is likely that many of them were contaminated by *M. circinelloides*. Despite careful isolating and subculturing of samples, it is still possible for contamination to occur. This appears to have occurred with the three frogs from which *M. circinelloides* was isolated. In the case of isolates that originally reacted positively with (-) mating strains of *M. amphibiorum*, and then subsequently didn't after several subculturings, it seems likely that some spores of *M. circinelloides* were present at the time of original isolation. Over the course of storage, conditions have favoured the *M. circinelloides*, and it has dominated the culture.

In contrast to the high variability present in *M. circinelloides*, the isolates of *M. amphibiorum* differed little in their ITS sequences. All of the Tasmanian isolates sequenced were identical, regardless of location or year in which the animal was caught. The isolates Tas3 and Tas4 were from animals captured at Cressy in 1994, whereas Tas1 was from an animal captured at Glengarry (approximately 60 kilometres away) in 1996, and Tas2 from an animal near Cressy in 1999. All of these isolates were (+) mating types, and differed by only two nucleotides from the other two (+) mating types. These latter two isolates, one from a cane toad captured in Queensland in 1994, the other from a green tree frog in West Australia in 1998, were identical. Interestingly, the (-) mating type, isolated from a cane toad in Queensland in 1994, differed by 8

nucleotides from the other mainland isolates, and by ten nucleotides from the Tasmanian isolates.

There are several possible explanations for the differences between (+) and (-) mating types. It may be that mating type is reflected in the ITS sequence, specifically in the nucleotides located between bp577 and bp587, where all of the differences between WA1, Q2 and Q1 occurred. It was also in this area where eight of the ten differences between the Tasmanian isolates and Q1 occurred. Unfortunately, the literature makes no mention of mating type and ITS sequences. A much larger sample of both (+) and (-) mating types would be required to resolve this point.

Another possibility is that pathogenicity and/or virulence is reflected in, but not caused by, differences in the ITS sequence. In general, the groupings based on ITS sequences reflected the groupings found in pathogenicity trials (chapter two). In those trials, cane toads infected with Tasmanian isolates resulted in significantly higher numbers of sphaerule-like bodies (slb) in liver sections than Q2, WA1 or Q1, and Q2 and WA1 had approximately five times more slb than Q1. Thus, results from the pathogenicity trials are reflected in part in ITS sequences.Differences in pathogenicity and/or virulence between strains of other fungal pathogens are not uncommon, and these differences are sometimes reflected in ITS sequences (Xue *et al.* 1992, Askary *et al.* 1998).

The data support the hypothesis that *M. amphibiorum* represents a true pathogen, and not an opportunistic saprobic environmental fungus. Opportunistic saprobic fungi result in an affected host carrying numerous strains, based on ITS sequencing, of the fungus (Debeaupuis *et al.* 1997), whereas truly pathogenic fungi tend to have less variability in their ITS sequences, and usually only one strain is isolated from one host (Bunting *et al.* 1995, Silva *et al.* 1995). The degree of variability in opportunistic fungi is demonstrated by the 429 different strains of *Aspergillus fumigatus* that have been isolated from hospital environments, all of which are capable of causing disease in compromised hosts (Reiss *et al.* 1998).

The similarity in sequences between the isolates of *M. amphibiorum* may be explained in several ways. It is possible that this species has little variability in these regions. Conversely, the lack of variation may indicate that the species was introduced into Australia, and that only a limited number of sequence types were introduced. Cane toads are regularly infected by *M. amphibiorum*, but do not seem to suffer the same high mortality as other amphibian species. This may be indicate that cane toads have developed with the fungus. It is possible that *M. amphibiorum* was introduced into Australia in cane toads in 1935, when this species was brought to Australia to help

control pests in cane fields (Barton 1997). This seems unlikely, given that mainland platypuses are unaffected by the fungus. If the introduced isolates of *M. amphibiorum* were pathogenic to platypuses, then infection in mainland platypuses could be expected.

The identical sequences in Tasmanian isolates does support the hypothesis that this isolate has been introduced into the state. Although only four isolates were sequenced, they were taken from animals in different areas at different times. Although it cannot be stated with certainty, it seems likely that all of these isolates share a common ancestor introduced into the state in the 1980's. The most probable method of introduction was in green tree frogs from Queensland. These frogs are known to be susceptible to the fungus, and are regularly found in Tasmania in bunches of bananas from Queensland. Although cane toads in Queensland are known to be infected by *M. amphibiorum* (Speare *et al.* 1994), they are unlikely to have been responsible for the introduction of the fungus into Tasmania. Cane toads are large, ground dwelling amphibians and are not found in fruit consignments.

Another possibility is that the Tasmanian isolates represent a strain of the fungus that has mutated. There may be many strains of *M. amphibiorum* non pathogenic to platypuses in Tasmania. The identical sequences seen in Tasmanian isolates would then be reflecting the isolation of only one strain of mutated *M. amphibiorum* from platypuses.

In conclusion, ITS sequencing is a reliable method for distinguishing between isolates of *M. amphibiorum* and *M. circinelloides*. The high variability between isolates of *M. circinelloides* from the same disease outbreak in West Australia, and the contamination of two cultures by this fungus, suggests that its isolation from ulcerated platypuses represents an accidental isolation of a ubiquitous fungus. It seems unlikely that tracing the geographic origins of isolates of *M. amphibiorum* will be possible using ITS sequencing, given that an isolate from Queensland was identical to one from West Australia. Of course, it is possible that the West Australian isolate arrived in that state from Queensland. Although the sample number is low, at present the isolates can be divided in to three groups, based on sequencing data. Group one comprises Tasmanian (+) mating types, which are capable of causing long-lasting disease in cane toads. Group two comprises (+) mating types with the sequences found in the West Australian and Queensland isolates, and which cause less persistent disease in cane toads, and Group three comprises the Queensland (-) mating type which apparently results in no long term disease in cane toads. This categorisation of the isolates is supported by the pathogenicity trials discussed in Chapter 2.

Chapter 2

Possible differences in pathogenicity between cane toad, frog and platypus derived isolates of *Mucor amphibiorum*, and frog and platypus derived isolates of *Mucor circinelloides*

2.1 Introduction

A possible explanation for the presence of ulcerative mycosis in Tasmanian, but not mainland platypuses, is that the strain of *M. amphibiorum* found in Tasmania is either more pathogenic or virulent than its mainland counterparts. Virulence and pathogenicity can vary markedly within fungal species, with some species containing both pathogenic and non pathogenic strains. It has also been hypothesised that *M. amphibiorum* is not the only fungus capable of causing ulcerative mycosis in the platypus. The closely related fungus *Mucor circinelloides* is capable of producing sphaerule-like bodies (slb) in *in vitro* experiments, and this fungus has also been isolated from the ulcer of an infected animal (Stewart *et al.* 1999). When amphibians were first reported as being infected by a dimorphic *Mucor* species, it was noted that the species responsible closely resembled *M. circinelloides* (Frank et al 1974).

Determining the pathogenicity of a fungal species is complicated. By necessity, a fungal culture grown under laboratory conditions differs from one growing in host tissues. Because pure cultures are required in the laboratory, isolates are grown on media that support growth of the desired species. Repeated sub culturing of such isolates can result in changes in growth rates, morphology and pathogenicity (Samsinakova and Kalalova 1983). Also, in the natural environment, species can interact, with competition resulting in different growth profiles in comparison to laboratory monocultures. Thus, any experimental infection of an animal with a cultured fungal pathogen will only approximate natural conditions.

When dealing with human pathogens, murine models are often used to determine the effects of the pathogen on mammalian species. Although there are differences between murine and human immune responses, in general these models are robust, and pathogenicity demonstrated in mice will often be reflected in humans. When dealing with platypus, two specific problems arise. It is not possible to use the platypus itself as an experimental model. Keeping sufficient platypus in captivity for such an experiment would be prohibitively expensive, and it's likely that such a model would founder before it started, given the propensity for platypus to suffer from stress when kept in captivity (Whittington 1991; 1993). As most fungi are opportunistic, the ability of an isolate to cause disease in a captive, stressed animal would more likely reflect the poor state of health of the animal, rather than pathogenicity of the fungus. Secondly, it would not be publicly acceptable to experimentally infect platypuses with an incurable mycosis.

If the platypus was similar to other mammals, a murine model would be acceptable for determining the pathogenicity of different *Mucor amphibiorum* isolates. However, the platypus has a body temperature of 32°C, which in part explains its susceptibility to infection by *M. amphibiorum*. Most other mammals are probably unaffected by *M. amphibiorum* because their body temperature is high enough to prevent growth of the fungus. The inability of most strains of the fungus to grow at temperatures above 36°C precludes the use of murine models for pathogenicity trials. Frank *et al.* (1974) tried a range of mammalian hosts for experimental infection without success.

Fortunately, an experimental model based on amphibians was considered feasible. The fungus is known to affect many amphibian species, and differences in pathogenicity should be reflected in differences in severity of disease in amphibians. Although Australian native frogs are known to become infected by the fungus, they were not considered suitable for pathogenicity trials for several reasons. Collecting enough frogs for a trial would be difficult. Also, the exact nutritional and general maintenance requirements of many native Tasmanian species is not known. Finally, infection by *M. amphibiorum* causes rapid mortality in many native species.

It has been known for some time that free ranging cane toads can be infected with *M. amphibiorum*, with 0.71% of a large sample of the wild population of cane toads in Queensland being infected (Speare *et al.* 1994). The infection does not cause rapid death in infected individuals, but the severity of the disease can be determined by examining pathological changes in major organs.

The fungus can infect the liver, spleen, kidneys, urinary bladder, heart, lungs, subcutaneous lymph spaces, skin, gastrointestinal tract, voluntary muscle, bone, cranial cavity and oral cavity. In infected wild toads, the liver is always implicated, with approximately half the animals also exhibiting granulomas in the heart (Speare *et al.* 1997).

The cane toad was considered a suitable model for the trial, as it was susceptible to the disease, did not suffer rapid mortality, and was available in large numbers for a relatively small price. Also, the care and maintenance of cane toads is easy and inexpensive. Three major organs, the liver, heart and lungs, were selected as the organs to be examined for infection.

2.2 Materials and methods

The trial was split into two parts. Firstly, a "sighter" trial was performed to determine an appropriate non-lethal dose of spores that would result in pathological changes in toad organs. The second experiment involved infecting 5 groups of 10 toads with different isolates of *M. amphibiorum*, and one of *M. circinelloides*.

2.2.1 Preparation of inocula

The isolates of *M. amphibiorum* had been stored in distilled water, and periodically re-cultured, tested for purity, and returned to distilled water. The isolates were taken from storage and grown on fresh Sabouraud's agar with added antibiotic. Once they had reacted positively in a mating reaction with their mating-type opposite, and were found to be bacteria-free, they were each sub-cultured to five Sabouraud's agar plates containing no antibiotic. The cultures were grown for five days at 25°C. Approximately 30 ml of sterile distilled water was added to each plate. The cultures were gently agitated with a glass pipette to rupture the sporangia and release the sporangiospores. The resulting fluid was removed and placed in a centrifuge tube, and the spores counted using a haemocytometer. The spores were washed and centrifuged three times in sterile distilled water at 1000g, and then resuspended in physiological saline at four concentrations; $1x10^6$ spores/ml, $5x10^6$ spores/ml, $1x10^7$ spores/ml and $5x10^7$ spores/ml. For each experiment, spores were harvested on the day prior to the inoculation of the cane toads. The isolate of *M. circinelloides* had been stored and recovered as per the other isolates, and its identity confirmed using the key of Schipper (1978).

2.2.2 Description of fungal isolates used

The five isolates of *M. amphibiorum* were designated Q1, Q2, Tas1, Tas2 and WA1, and the isolate of *M. circinelloides* Tas5 (Table 2.1).

Isolate	Species	Source	Location	Mating type	Year
Q1	*M. amphibiorum*	Cane toad	Queensland	(-)	1994
Q2	*M. amphibiorum*	Cane toad	Queensland	(+)	1994
Tas1	*M. amphibiorum*	Platypus	Tasmania	(+)	1996
Tas2	*M. amphibiorum*	Platypus	Tasmania	(+)	1999
Tas5	*M. circinelloides*	Platypus	Tasmania	na	1995
WA1	*M. amphibiorum*	Slender tree frog	West Australia	(+)	1998

Table 2.1: Details of *M. amphibiorum* and *M. circinelloides* isolates used in pathogenicity trials.

Both Queensland isolates were taken from euthanased cane toads by Speare *et al.* (1994). Tas1 was isolated from a living, young, ulcerated female platypus captured at Glengarry. Tas2 was taken from a dead, ulcerated male platypus found dead on the banks of Brumby's Creek. Tas5 was isolated from a moribund male platypus from Deloraine. WA1 was isolated from a dead slender tree frog (*Litoria adelensis*) from the Perth Zoological Gardens (Creeper *et al.* 1998).

2.2.3 Cane toads

Cane toads of approximately 100mm in length were purchased from a commercial supplier (Peter Krauss) in Queensland. The toads supplied were mixed males and females. They were housed in wire cages, and supplied with constantly running water. Paper pellet litter was provided, and changed when necessary. The ambient temperature (22°C) was such that the toads did not feed during the course of the experiment.

2.2.4 "Sighter" trial

For the first experiment, the cane toads were randomly divided into four groups of 10 animals, and tagged around the upper right forelimb with coloured electrical ties. The animals were injected intraperitoneally with one of four spore concentrations, with each animal receiving 100ul of inocula. Thus the lowest treatment animals received $1x10^5$ spores, the next treatment $5x10^5$ spores, then $1x10^6$ spores, and the highest treatment $5x10^6$ spores. Spores for this trial were harvested from Tas1.

After 30 days, the animals were euthanased by placing in a shallow bath containing 2% chloral hydrate. The liver, heart and lungs were taken from each animal and placed in 10% formol saline. Samples were fixed and embedded in paraffin wax before sectioning at 4 um

and staining with H and E. A random field of view was selected for liver sections, and viewed microscopically at x400. The field was ?x?. The presence or absence of sphaerule-like bodies (slb) was noted, and the slb enumerated. As heart and lungs sections were generally only lightly infected, the entire section was examined for the presence of slb.

2.2.5 Pathogenicity trial

For the second experiment, six groups of ten randomly selected cane toads were injected intraperitoneally with $5x10^6$ spores from one of the six fungal isolates previously described (Table 2.2) . Each treatment group was housed in a separate cage, to avoid possible cross-contamination. The animals were euthanased after ten weeks, and liver, heart and lung removed and treated as per the "sighter" trial. The fixed and stained sections were viewed microscopically at x400. For liver sections, a random field was selected, and the granulomas and slb counted within the field. As with the "sighter" trial, granulomas and slb were counted for the entire heart and lung sections.

Cane toad group	Isolate used for inoculation
One	Q1
Two	Q2
Three	Tas1
Four	Tas2
Five	Tas5
Six	WA1

Table 2.2: Details of isolates used to inoculate the different groups of cane toads in Experiment two

Prior to Experiment two commencing, the different spore isolates used were diluted 1:10 in distilled water, and 100ul of the solution spread over Sabouraud's agar. These plates were incubated at 25°C and examined after two days for growth, to ensure that each isolate was viable.

In both experiments, sections of liver from infected animals were rubbed onto Sabouraud's agar, and the plates incubated for 48 hours at 25°C. Sub cultures were taken, transferred to fresh Sabouraud's agar, and incubated at 25°C for 5 days. Sections of each of the cultures were then tested against both (+) and (-) tester strains.

Prior to both experiments, five untreated animals were euthanased, and their hearts, lungs and livers removed and fixed for histology after streaking liver sections onto Sabouraud's agar and incubating as previously described.

2.2.6
Statistical analyses

Data were analysed with a one-way analysis of variance using the JMP 3.2.1 statistical package. ANOVA assumption of normality and homogeneity of variances were tested using the Shapiro-Wilk W test and Bartlett's test respectively. Non-normal data was log 10 transformed. Significant differences were highlighted as multiple comparisons of means (Tukey-Kramer HSD test) were performed.

2.3 Results

2.3.1 Experiment one

Due to the loss of tags in this experiment, only eight animals from the $1x10^5$ group, nine from the $5x10^5$ group, seven from the $1x10^6$ group and nine from the $5x10^6$ group could be used.

All four dose rates resulted in at least some *M. amphibiorum* infection in the cane toads (Table 2.3). At the lowest dose rate, granulomas and slb were rare in sections of liver, and not present in either sections of heart or lung. As the dose rate increased, so too did the number of slb seen in sections of heart, lung and liver (Figure 1). Typically, granulomas were composed of epithelioid histiocytes and fibroblasts. Occasionally lymphocytes, multinucleate cells and eosinophils were also present. Fibroblasts were more prevalent at the periphery of the granulomas. Some, but not all granulomas, contained slb. The slb were present in two forms. Firstly, there were small, clear slb up to 10um in diameter. These were present in liver sections from animals exposed to all dose rates. The second type of slb were larger (up to 50um in diameter), contained numerous daughter cells, and were only observed in liver sections from animals exposed to the highest dose rate. Both types of slb were seen in granulomas, and also in areas of tissue where they were apparently eliciting no host response. A granuloma was only seen in one heart section from an animal exposed to the highest dose rate, although slb were seen in three other heart sections. At all dose rates, liver

sections contained slb that were apparently degenerate, with the cell wall disrupted and the contents lacking a defined shape consistent with a viable slb.

	Percentage of Animals Affected			
Dose	$1x10^5$ spores	$5x10^5$ spores	$1x10^6$ spores	$5x10^6$ spores
Heart	0	11	14	33
Lung	0	11	28	78
Liver	25	56	86	100

Table 2.3: The percentage of cane toads with slb in various organs as a result of being injected with four different doses of *M. amphibiorum* spores

2.3.2 Pathogenicity trial

2.3.2.1 Liver granulomas

The Queensland isolate of *M. amphibiorum*, Q1, produced significantly fewer granulomas in liver sections than the other Queensland isolate (Q2), the West Australian isolate (WA1), and one of the Tasmanian isolates (Tas1). The number of granulomas produced by the other Tasmanian isolate, Tas2, was not significantly different to either Q1 or the other isolates (Figure 2.2). The size of the granulomas produced by the different isolates was not significantly different (Figure 2.3)

2.3.2.2 Sphaerule-like bodies in liver sections

The two Tasmanian isolates produced significantly greater numbers of slb in the liver than any of the other isolates (Figure 2.4).

2.3.2.3 Heart granulomas

There was no difference in the number of granulomas found in heart sections from toads infected with any of the isolates used (Figure 2.5), but Tas1 had a significantly greater number of slbs in the heart than either of the two Queensland isolates, or the West Australian isolate. The other Tasmanian isolate, Tas2, did not differ significantly from any of the other isolates (Figure 2.6).

2.3.2.4 Sphaerule-like bodies in lung sections

The Tasmanian isolate Tas1 had significantly greater numbers of slb in lung sections than Q1, but did not differ from the other isolates tested (Figure 2.7).

2.3.2.5 Sphaerule-like body morphology

Different sphaerule-like morphologies consistent with those observed in the "Sighter" trial were present in all organs. There was no difference in morphology between any of the isolates tested. All (+) mating types of *M. amphibiorum* produced a greater percentage of slb with daughter cells relative to simple slb than the (-) mating type (Figure 2.8).

2.3.2.6 Pathology of *M. circinelloides*-infected toads

There was no evidence of either granulomas, fungal hyphae or slb in any sections taken from the toads injected with *M. circinelloides* spores.

2.3.2.7 Pathology of non-infected toads

In both of the experiments, none of the organs taken from the five toads sacrificed prior to the experiments contained granulomas or slb.

2.3.2.8 Gross pathology of infected organs

The gross morphology of infected organs in both experiments was consistent with the findings of Speare *et al.* (1997), with organs, especially the liver, containing lesions which appeared as lemon-coloured nodules up to 5mm in diameter. There was no evidence of skin lesions in any of the animals examined.

2.3.2.9 Growth on Sabouraud's agar inoculated with liver sections from infected toads

The Sabouraud's agar plates inoculated with liver sections from infected toads in both experiments all produced good growth of *M. amphibiorum*, and the identity of the isolates was confirmed in mating trials. Sabouraud's agar streaked with liver sections from *M. circinelloides* infected toads did not produce growth of this isolate. Sabouraud's agar plates streaked with liver sections from uninoculated cane toads from both experiments did not produce growth of either *M. amphibiorum* or *M. circinelloides*.

2.3.3 Growth of *M. amphibiorum* and *M. circinelloides* on test plates

The plates inoculated with the 1:10 dilutions of the six fungal isolates used all produced good growth after two days incubation.

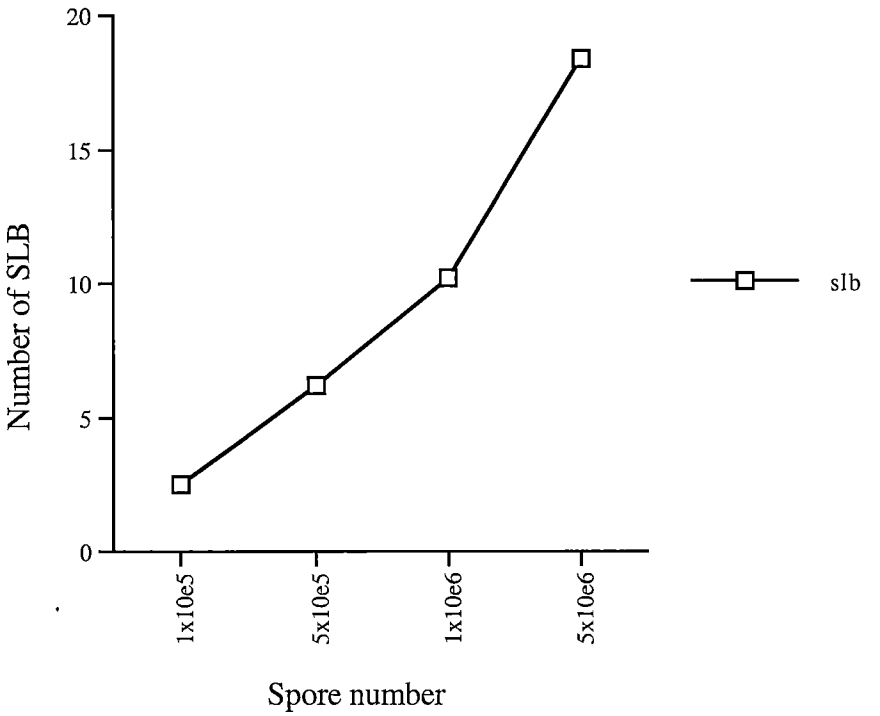


Figure 2.1: The effect of increasing numbers of *Mucor amphibiorum* spores on the number of sphaerule-like bodies in the livers of experimentally infected cane toads

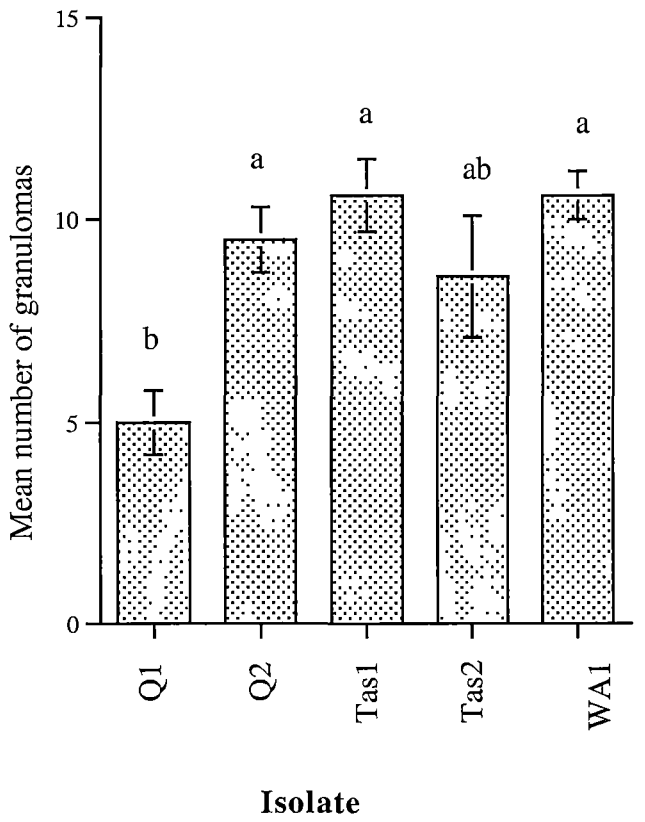


Figure 2.2: The mean number of granulomas in liver sections of cane toads experimentally infected with the fungus *Mucor amphibiorum*.

(Error bars show standars error. Different superscripts indicate means that are significantly different at p=0.05)

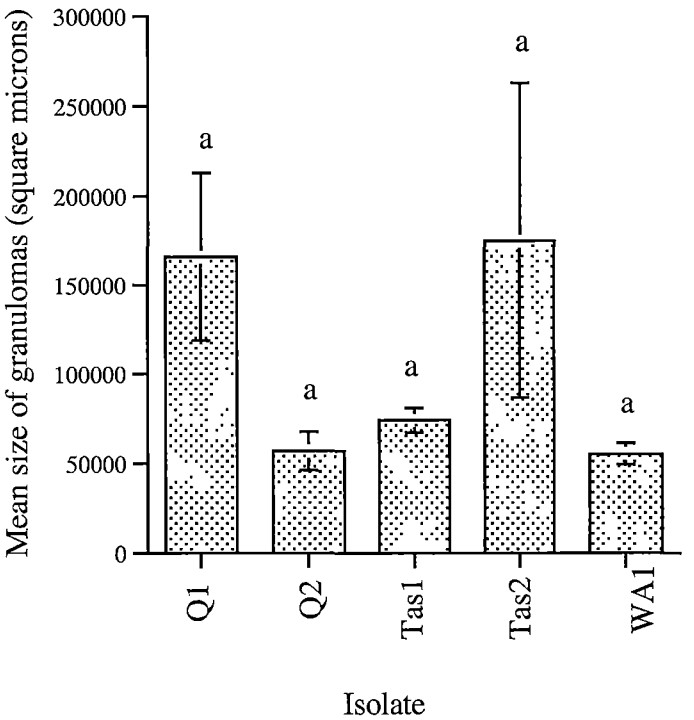


Figure 2.3: The mean size of granulomas in the livers of cane toads experimentally infected with the fungus *Mucor amphibiorum.*

(Error bars show standard error. Different superscripts indicate means that are significantly different at p=0.05)

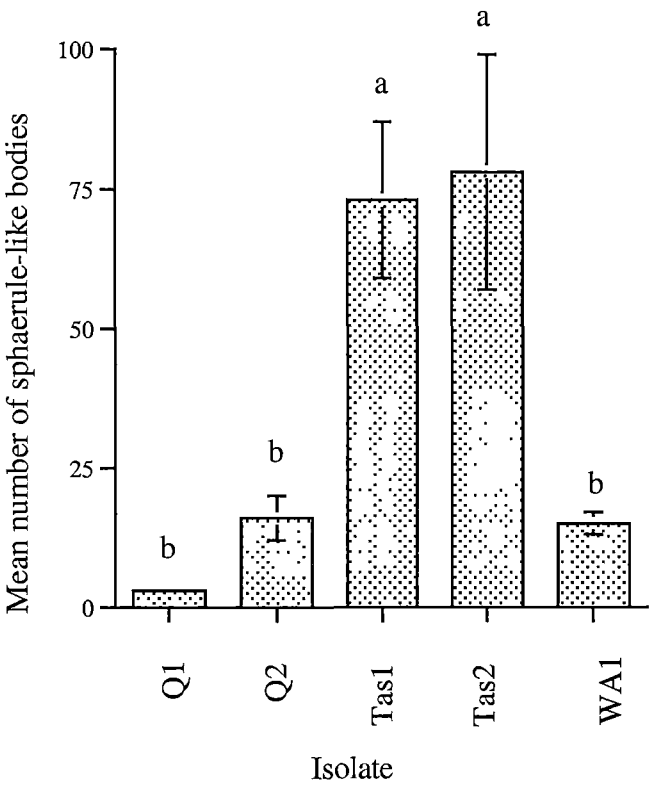


Figure 2.4: The mean number of sphaerule-like bodies present in liver sections of cane toads experimentally infected with different isolates of the fungus *Mucor amphibiorum.*

(Error bars show standard error. Different superscripts indicate means that are significantly different at $p<0.05$)

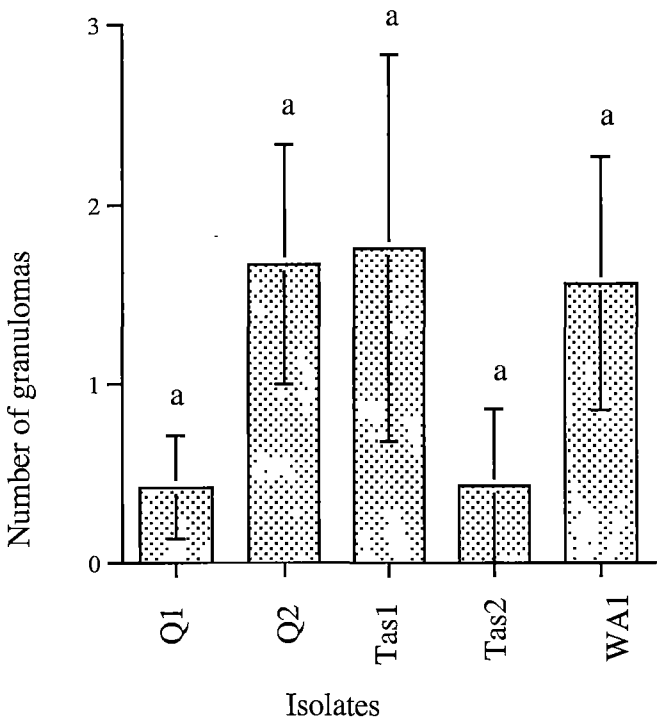


Figure 2.5: The mean number of granulomas in heart sections of cane toads experimentally infected with the fungus *Mucor amphibiorum.*

(Error bars show standard error. Different superscripts indicate means that are significantly different at p=0.05)

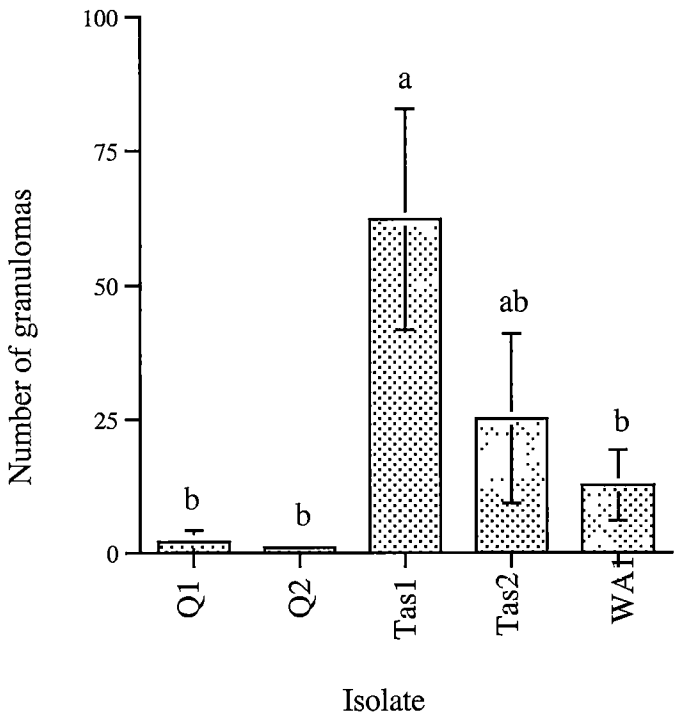


Figure 2.6: The mean number of sphaerule-like bodies in heart sections of cane toads experimentally infected with the fungus *Mucor amphibiorum*.

(Error bars show standard error. Different superscripts indicate means that are significantly different at p=0.05)

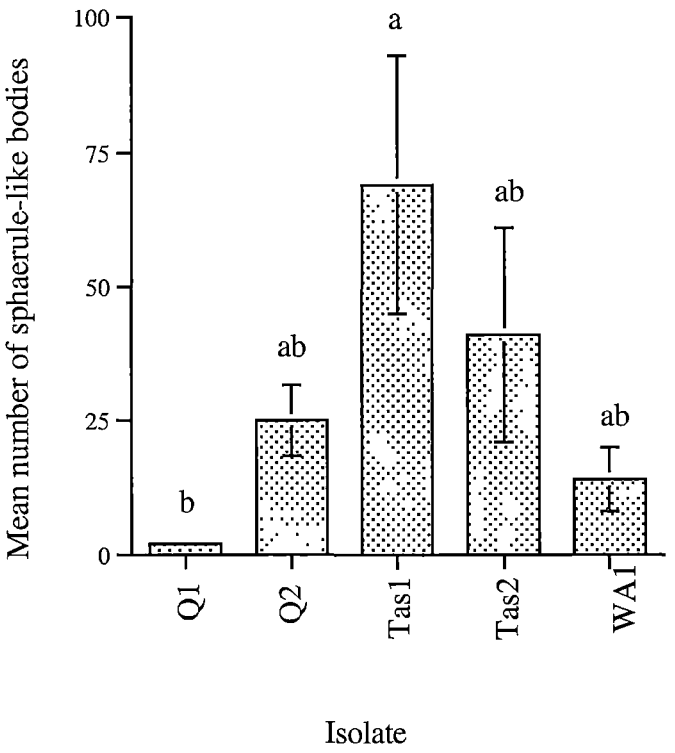


Figure 2.7: The mean number of sphaerule-like bodies present in lung sections of cane toads experimentally infected with different isolates of the fungus *Mucor amphibiorum*.

(Error bars show standard error. Different superscripts indicate means that are significantly different at $p<0.05$)

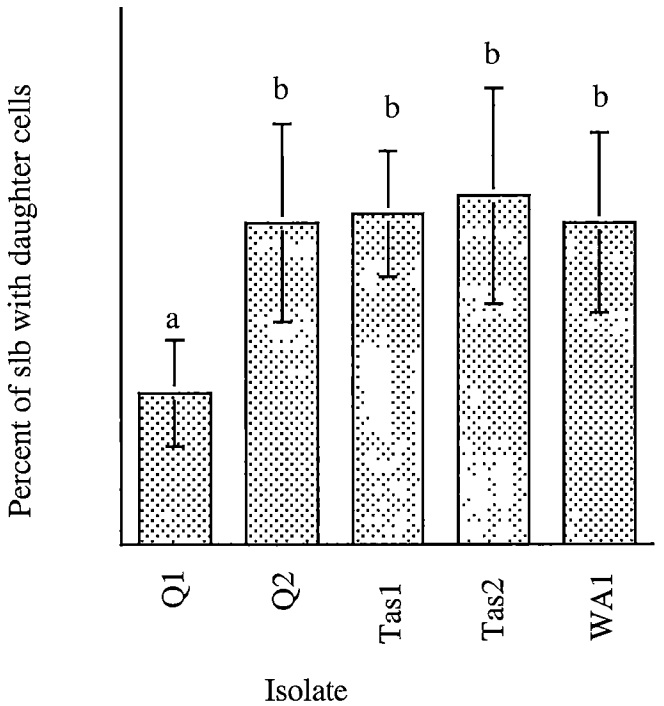
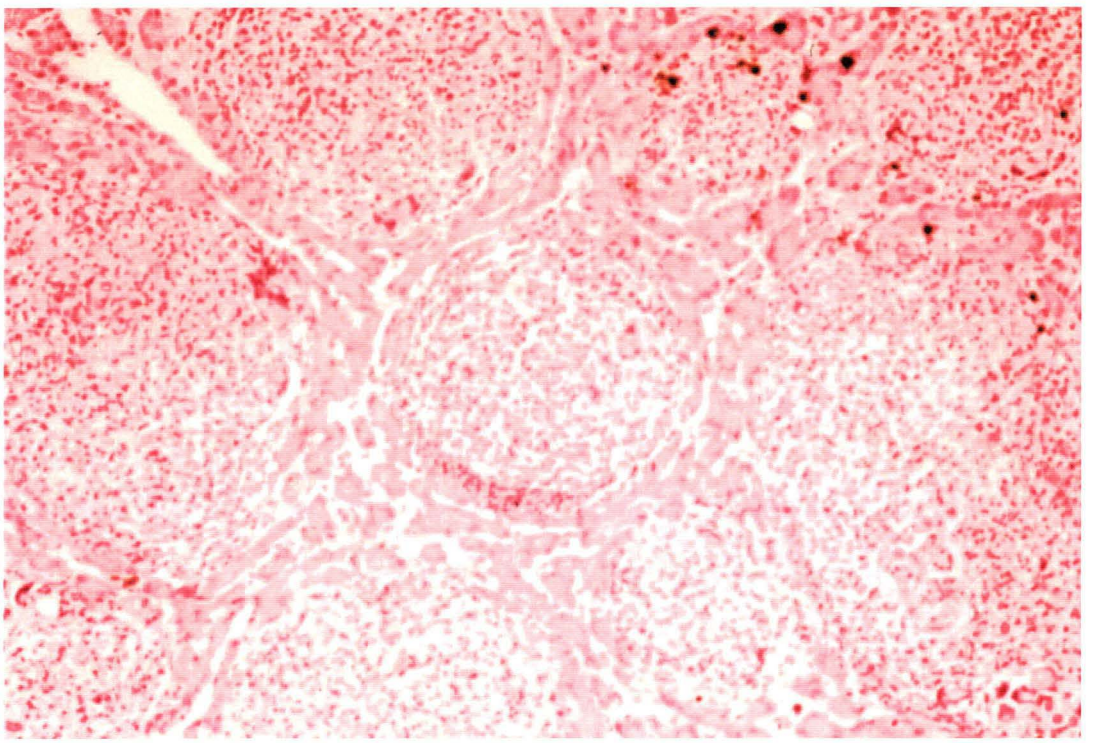


Figure 2.8: The percentage of sphaerule-like bodies possessing daughter cells in liver sections of cane toads experimentally infected with different isolates of the fungus *Mucor amphibiorum*

(Error bars show standard error. Different superscripts indicate means that are significantly different at p=0.05)



Scale bar = 100um

Figure 2.9: Granulomas in a liver section of a cane toad infected with the Queensland (-) mating type isolate of *M. amphibiorum* Q1. Note the absence of sphaerule-like bodies.

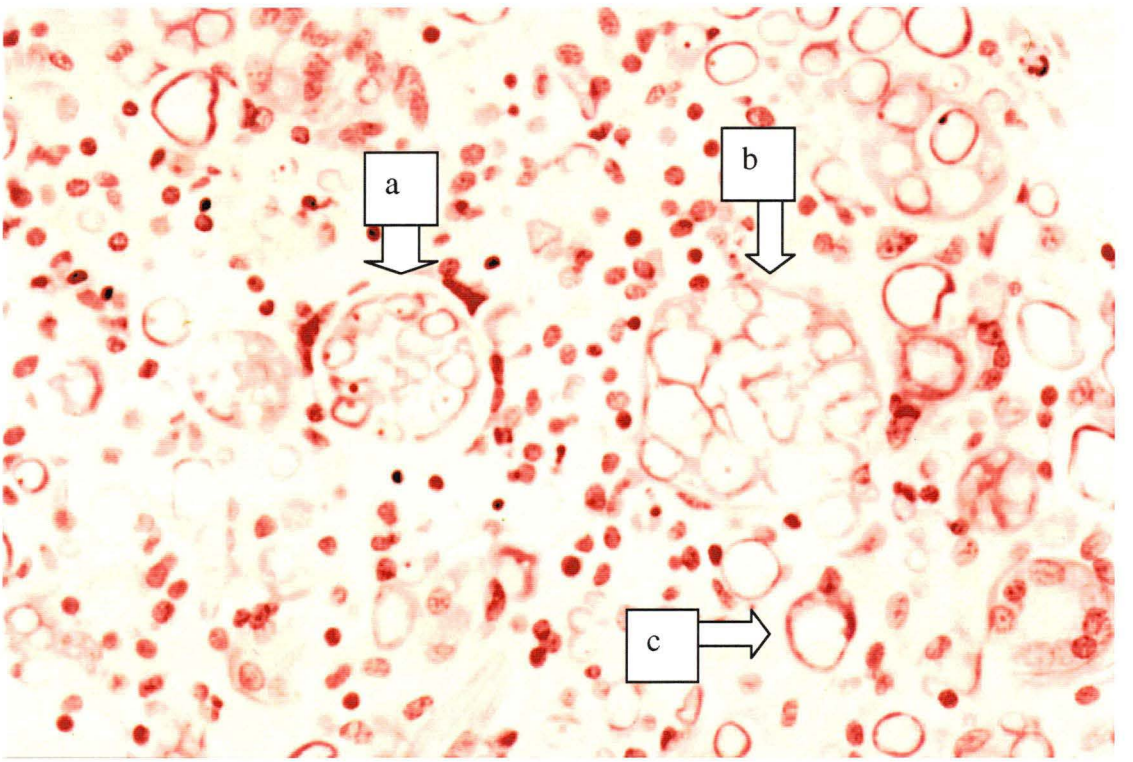


Figure 2.10: Liver section of cane toad infected with platypus-derived *M. amphibiorum* isolate Tas1. Depicted are a) a sphaerule-like body containing daughter cells, b) a sphaerule-like body that has ruptured, releasing daughter cells, and c) a daughter cell.

2.4 Discussion

Prior to commencing the pathogenicity trial, it was necessary to determine a suitable non-lethal dose rate of spores that would result in pathological changes in cane toad organs. Such trials have not been performed in Australia before. Frank *et al.* (1974) infected a range of amphibians with the original isolates of *M. amphibiorum*, but the concentration of spores used is not specified. The sighter trial could have been performed with either the Tasmanian, platypus-derived isolates of *M. amphibiorum*, or the mainland, cane toad- or frog-derived isolates. The Tasmanian isolate was chosen because at the time this isolate had just been re-cultured and tested for purity. For such a trial, any of the isolates would have been equally appropriate. Determining the correct dose range could not be based on previous studies dealing with the same species of fungus. However, mycotic infections have been induced in mammalian species using other pathogenic fungi, with dose rates being generally in the range of $1x10^5$ spores/conidia per animal to $5x10^6$ spores/conidia per animal (Sondhi *et al.* 1999, Mayayo *et al.* 1998, Iovannitti *et al.* 1999).

The liver, heart and lung were chosen as target organs because of their ease of removal, and the prevalence of slb in these target organs in wild, infected toads (Speare *et al.* 1997). The gross morphology of infected organs was consistent with the findings of Speare *et al.* (1997), with organs, especially the liver, containing lesions which appeared as lemon-coloured nodules up to 5mm in diameter. The severity of the infection appears to be dose-dependant, with a steady increase up to $1x10^6$ spores, then a sharper increase between $1x10^6$ spores and $5x10^6$ spores.

The prevalence of lesions in the liver and lungs of animals infected with the highest dose rate also accorded closely with that found by Speare *et al.* (1997) in wild animals. In the infected animals they examined, 100% of animals presented with granulomas in the liver, and 74% with granulomas in the lungs. In this study, granulomas were present in 100% of livers examined, and 78% of lungs. The wild caught toads exhibited a higher infection rate in the heart (54%) compared to the experimentally infected animals (34%). Presumably infection rates of different organs is not only a function of initial exposure, but also of time.

The spread of the fungus throughout tissues was rapid. At the high dose rate, all animals possessed slb in the liver. The large slb containing daughter cells were presumably spores that had developed into mother cells. The smaller cells that did not possess internal structures may either have been spores that had not yet differentiated, or daughter cells released when a mother cell had ruptured. Both of these cell types were

also observed by Speare *et al.* (1994) in cane toads, and by Creeper *et al.* (1998) in slender tree frogs (*L. adelensis*) and white-lipped frogs (*L. infrafrenata*). When grown *in vitro* on horse blood agar, spores of *M. amphibiorum* formed slb bodies within a week at 20°C, 25°C and 30°C, and both types of slb were noted. Given the rapid growth of *M. amphibiorum in vitro*, both in the mycelial form on Sabouraud's agar, and as slb on horse blood agar, it is likely that *in vivo* many spores differentiate into slb within seven days. Frank *et al.* (1974) reported both high mortalities and slb in amphibians within 10 days of infection with *M. amphibiorum*.

It seems likely that at the lowest dose rate the cane toads' immune system could efficiently eliminate the spores. In sections of liver from animals exposed to the lowest rate, granulomas without slb were seen that presumably had been the site of infection by spores and/or slb. At the higher rates, the immune system is incapable of effectively eliminating all spores, and the rapid formation and rupture of slb would ensure that the disease was quickly established.

In cane toads, the liver is the organ most likely to show signs of infection after intraperitoneal injection of a pathogen. This is not surprising, given that all blood from the gut first passes through the liver. In ulcerated platypus, the livers examined have not shown any evidence of infection, presumably due to both the higher temperature of internal organs relative to the skin, and the different route of infection. However, given the size of the platypus liver, it is possible that light infection in the liver would not normally be detected, unless serendipitously. Also, the platypus immune system may well be capable of dealing with *M. amphibiorum* when spores of the fungus are present in low numbers, even if the higher temperature of the internal organs themselves did not prevent growth.

In the platypus, ulceration of the skin is common, with occasional lesions being found in the lungs. Both of these sites are at a lower than core temperature. The description of cane toads with elevated papules up to 8mm in diameter on the skin, some with ulcerated centres (Speare *et al.* 1994), is consistent with observations of ulcerated platypus. In the toads, microscopic skin lesions were seen in the stratum spongiosum, often in association with blood vessels. In cane toads, it seems that the disease spreads both haematogeneously, and via direct extension within organs. No skin ulceration was observed in experimentally infected animals, but this may be due to the relatively short period of observation.

The second experiment demonstrated that there are differences in the effect of *M. amphibiorum* infection on cane toads, depending on where the fungal isolate was sourced. Although all isolates elicited a good immune response in the three organs selected, it seems that the Tasmanian isolates posed a greater challenge to the toads' immune system. Unlike the experimental infection carried out by Frank *et al.* (1974), where mortalities were noted in amphibian species within 10 days of infection, no mortalities occurred in any of the treatment groups. The susceptibility of native species to *M. amphibiorum*, and the less severe effects on cane toads, suggests that cane toads are inherently more resistant to the disease. The ability of the cane toads to survive with heavy infections, and the mobility of the species, may have important implications for wildlife conservation in areas in which cane toads are likely to spread.

The size, shape and description of the bodies found in tissues of all cane toads was the same as that given by Speare *et al.* (1994) for wild caught, infected toads. Apparently, spores that have lodged in the tissue of the toads at some point become committed to developing into slb. As noted by Frank *et al.* (1974), these slb are capable of growing in the hyphal form when re-inoculated onto Sabouraud's agar. Insofar as morphology and growth are concerned, there are no differences between mainland and Tasmanian isolates. The inability of *M. circinelloides* to form slb *in vivo* was interesting. This species is capable of forming slb on horse-blood agar at temperatures between 20°C and 30°C (Stewart 1998). Whether this species is incapable of infecting amphibians and mammals, or whether the cane toad is an unsuitable host, could not be determined. It is possible that the dose rate given was not high enough to induce disease in the toads. However, it seems likely that it is not implicated in disease in platypuses, and that given its ubiquity, its previous isolation from an ulcerated platypus represented an environmental contamination.

The severity of the infection in the cane toads did not seem to be influenced by the time since isolation of the isolate. In fact, the older of the two Tasmanian isolates (Tas1), produced significantly greater numbers of slb in the heart and liver than the more recently isolated West Australian isolate (WA1), and greater numbers of slb in the lung and heart than the more recently isolated Tasmanian isolate (Tas2). The two Queensland isolates produced similar results.

If the number of slb in the liver is used as a measure of severity of infection, it can be stated that the two Tasmanian isolates are more capable of producing large numbers of infective *M. amphibiorum*. This has obvious epidemiological implications. The heart and lung results were not so strikingly different, although there were significant

differences between Tas1 and mainland isolates for heart sections, and between Tas1 and Q1 for lung sections. The trend was for Tasmanian isolates to produce greater numbers of slb in these organs as well. Statistically, the two Tasmanian isolates were not different from each other for any of the parameters measured.

It was interesting to note that all the (+) mating types, regardless of source, had a greater percentage of slb with daughter cells relative to simple slb than the (-) mating type. It has been reported that (+) mating types of other *Mucor* species are more pathogenic than their (-) mating type counterparts (Michailides and Spotts 1986). The seriousness of infection may be related to a greater ability of (+) mating types of *M. amphibiorum* to rapidly produce mother cells from spores and/or daughter cells.

Statistically, mating type did not appear to affect the ability of an isolate to infect a cane toad. However, the propagule responsible for infection, the sphaerule-like body, occurred in statistically greater numbers in the livers of cane toads inoculated with Tasmanian, platypus-derived isolates. The other two (+) mating types, Q2 and WA1, produced approximately five times more slb than the (-) mating type Q1, although this was not statistically significant. These data suggest that (+) mating types may be capable of inducing greater rates and intensities of infection than the (-) mating type. Increased pathogenicity in (+) mating types has been observed in other *Mucor* spp (Michailides and Spotts 1986), although this phenomenom appears to be species specific (Vagvoelgyi *et al.* 1996). It is interesting to note that all isolates of *M. amphibiorum* cultured to date from platypuses have been (+) mating types. Also, the majority of isolates collected by Speare *et al.* were (+) mating types. It is possible that platypuses are infected by (-) mating types, but are easily capable of fighting the infection, possible due to the inability of that mating type to rapidly produce slb. Conversely, there may be physiological factors peculiar to the platypus that lend them to infection by only (+) mating types.

The results lead to some interesting hypotheses. It is tempting to state that Tasmanian, platypus-derived isolates of *M. amphibiorum* are more able to produce heavy environmental contamination with consequent greater rates and intensities of infection in susceptible hosts. However, it is also possible that the mainland isolates are as pathogenic as the Tasmanian isolates when conditions are suitable for those isolates. In Queensland, the average temperature is considerably higher than in Tasmania. During the course of the experiment, the temperature was a constant 22°C. The cane toads, accustomed to higher temperatures, may have succumbed to an infection that, under normal conditions, would have been insignificant. It would be interesting to determine

whether pathogenicity of isolates is, in part, a function of ambient temperature. It is unlikely that isolates differ from each other as far as their growth in response to temperature is concerned. Previous experiments have shown that the growth rate of all isolates tested is the same at 25°C, and vary little at a range of incubation temperatures from 20°C through to 35°C (Stewart *et al.* 1999).

Another possibility raised by the results is that the Tasmanian isolates are more pathogenic to cane toads because the toads have not previously been exposed to these particular strains of the fungus. It may be that these strains are native to Tasmania, and thus the Queensland cane toads are susceptible because they have not previously been exposed. However, given the sudden appearance of the disease in Tasmania, and its apparent spread, this seems unlikely.

It is also possible that *M. amphibiorum* does occur naturally in Tasmania, but is not pathogenic, and that a mainland isolate has been introduced into the state, with its pathogenicity increasing through constant passaging in the platypus. It is not uncommon for the passaging of a pathogen through a host to lead to increased pathogenicity (pers. comm. Munday). Cane toads would then be faced with a more pathogenic strain of a naturally occurring Queensland isolate. Although this possibility cannot be ignored, it has several weaknesses. Given the large number of amphibians that find their way into Tasmania via shipments of bananas and other fruit, it seems highly unlikely that only one of them has been infected with *M. amphibiorum.* A more likely scenario is that *M. amphibiorum* is not native to Tasmania, but has been regularly introduced into the state by amphibians, but is normally not capable of causing disease in platypuses. The relatively rapid clearance of the infectious stages of the fungus from the organs of cane toads injected with mainland isolates in comparison to Tasmanian isolates, and the formation of granulomas in all treatment groups, suggests that the severity of the disease (that is, the host response), does not differ between any of the isolates, but that the fecundity of the Tasmanian isolates results in the host immune system being overwhelmed. Only a small number of mainland isolates were tested. It is possible that a screening of a larger number of mainland isolates would uncover other, equally fecund isolates.

For platypuses to become infected with the fungus, they would first have to be exposed to a sufficient number of spores. Once the spores had entered the platypus, probably via inhalation, they would then have to produce slb with daughter cells. These bodies would have to rupture, releasing the daughter cells for the cycle to continue. Any spores that were incapable of rapidly differentiating into daughter-cell bearing slb would

presumably be destroyed by the platypuses' immune system. For the disease to become established and then spread, it would need not only to infect an individual; it would also be necessary for that individual to infect other animals. A likely hypothesis to explain how this happens involves the establishment of the fungus in the burrows of platypuses. An ulcerated animal entering a burrow would deposit slb and daughter cells on the floor and ceiling of the burrow. Some of these slb, growing *in vitro*, would differentiate into the morphology commonly associated with the species, exhibiting aerial mycelia and sporangiospores. The mature fungus would then release spores into the burrow, which would be inhaled by other platypuses. This means of dispersal and spread would explain the slow spread of the disease from the original point of infection. The platypus burrow should provide excellent growing conditions for the fungus, having high humidity and constant temperature. Platypuses commonly use several burrows within their territories for rest periods. They may use these burrows for short periods (several hours), or may spend up to three days in them. Within a 24 hour period, a platypus has been seen to use three separate burrows. In a month, another was observed using 11 different burrows (Grant 1995). Not only do platypuses use different burrows for resting, they can also share burrows with other platypuses (Serena 1994). As platypuses have overlapping territories, and can travel up to 10km in a 24 hour period (Serena *et al.* 1998), it is likely that infected animals make use of burrows in areas inhabited by healthy individuals.

The importance of dose rate in infection was demonstrated in the first experiment. At low dose rates, the presence of granulomas without any evidence of slb suggests that at a low exposure the invading spores are easily destroyed. At all dose rates small, non-daughter bearing slb were observed. However, the large daughter-bearing cells were only seen at the highest dose rate. The apparent inability of amphibian-derived spores to produce high numbers of daughter-bearing slb at dose rates that result in high numbers of such cells from platypus-derived spores may explain the successful infection of platypus by the latter isolates.

In conclusion, both (+) and (-) mating types of *M. amphibiorum* seem equally capable of infecting cane toads, but the severity of the disease is apparently increased for (+) mating types from the mainland, and is statistically greater for platypus-derived isolates. Also, the Tasmanian isolates resulted in statistically greater numbers of slb in the liver. As these bodies are the infective morphology of the fungus, it is reasonable to conclude that infection with Tasmanian platypus-derived isolates is more likely to overwhelm a hosts defences than infection with a mainland isolate.

Chapter 3

Organochlorines in tailfat samples of Tasmanian platypuses

3.1 Introduction

The increased industrialisation of the twentieth century has seen a concomitant rise in the release of xenobiotics into the environment. Many of them are by-products of industrial processes, and are released incidentally. Others, such as polychlorinated biphenyls (PCBs), have been important components in many industrial processes. Plastics, paints and coolants for electrical transformers have all relied heavily on PCBs for their manufacture. Although first synthesized at the end of the 19th century, commercial production of PCBs did not start until 1929, with large scale production beginning in 1945. They were first detected in the environment in 1966 (Jensen 1966), and their production was banned in Western Europe and the U.S.A. in the 1970's, and in Russia and Eastern Europe in the early 1990's. It has been estimated that worldwide production of PCBs was about 1.3 million tons over the period 1929 to 1977 (Giesy and Kannan 1998).

Commercial PCBs were popular in many processes because of their stability. Water, acids and alkali do not hydrolyse them, and they can withstand temperatures up to 600°C without decomposing. It is this stability that also contributes to their long-term adverse health and environmental effects. Much of the PCBs produced is still in existence. Electrical equipment produced prior to the 1970's contain PCBs in the coolant oil, and environmental compartments such as landfills, spillage sites and aquatic sediments can be areas of PCB contamination (Lawton *et al.* 1985). The persistence of PCBs, and their ready transportation from localised or regional sites of contamination to remote areas has led to their presence in almost every compartment in the environment (Giesy and Kannan 1998). It has been estimated that there are millions of kilogrammes of PCBs still in existence in environmental compartments (Lawton *et al.* 1985).

The PCBs are a class of compounds having paired phenyl rings with various degrees of chlorination. There are 10 positions on the biphenyl ring that can be chlorinated, resulting in a possible 209 congeners (PCBs with different numbers of chlorines). Of these, 135 have been found in environmental samples (Erickson 1986). All PCBs are highly chlorinated, with most commercial mixtures containing between 18% and 68% chlorine on a per weight basis. Many of the commercial PCB mixtures were also inadvertently contaminated with chlorinated dibenzofurans and naphthalenes (Giesy and Kannan 1998). As a general rule, the more highly chlorinated a PCB congener, the more toxic it is. Both the number and the position of the chlorine atoms can result in differences in enzyme induction and toxicity in exposed animals.

Mechanistically, PCBs can be separated into 3 groups; estrogen-like PCBs, phenobarbitol-like PCBs and dioxin-like PCBs. Estrogen-like PCBs tend to be short-lived. They react with with estrogen receptors and induce a cascade of events similar to that of endogenous estrogen. There is some evidence that the cytochrome P-450 enzyme system hydroxylates some PCBs, producing metabolites that can interact with estrogen receptors. Phenobarbitol-like PCBs are so named because, like phenobarbitol, they induce CYP 2B isozymes (Hu and Bunce 1999).

The most toxic are "dioxin like" PCBs, so called because they are approximate isostereomers of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), bind with high affinity to the Ah receptor, and induce 3-methylcholanthrene-inducible p-450 isozymes. Of the 74 gene families of cytochrome P-450 so far described, 14 families and over 400 individual genes have been identified for a broad range of species, including mammals, birds, reptiles, fish, arthropods, molluscs, plants, fungi and bacteria (Hu and Bunce 1999). In endogenous pathways of animals, P-450 synthesizes and degrades steroids, prostaglandins, fatty acids and other biological molecules (Stegeman *et al.* 1992). The toxicity of organic chemicals can be altered by P-450, resulting in either a nontoxic compound, or, in some cases, a compound with drastically increased toxicity. P-450 is involved in the hydroxylation of PCBs. Coplanar PCBs are usually hydroxylated in the para position in the least chlorinated ring. The rate of metabolism generally decreases with increasing chlorine substitution (Hu and Bunce 1999). Many halogenated liphophilic compounds, such as polychlorinated dibenzo-*p*-dioxins (PCDD), are metabolised to more hydrophilic compounds that are more easily excreted. However, some PCBs differ in that their metabolites are selectively retained.

The toxic effects of PCBs are similar to those elicited by TCDD and halogenated aromatic hydrocarbons (HAH). Toxicity is related to the affinity of the individual isomers for binding to the aryl hydrocarbon (Ah) receptor. The toxic effects of PCBs in different tissues is in part determined by the presence of high affinity Ah receptors in the tissue. Mice that express high affinity Ah receptors are more prone to humoral immunosuppression by Ah receptor-binding PCB isomers than mice with low affinity receptors (Kerkvliet *et al.* 1990, Tryphonas, 1994). The potency of a congener to suppress cytotoxic T cell response is directly correlated with the affinity of the congener for the Ah receptor (Kerkvliet *et al.* 1990).

Organochlorines such as PCBs can have a variety of effects on different organs. The thyroid gland is especially sensitive to some PCBs. Morphological changes have been observed in the thyroid gland of rats exposed to PCBs (Collins *et al.* 1977), and plasma retinol and thyroxin concentrations drop significantly in seals fed a diet of fish high in PCBs caught in the highly polluted Wadden sea (Brouweret *et al.* 1989). Reduced plasma retinol concentration is indicative of an induced vitamin A deficiency, which may result in an increased susceptibility to viral infections and reproductive disorders. PCBs and dioxins and the active thyroid hormones T3 and T4 show similar structural properties that appear important in molecular recognition in biochemical systems.

The adrenal glands of animals can also be adversely affected by PCBs. Grey seals and ringed seals in the Baltic sea suffer from adrenal hyperplasia, which has been associated with high loads of organochlorines, especially PCBs, in the populations (Bergman and Olsson 1985), and Beluga whales in the St. Lawrence estuary suffer lesions of the adrenal cortex, which are also associated with high PCB loads (DeGuise *et al.* 1995).

Organochlorines are capable of causing neurological damage, with children exposed to high levels of PCBs showing intellectual impairment (Tilson and Kodavanti 1997). Children exposed to lower, but consistent prenatal levels of PCBs consistently scored lower than non-exposed children on a number of psychological tests, including reduced psychomotor performance (Rogen and Gladed 1992). Monkeys exposed to levels of PCBs found in human breast milk showed behavioural impairment in comparison to non-exposed animals (Rice 1999).

Much effort has recently gone into understanding the possible immunosuppressive properties of many of the organochlorines. Thymic atrophy is common in many species experimentally exposed to organochlorines. Coplanar PCB congeners in particular are associated with lymphoid depletion in chicks (Andersson *et al.* 1991), reduced natural cell function in rats (Exon *et al.* 1985, Smialovicz *et al.* 1989) and reduced T cell mediated cytoxic activity in mice (Kerkvliet *et al.* 1990). The effects of some PCBs can be species specific. Harbour seals fed fish high in PCBs from the Baltic sea had reduced NK cell activity, and a reduced response to T cell mitogens, whereas rats fed the same diet showed no discernible effect (Ross *et al.* 1996). Non-human primates exposed to PCBs in their diet show an increase in NK cell activity (Tryphonas *et al.* 1991).

Gauging the effects of organochlorines on the immune system is complicated by the fact that not only are there species differences in susceptibilty to the effects of the organochlorines, but also that *in vivo* and *in vitro* effects often differ. A decreased lymphocyte proliferative response has been observed in response to T dependant mitogens in bottlenose dolphins with high levels of PCBs and DDT from Florida (Lahvis *et al.* 1995). The same effect was noted in harbour seals fed fish from the Baltic sea (Ross *et al.* 1996). However, rat lymphocytes directly exposed to levels of PCBs found in the wild in fish from the St Lawrence river (500ng/g) resulted in a greater stimulation index than for unexposed lymphocytes (Omara *et al.* 1997), whereas lymphocytes from rats fed contaminated fish from the Baltic (7135ng/g) had the same stimulation index as rats fed a non-contaminated diet (Ross *et al.* 1996). Lymphocytes from seals fed fish from the polluted Baltic had a reduced response to T cell mitogens (Swart 1997) whereas PCBs added directly to the medium in a proliferation assay had no effect on human lymphocytes (Fernlof *et al.* 1997). Other studies have found suppression of both B cell- and T cell-mediated imune function at doses of organochlorines which do not produce overt signs of toxicity (Svensson *et al.* 1994). Natural killer cell activity has also been shown to be highly variable, depending on species tested and method used (Svensson *et al.* 1994, Ross *et al.* 1996, Tryphonas 1994, Smialowicz *et al.* 1989). Many of the differences observed between *in vivo* and *in vitro* effects is probably due to the complexity of many of the PCB mixtures released into the environment, and the inter-reaction of these mixtures with other toxicants. Different congeners have different toxicities, and there is some evidence that the toxicity of complex mixtures such as Aroclor 1254 (a common PCB mixture) is not due to the summed effect of each of the chemicals, but rather to only a few of the constituents acting synergistically (Sargent *et al.* 1989).

The exact process by which PCBs affect the immune system is not fully understood. While some PCBs seem capable of directly inducing apotosis in lymphocytes (Yoo *et al.* 1997), it is likely that many PCBs affect the immune system indirectly by altering hormone levels. Circulating levels of endogenous immunomodulators such as testosterone, prolactin and thyroid hormones have been shown to be altered by exposure to organochlorines (De Krey *et al.* 1994). The effect of many xenobiotics is species specific. For example, deer mice are 10 times more sensitive to PAH than laboratory mice (Dickerson *et al.* 1994), and gulls are 10 to 50 times more sensitive to the teratogenic effects of many organochlorines than domestic fowl, Japanese quail or finches (Fry *et al.* 1987).

Despite the strong laboratory evidence that PCBs can adversely affect certain immune parameters, linking high loads of PCBs in animals with disease outbreaks is difficult. Morbillivirus outbreaks in seals and dolphins have been linked with high organochlorine loadings in affected animals. In a morbillivirus outbreak that affected harbour seals in Europe, animals that died had significantly higher concentrations of organochlorines in blubber than those that survived (Aguilar and Borrell 1994). Similarly, dolphins that had died from a morbillivirus infection had significantly higher concentrations of organochlorines in blubber than live animals (Hall *et al.* 1992). In neither case was it possible to draw firm conclusions about a causative role for organochlorine in the outbreaks, as it was impossible to control for factors such as sampling methods and virus exposure. Also, many of the dead animals were in poor condition, with little blubber, which may have lead to organochlorines being concentrated in the remaining fat (Hall *et al.* 1992).

Determining "safe" levels of PCBs and organochlorines for different species is complicated by the ability of different species to metabolise the toxicants. Small cetaceans, seals and mink have relatively low function of MC (3-methylcholoanthrene)-type enzymes and no function of PB (phenobarbital)-type enzymes in comparison to many other animals, which leads to accumulation of lower chlorinated biphenyls (tri-, tetra- and pentachlorobiphenyls) in these animals (Tanabe 1988). The inability of these species to efficiently metabolise certain congeners can lead to bioaccumulation far in excess of other animals.

Although determining safe levels for species is problematic, it is certain that the further up the food chain an animal is, the greater its potential intake of PCBs. Biomagnification results in tissue levels of PCBs being an order of magnitude higher in tissue than in soil in some species (Dickerson *et al.* 1994). In aquatic environments, the biomagnification can be even greater. The preferential biomagnification from lower to higher trophic level is well demonstrated in the North Pacific, with the dolphin as the top order predator. In that case, the bioconcentration factor (concentration ratio of PCBs in organisms to water) can be as high as 10^7 (Tanabe *et al.* 1984). The biomagnification factor also results in greater biomagnification of higher chlorinated congeners. In the case of the otter, the biomagnification factor (from fish to otter) for tetrachlorobiphenyls is 0.02, for most pentachlorobiphenyls between 9 and 15, for most hexachlorobiphenyls between 12 and 30, 14 for most heptachlorobiphenyls and 21 for most octachlorobiphenyls (Leonards *et al.* 1997). The effect of different PCB congeners on different species is also affected by the uptake efficiency of the species in question.

Determining the risk that different PCB mixtures pose to the environment is not only problematic due to the difference in accumulation and effect on different species, but also by the effect of the environment upon the PCB mixtures. Degradation rates of individual congeners differ, and there is a tendency for less toxic congeners to be selectively removed, resulting in a greater concentration of more toxic congeners (Giesy and Kannan 1998).

Despite the difficulties in quantifying the effects of PCBs on different species, there is no doubt that they are capable of adversely affecting the health of all species examined. Their deleterious effects seem to be associated with an increased incidence of disease in some species, and with population decline in others . Because of this, and the ability of PCBs to act as immunomodulators, it was decided to sample platypuses from throughout Tasmania to determine whether there was any correlation between levels of PCBs and incidence of the disease ulcerative mycosis.

Included in the study are the concentrations of DDT and lindane found in tailfat samples of platypuses. The primary emphasis is on PCBs, as these can be powerful immunomodulators. DDT is more commonly associated with shell-thinning in bird and reptile eggs. Its estrogen-like effects can result in skewed sex ratios in birds, due to testicular feminisation, and to reduced hatching rates. Offspring can also be smaller and less healthy than non-contaminated hatchlings. Although its estrogen-like effects are well documented, there is also some evidence to suggest that it may act as an immunomodulator in some cases. It has been linked with reduced natural killer cell numbers in the blood, and with reduced proliferative response in lymphocytes from dolphins (Lahvis *et al.* 1995). However, high levels are required before immunosuppressive effects are noted, with rabbits requiring 0.18mg/kg for up to 8 weeks before histopathological lesions were noted in lymphoid organs (Street and Sharma 1975).

Also included in the study is Lindane. Lindane is the gamma isomer of 1, 2, 3, 4, 5, 6 hexachlorocyclohexane (HCH), and is a widely used organochlorine insecticide for agriculture. Like many other organochlorines, it is highly liphophilic and extremely stable (Saha and Banerjee 1993). There is evidence to suggest that it can act as an immunomodulator. Both mice and rats exposed to diets containing Lindane show inhibition of specific antibodies against *Salmonells typhi*, and reduced IgG and IgM fractions of serum immunoglobulin (Saha and Banerjee 1993, Meera *et al.* 1992). Lymphocyte proliferation is also affected by exposure to Lindane, with an initial increase in proliferation, followed shortly after by a sharp fall (Meera *et al.* 1992).

3.2 Materials and Methods

3.2.1 Animals

Platypuses were obtained from all areas of occurrence of platypuses in Tasmania cited by Connolly and Obendorf (Connolly and Obendorf 1998) except the central highlands and the eastern and western sectors (Fig 3.1). In total, 56 animals were sampled, comprising 38 males and 18 females (Table 3.1)

3.2.2 Collection of tailfat samples

Fat samples were obtained from both living animals, and accidentally killed animals. Living animals were anaesthetised with isofluorane, and a small section of fat (0.1 - 0.5g) excised using a clean scalpel blade. The wound was then closed with two absorbable sutures. The tailfat of accidentally-killed animals was excised with a clean scalpel blade. All fat samples were wrapped in aluminium foil and frozen at -20C until prepared for analysis.

3.2.3 Analytical procedure

Firstly, the samples were freeze dried for 3 days in a Dynavac® freeze drier. They were then prepared for analysis by homogenising in a mixture of acetone and cyclohexane (1:1) followed by ultrasonication for 10 minutes. After removal of the acetone, the hexane was evaporated and the fat content determined gravimetrically. The fat was redissolved in about 1mL of hexane and cleaned up with sulphuric acid (Sodergren 1987). The organochlorine residues were separated and measured by gas chromatography (Varian 3500) with electron capture detection on a fused capillary column (DB-5, 60-m). The column temperature was kept at 110°C for 1 minute, increased by 6°C/minute to 240°C and kept there for 60 minutes. The injector and detector temperatures were 220°C and 290°C respectively. Hydrogen was used as carrier gas and nitrogen as make-up gas. Organochlorine concentrations were calculated from the peak area of the sample to the corresponding external standard. PCBs with IUPAC numbers 30, 204 and 209 were used as internal standards while Clophens A50 and A60 served as external standards (Ballschmiter and Zell 1980). Concentrations of individually resolved peaks of PCB isomers and congeners were summed to obtain total PCB concentrations. Results were reported as ppb PCBs, Lindane and DDT in tailfat lipid.

A subsample of 43 animals were further assayed (Table 2) for 28 PCB congeners as shown in Table 3. The results were reported as the proportion of each congener relative to IUPAC number 153.
Analyses were performed at Lund University, Sweden, by Professor Anders Sodergren of the Department of Ecology.

3.2.4 Comparisons and statistical analysis

Because of the wide disparity found in lipid content of tailfat tissue from platypuses, the results were standardised to 50% lipid equivalent by multiplying the organochlorine level recorded by the tailfat lipid percentage, then dividing by 50%.
The mean and standard deviation was calculated for PCB, Lindane and DDT in males and females separately, for endemic and non-endemic areas (Table 3.1), and for ulcerated and non-ulcerated animals. In all tables, endemic areas are in bold font, and non-endemic areas in normal font.
A two-tailed student t-test with a P value <0.05 as significant was used to compare results from male and female platypuses, and from ulcerated and non ulcerated animals.
Regional data were analysed with a one-way analysis of variance using the JMP 3.2.1 statistical package. ANOVA assumptions of data normality and homogeneity of variances were tested using Shapiro-Wilk W test and Bartlett's test respectively. Where necessary, data were log10 transformed. If data were still not normal, the ANOVA was performed on the untransformed data. A significance level of $P<0.05$ was adopted.

Regional congener patterns were expressed graphically as the percentage of animals in which individual congeners were detected in each area. The sample size in Site 3 and Site 6 was small, and the results were not graphed (Table 3.2). Congener patterns were determined for 3 ulcerated platypuses. Two were from Site 2, and 1 from Site 3. The animals were all male.

Region	Activity	Male platypus	Female platypus
1: Northwest Tasmania	Intensive agriculture, some industry	8	1
2: Central N Tasmania	Agriculture, some industry	20	8
3:Upper Blessington	Agricultural	2	-
4: Central South Tasmania	Agricultural	4	5
5: Lake Pedder	Hydroelectric development, remote	3	2
6: King Island	Pastoral, remote	1	2

Table 3.1 Details of animals from which tailfat samples were obtained

Region	Sample number	Male	Female
1. Northwest Tasmania	5	5	0
2. Central N. Tasmania	22	17	5
3. Upper Blessington	2	2	0
4. Central South Tasmania	8	3	5
5. Lake Pedder	5	3	2
6. King Island	2	1	1

Table 3.2 Details of platypuses from different sites for which conger patterns were produced

The 28 congeners tested were divided into 6 groups, comprising tetrachlorobiphenyls, pentachlorobiphenyls, hexachlorobiphenyls, heptachlorobiphenyls, octachlorobiphenyls, and nonachlorobiphenyls (Table 3.3). The percentage that each of these contributed to the total PCB load was calculated.

Biphenyl	Congener (IUPAC number)
Tetrachlorobiphenyl	52
Pentachlorobiphenyl	87, 90, 92, 95, 101, 110
Hexachlorobiphenyl	128, 132, 136, 138, 141, 149, 151, 153
Heptachlorobiphenyl	170, 174, 176, 177, 180, 183, 187, 192
Octachlorobiphenyl	194, 196, 201, 202
Nonachlorobiphenyl	206

Table 3.3 Details of congeners comprising different groups of biphenyls

3.3 Results

3.3.1 Lipid in tailfat tissues

The mean lipid content in tailfat tissues from all animals was 43 ± 16% with a range of 4.3 to 73%. There was no statistical difference between male and female platypuses, with mean lipid content in males being 43 ± 15%, and 44 ± 18% in females. A repeat sample was obtained from only one animal, with an initial tailfat lipid content of 38%, and 1.8% when the animal was found dead five months later.

3.3.2 PCBs, DDT and Lindane in tailfat lipid

3.3.2.1 PCB in tailfat lipid

The mean PCB concentration in tailfat lipid from all animals at the first sampling was 1118 ± 3067 ppb, with a range of 0 to 17335 ppb.

There was no significant difference between the mean concentrations of PCBs in male and female platypuses. The mean concentration for male animals was 1276 ± 3506, and for females 715 ± 2327.

Region	Mean PCB (ng/g)	Standard deviation	Range
1. Northwest Tasmania	3146	6349	0-17335
2. Central N. Tasmania	789	2146	16-7204*
3. Upper Blessington	105	14	92-119
4. Central South Tasmania	201	133	21-462
5. Lake Pedder	1978	1871	25-4221
6. King Island	100	95	11-201

Table 3.4 Mean, standard deviation and range of PCBs in tailfat samples from endemic and non-endemic sites. (* Only initial result for re-sampled animal included)

There was no statistical difference between PCB levels in tailfat samples from platypuses in the two endemic and four non-endemic sites. The range of levels was great, especially in sites 1 and 2 (Table 3.4)

3.3.2.2 DDT in tailfat lipid

The mean DDT concentration in tailfat lipid from all animals at the first sampling was 588 ppb ± 1916, with a range of 29 to 13754 ppb.

There was no significant difference between the mean concentrations of DDT in male and female platypuses. The mean concentration for male animals was 715 ± 2327, and for females 326 ± 297.

Region	Mean DDT (ng/g)	Standard deviation	Range
1. Northwest Tasmania	2171	4575	65-13754
2. Central N. Tasmania	308	340	19-827
3. Upper Blessington	14	2	23-16
4. Central South Tasmania	338	222	29-601
5. Lake Pedder	146	124	40-336
6. King Island	48	11	36-57

Table 3.5 Mean, standard deviation and range of DDT in tailfat samples from endemic and non-endemic sites

There was no statistical difference between PCB levels in tailfat samples from platypuses sampled in the two endemic and four non-endemic sites. The range of levels was great, especially in sites 1 and 2 (Table 3.5).

3.3.2.3 Lindane in tailfat lipid

The mean lindane concentration in tailfat lipid from all animals at the first sampling was 24 ± 131 ppb, with a range of 0 to 964 ppb.

There was no significant difference between the mean concentrations of lindane in male and female platypuses. The mean concentration for male animals was 33 ± 159, and for females 5 ± 19.

Region	Mean lindane (ng/g)	Standard deviation	Range
1. Northwest Tasmania	132	315	0-964
2. Central N. Tasmania	4	16	0-83
3. Upper Blessington	0	0	0
4. Central South Tasmania	2	3	0-10
5. Lake Pedder	0	0	0
6. King Island	0	0	0

Table 3.6 Mean, standard deviation and range of Lindane in tailfat samples from endemic and non-endemic sites

There was no statistical difference between Lindane levels in tailfat samples from platypuses sampled in the two endemic and four non-endemic sites. The range of levels was great, especially in sites 1 and 2 (Table 3.6)

3.3.2.4 PCB, DDT and lindane in tailfat lipid of ulcerated platypuses

The four ulcerated platypuses had a mean PCB concentration in tailfat lipid of 209 ± 229, with a range of 51-549 ppb. The DDT level was 96 ± 67, with a range of 21-184 ppb. The lindane level was 5±10, with a range of 0-19. These levels were not significantly different from those for non-ulcerated animals from either the endemic or non endemic area.

3.3.3 Congener patterns

3.3.3.1 Congener patterns in male and female platypuses

Twenty seven of the 28 congeners tested for were present in both male and female platypuses. Congener IUPAC number 87 was not detected. A high percentage of tailfat samples from both males and females contained heavily chlorinated congeners, with a

smaller percentage containing lower chlorinated congeners (Figs 3.2, 3.4). In both males and females, the majority of the biphenyls detected were comprised of pentachlorobiphenyls and hexachlorobiphenyls (Figs 3.3, 3.5).

3.3.3.2 Congener patterns in platypuses from different sites

Congeners with IUPAC numbers 52, 153, 138, 187 and 180 were detected in tailfat samples from Site 3. From Site 6, numbers 101, 151 and 180 were detected. Tailfat samples from the other sites contained most of the congeners tested (Figs 3.6, 3.8, 3.10), with exception of Site 5, which contained no tetrachlorobiphenyls or pentachlorobiphenyls (Fig 3.12). Samples from sites 1, 2 and 4 were predominantly contaminated with pentachlorobiphenyls, hexachlorobiphenyls and heptachlorobiphenyls (Figs 3.7, 3.9, 3.11). In Site 5, hexa- and heptachlorobiphenyls predominated (Fig 3.13).

3.3.3.3 Congener patterns in ulcerated and non ulcerated platypuses from the endemic area

Tailfat samples from ulcerated platypuses contained congeners from all of the 6 biphenyl groups, as did the non ulcerated animals. Penta- and hexachlorobiphenyls predominated in the non ulcerated animals (Fig 3.14), and hexa- and heptachlorobiphenyls predominated in ulcerated platypuses (Fig 3.15).

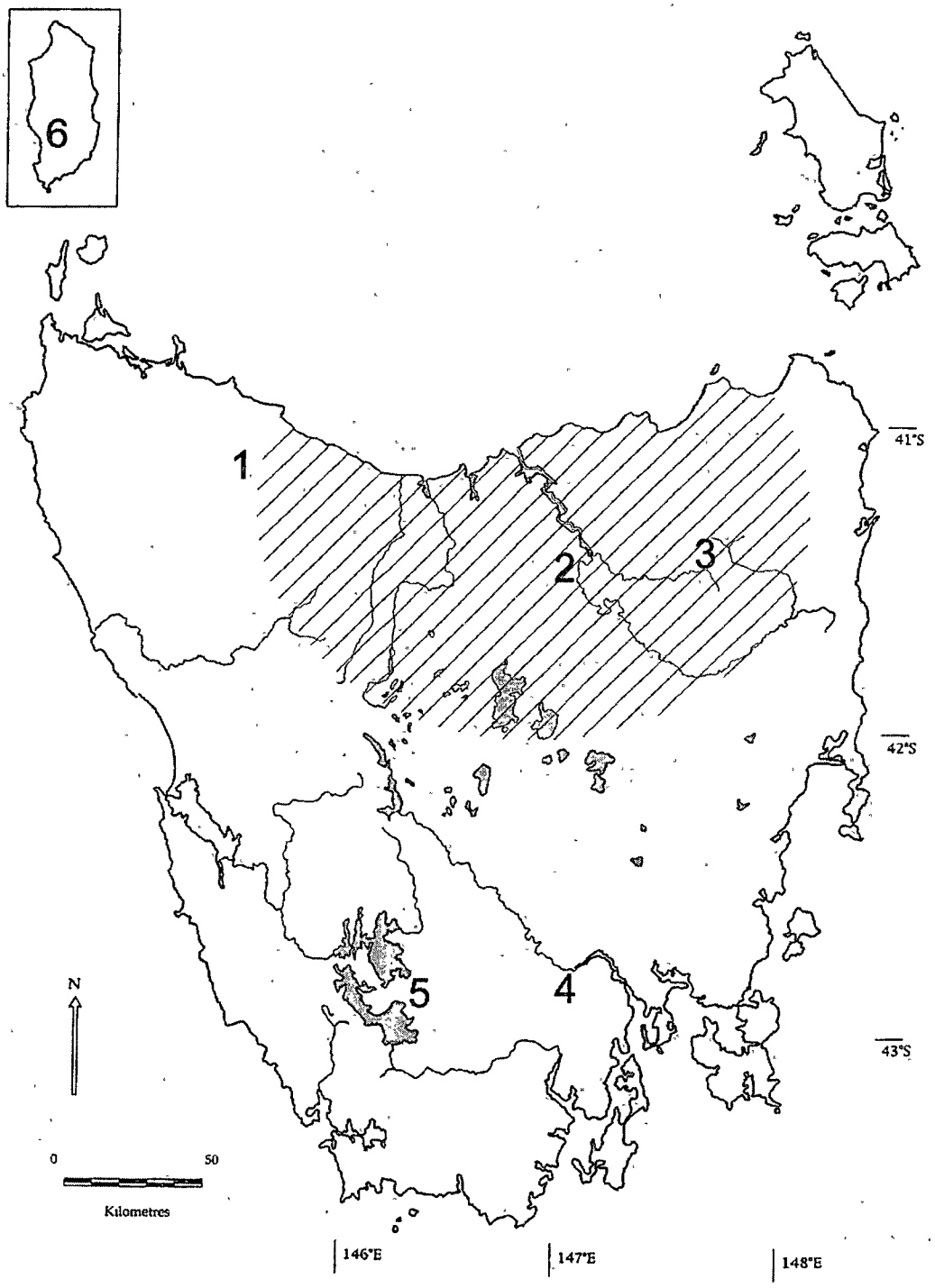


Figure 3.1: Map of Tasmania showing sampling sites. Site 1 = N.W. Tasmania, Site 2 = Central N. Tasmania, Site 3 = Upper Blessington, Site 4 = Central S. Tasmania, Site 5 = Lake Pedder, Site 6 = King Island. The shaded area indicates the endemic area for ulcerative mycosis.

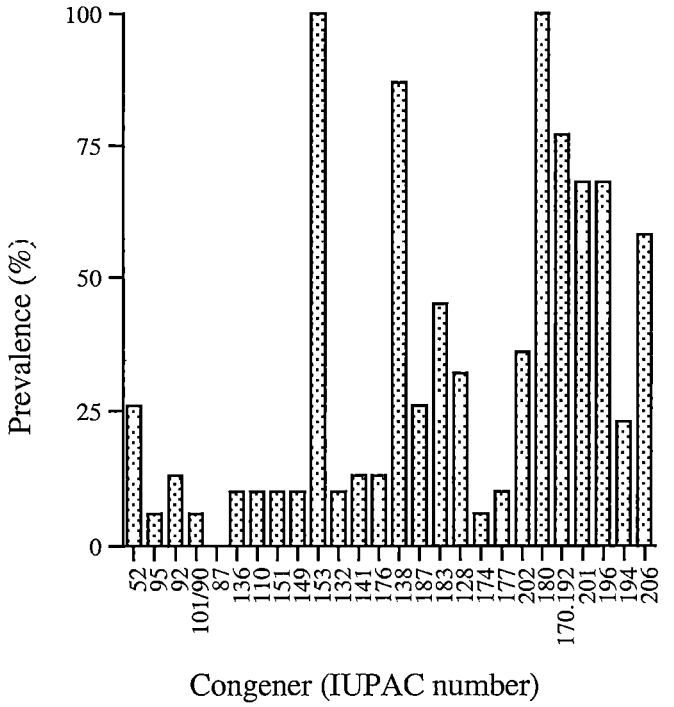


Figure 3.2: The percentage of male platypuses from all sites with different congeners detected in tailfat samples

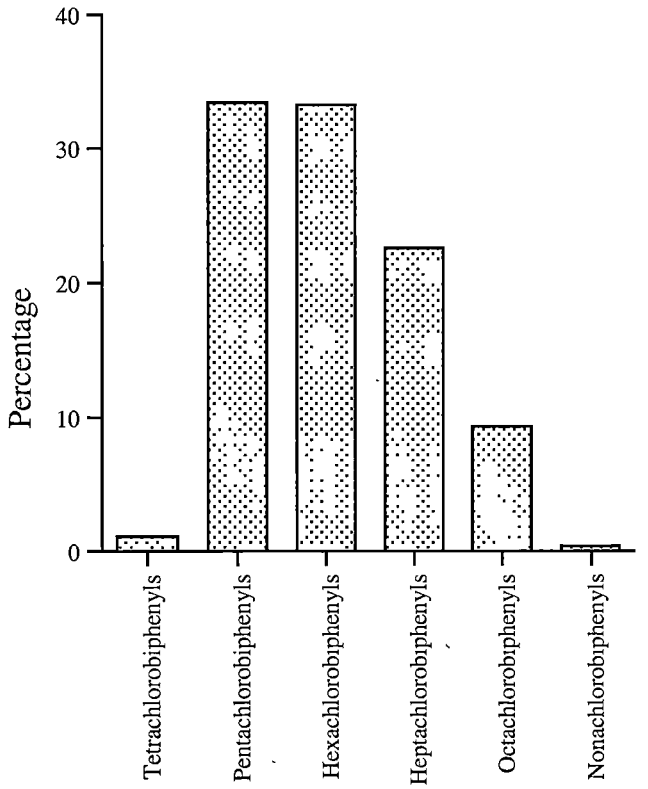


Figure 3.3: The percentage of different biphenyls in tailfat samples from males from all sites

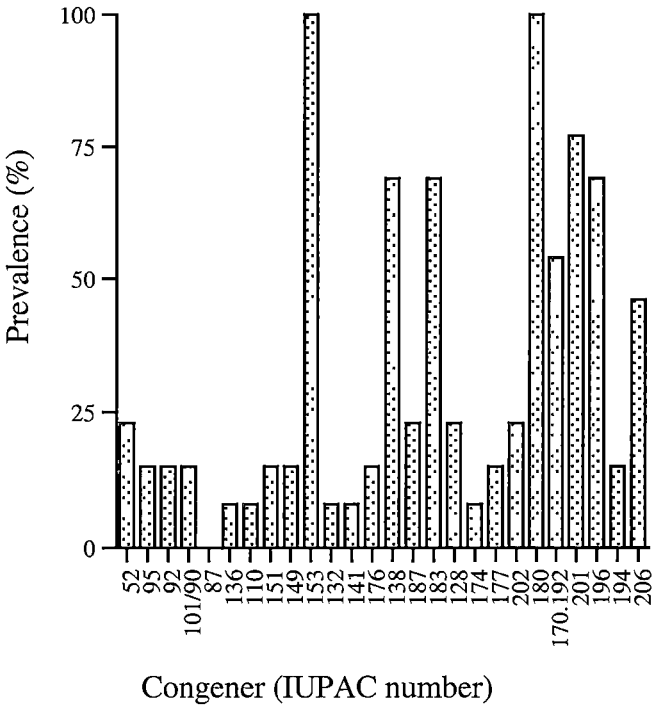


Figure 3.4: The percentage of female platypuses from all sites with different congeners detected in tailfat samples

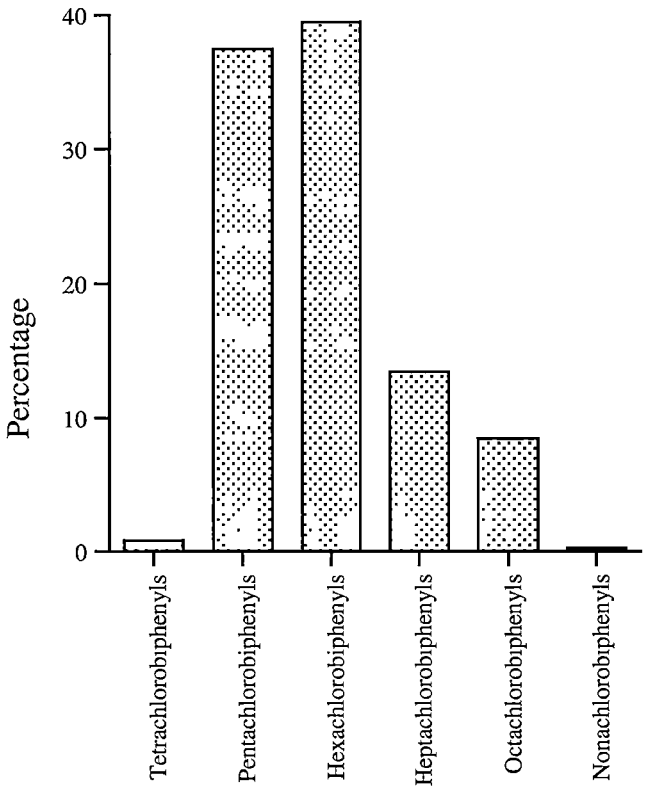


Figure 3.5: The percentage of different biphenyls in tailfat samples from females from all sites

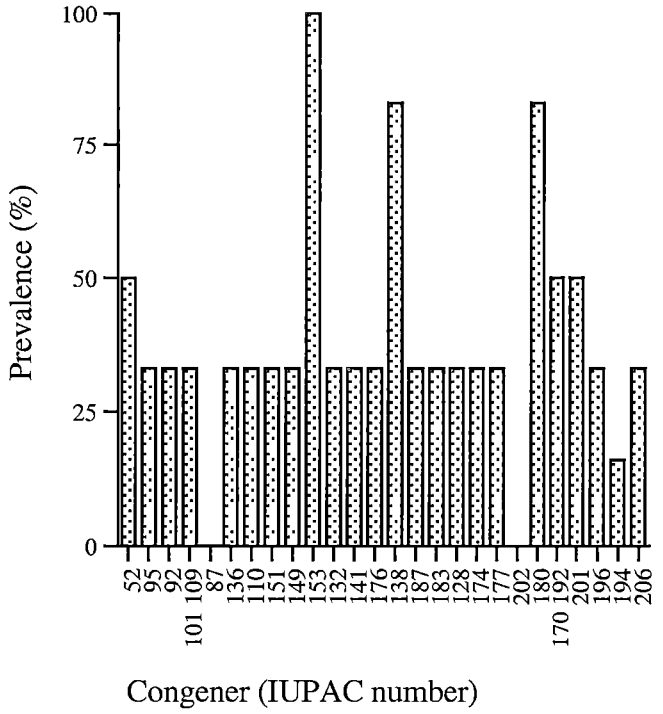


Figure 3.6: The percentage of platypuses from Site 1 with different congeners detected in tailfat samples

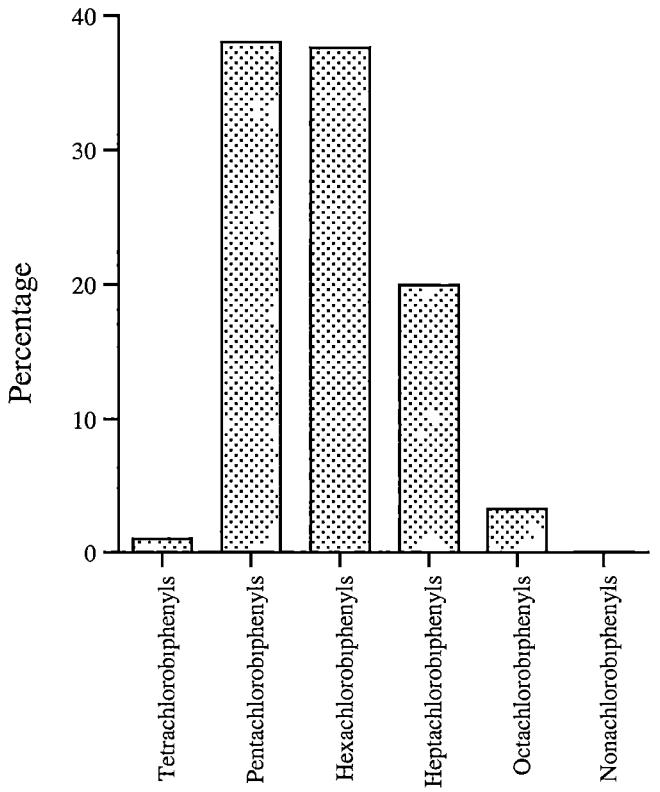


Figure 3.7: The percentage of different biphenyls in tailfat samples from platypuses from Site 1

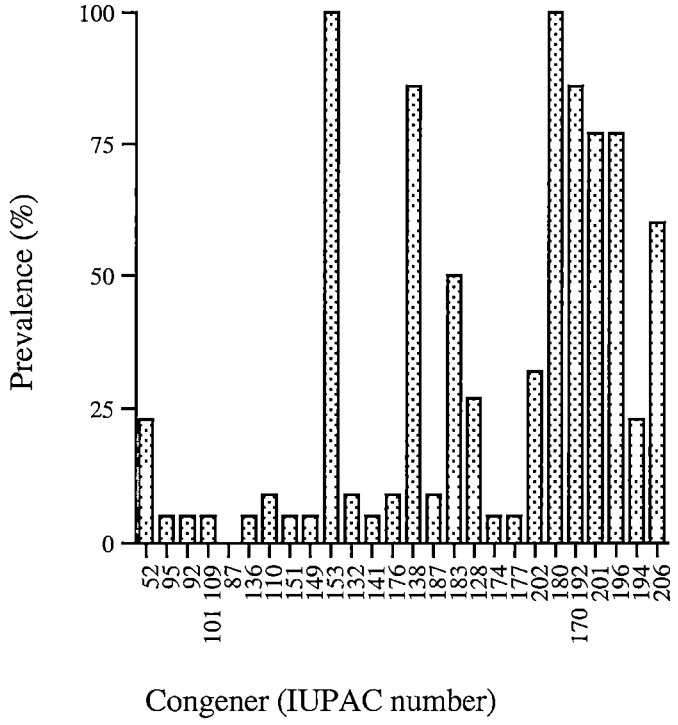


Figure 3.8: The percentage of platypuses from Site 2 with different congeners detected in tailfat samples

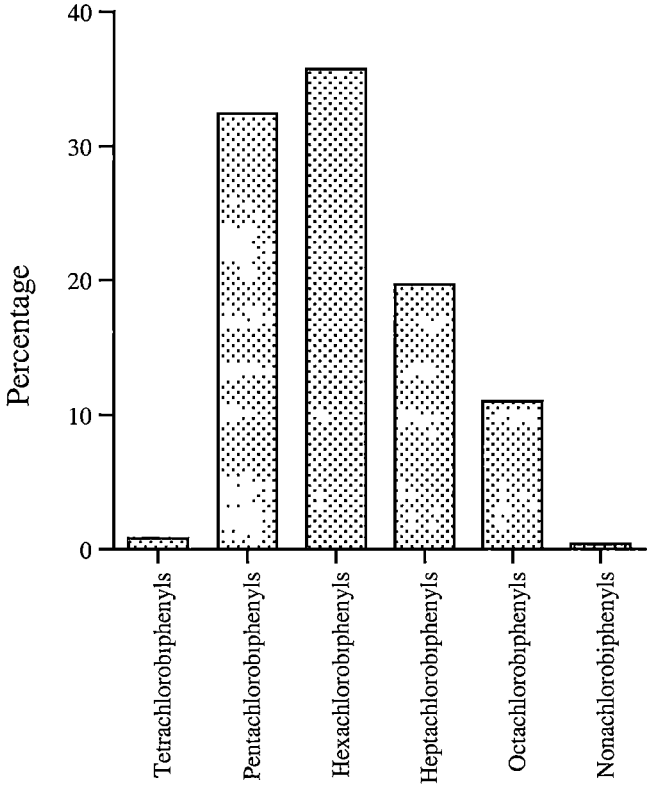


Figure 3.9: The percentage of different biphenyls in tailfat samples from platypuses from Site 2

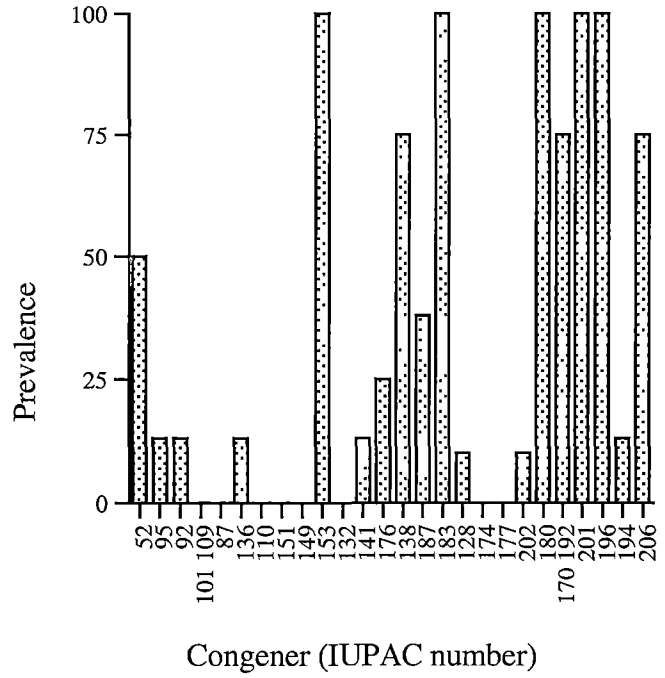


Figure 3.10: The percentage of platypuses from Site 4 with different congeners detected in tailfat samples

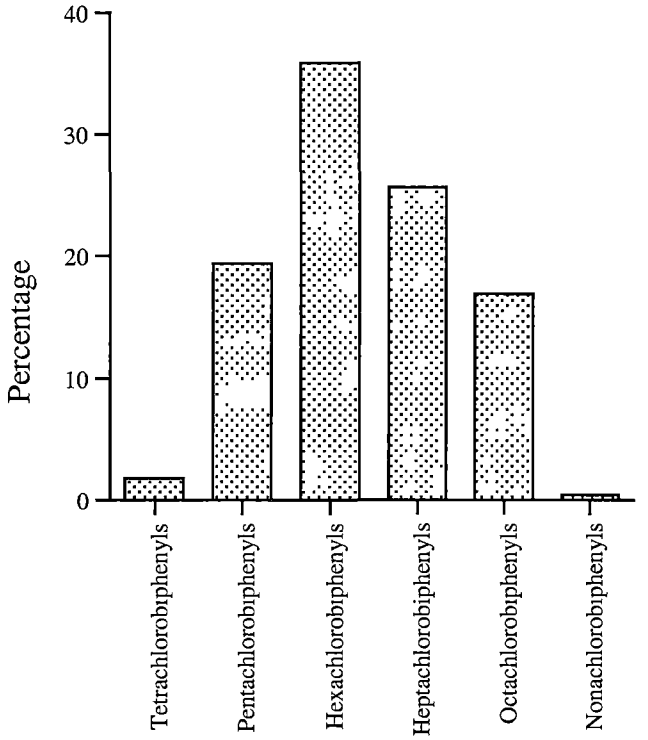


Figure 3.11: The percentage of different biphenyls in tailfat samples from platypuses from Site 4

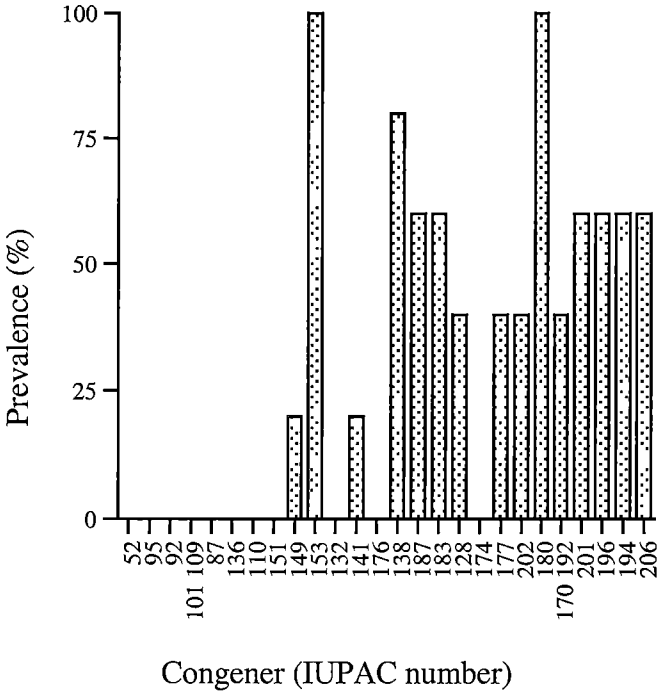


Figure 3.12: The percentage of platypuses from Site 5 with different congeners detected in tailfat samples

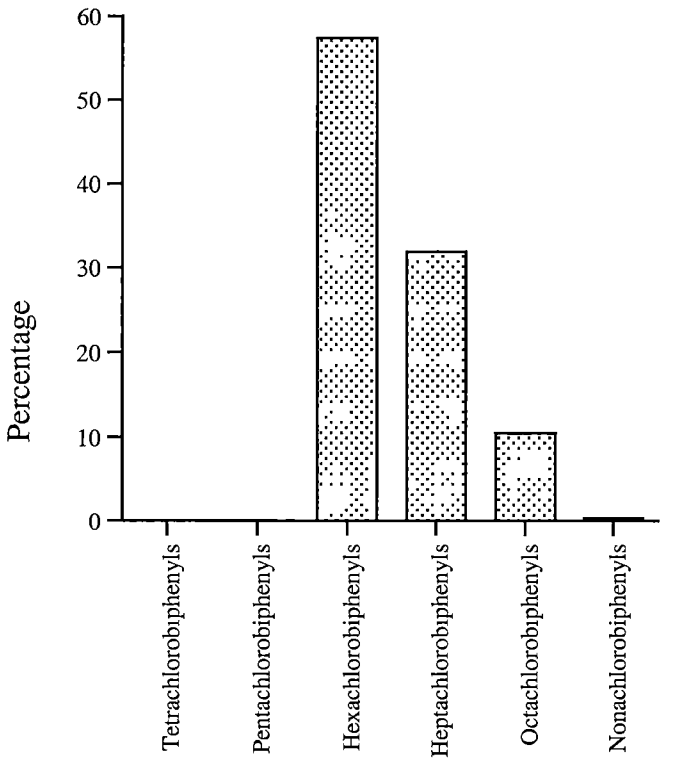


Figure 3.13: The percentage of different biphenyls in tailfat samples from platypuses from Site 5

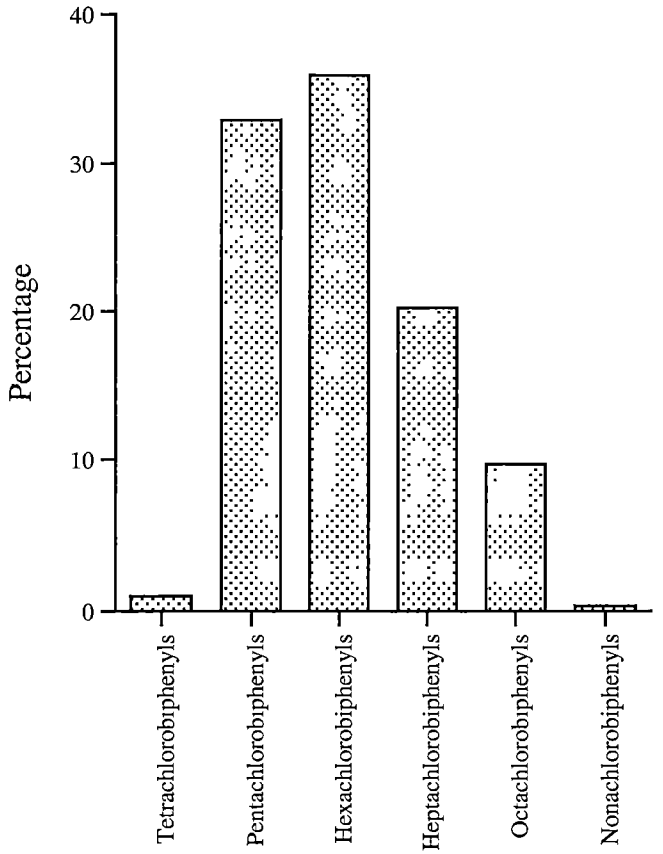


Figure 3.14: The percentage of different biphenyls in tailfat samples from healthy platypuses in the endemic area (Sites 1 & 2)

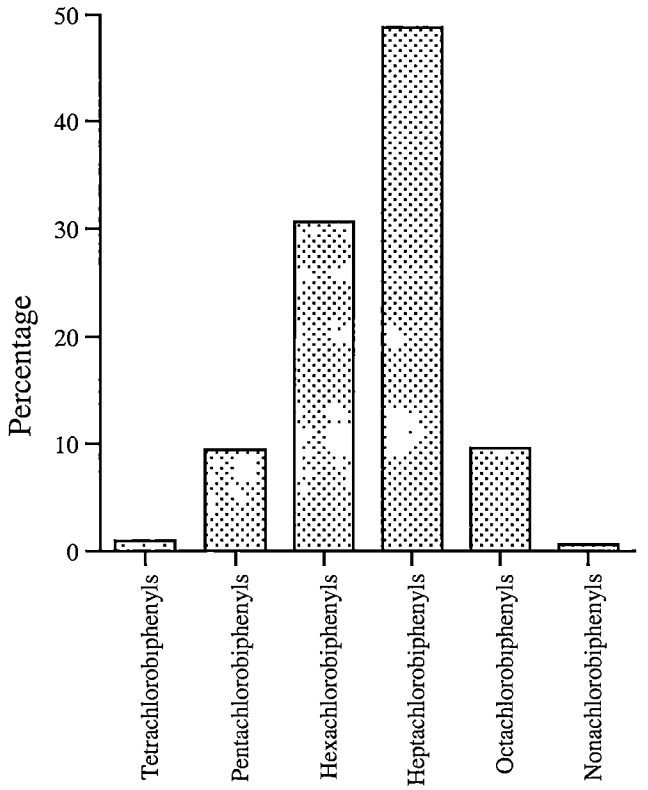


Figure 3.15: The percentage of different biphenyls in tailfat samples from ulcerated platypuses from the endemic area (Sites 1 & 2)

3.4 Discussion

The tailfat lipid content of male and female platypuses was not significantly different, which is in agreement with S. Munks (pers. comm.) that there is no significant difference in tail volume index (a crude measure of tailfat reserves) between male and female platypuses. Lactating females often have lower fat reserves than males and non lactating females. This was not supported by comparison of results for the breeding season (November to May [Connolly and Obendorf 1998] against other periods. This period also coincides with maximum food availability, especially of insect larvae. It may be that any fat loss due to lactation is offset by a ready abundance of food.

During times of fasting, large amounts of organochlorines can be released into the bloodstream (Ross *et al.* 1996), and these can cross the placenta, exposing the foetus to high levels. In eutherian animals, during times of fasting large amounts of organochlorines can be released into the bloodstream (Ross *et al.* 1996) and these can cross the placenta, exposing the foetus to high levels. The situation with the platypus is more complicated, as the platypus lays eggs and then suckles the young. During organogenesis eutherians are protected from the teratogenic effects of environmental chemical exposure by maternal metabolism (Bolton-Grob *et al.* 2000). Presumably, platypuses are more susceptible to these chemicals. When the platypus hatches from the egg, it is at a similar stage of development as a new-born marsupial (Grant 1995), and faces the same problems associated with toxicant exposure. During the period of post-organogenesis development of inter-organal regulatory networks, and of enzyme systems for xenobiotic metabolism platypuses, like marsupials, are exposed via maternal milk to xenobiotics (Bolton-Grob *et al.* 2000). In eutherians, xenobiotic metabolism of the prenate is modulated by maternal metabolism. Thus, it is likely that prenatal platypuses are more susceptible to lower levels of organochlorines than eutherians.

Organochlorines can also be concentrated in milk. Lactation in many species results in females having lower levels of organochlorines in fat reserves than males (Fromberg *et al.* 1999). Each breeding cycle that results in lactation clears significant quantities of organochlorines from the female. Studies of seals, porpoises, whales and walruses in the Faroes and in Greenland found that persistent organic pollutants such as organochlorines were in general 30 to 300% higher in male animals (Fromberg *et al.* 1999). Although dietary variation may account for some of the difference, the major factor appears to be lactational transfer in spring. There was no significant difference beween male and female platypuses in organochlorine concentration in tailfat. It is possible that few of the females

caught in this study had previously lactated. Female platypuses do not breed every year, and females that have not reached breeeding age would have comparable organochlorine levels to males of a similar age. The lack of significant differences between the sexes may in part be explained by fluctuating body condition. In many animals, the loss of body fat during times of starvation or increased metabolism (eg. associated with lactation) results in the concentration of organochlorines in the remaining fat. Although there was no significant difference between the sexes, the levels of organochlorines were lower in females, suggesting that lactational transfer could be occurring. Direct measurement of organochlorine levels in platypus milk would resolve the issue.

Organochlorines tend to increase in concentration in fat over time in many species; older animals have higher levels than juveniles. Accurately calculating the age of platypuses is not possible, beyond stating that an animal is a juvenile (determined by size and spur length), or old (determined by size, and state of spurs in males). In the wild, platypuses may not live long enough for significant differences between males and females to become obvious. In captivity, platypuses may live to 21 years of age, but in the wild it is estimated that maximum lifespan for a female is 13 years, and six years for a male (Jackson *et al.* 1999). It may also be that female platypuses do not transfer high levels of PCBs in milk. In other species, lactating females can excrete between 15% and 90% of PCB body burdens in their milk (Addison and Brodie 1977, Tanabe *et al.* 1981).

Although ageing platypuses accurately is difficult, it seems likely that PCBs do not accumulate substantially in the platypus in the long term. An obviously old platypus caught at Cressy had PCB levels comparable with younger animals. This has also been found in other species, especially the otter, where there is no correlation between age and concentration of PCBs in tissue (Kruuk and Conroy 1996).

The congener pattern between male and female platypuses was similar. Of the 28 congeners tested for, all but IUPAC 87 were present in at least some of both sexes (see figure 1 and 2). A higher percentage of all animals were contaminated with congeners above IUPAC 176, than below. The percentage biphenyl composition was also similar between sexes, with penta- and hexachlorobiphenyls comprising the majority of biphenyls detected. Normally, lower chlorinated PCBs are more readily cleared from the body than highly chlorinated ones. Low chlorinated congeners (di-, tri-, tetra) are predominant in surface water, plankton and fish in non-point source areas (Tanabe *et al.* 1984). This does not appear to be the case for the platypus. Although di- and trichlorobiphenyls were not tested, tetrachlorobiphenyls

were only present in very small quantities relative to penta-, hexa-, hepta- and octachlorobiphenyls. The greater percentage of higher chlorinated congeners suggests point source contamination in the sites tested.

In a study of the Chubu region in Japan, a comparison of levels of PCBs, and congener ratios in different species was made. This study sampled wild boar, black bear, Japanese macaque, Japanese marten, red fox, masked palm civet, raccoon dog, dog, pigeon, crow, cormorant, little egret, black-crowned night heron, black kite, peregrine falcon, lesser sparrow hawk, mountain hawk-eagle and golden eagle (Hoshi *et al.* 1998). The most dominant congener for fox and dog was IUPAC 180, and 153 for all other species tested. In Tasmanian platypuses, IUPAC 153 was most prevalent (found in 100% of animals tested), followed by IUPAC 180, present in 100% of females, and 96% of males tested. These 2 congeners were also prevalent in Scottish otters (Kruuk and Conroy 1996). The composition of congeners in different species reflects not only the PCB mixture to which the animal has been exposed, but also the differences of feeding habits and xenobiotic metabolising systems among species (Hoshi *et al.* 1998). It is likely that some of the differences observed in congener patterns between Tasmanian sites is due to dietary factors. The distribution of PCB congeners did not enable a decision to be made concerning the source of the PCBs. It is likely that both site 5 and 6 were contaminated with transformer oil, as the former is a hydroelectric development, and the latter, although having no industry, does have reticulated electricity. The different congener patterns between the two is probably explained by the differences in diet. Freshwater crayfish are a favoured prey species of the platypus. At site 5, crayfish are readily found attached to submerged trees. At site 6, crayfish tend to burrow, and are not readily accessible to the platypus. At this site platypuses feed mainly on insect larvae and oligochaetes. Crayfish are further up the food chain than insect larvae and oligochaetes, and this may explain the difference in congener profile.

Although statistically there was no difference in burdens of xenobiotics in tailfat samples between sites, this is probably due to the high variability of the assay results, and the low sample number in most areas. There were apparent differences between sites. The relatively high levels in sites 1 are 2 are easily explained by the industrial activities in those areas. The northwest of Tasmania has past and present industries such as paper pulp and pigment production, as well as hydroelectric plants which have used PCBs in transformer oil in the past. There is good evidence for significant industrial pollution with PCBs in central north Tasmania (Mondon *et al.* 2000), and there is anecdotal evidence that transformer oil was

used in the past in the central north to settle dust on roads. The levels at Lake Pedder, while superficially surprising, are easily explained by the proximity of the captured platypus to the hydroelectric generation village of Strathgordon. That the contamination was due to transformer oil is supported by the similar congener profile observed in animals caught at Cressy in Site 2. The Cressy sampling site is fed by water which has passed through a hydro-electric from the Great Lakes, a hydroelectric generating station. The low levels at site 3 and 4 were expected, with neither site being in close proximity to possible contamination sources.

The distribution pattern of PCB congeners was consistent with proximity of the different areas to possible sources of contamination. Site 1 incorporates not only agricultural areas, but is also in close proximity to heavy industry. This is reflected in the profile from Site 1, where all but 2 congeners were found in samples. The same pattern occurred in Site 2, although the percentage of animals contaminated with the different congeners was lower. Site 3 is located at the foothills of a mountainous national park, with no contamination sources nearby, and so had both lower overall levels of PCBs, and fewer congeners detected. Site 4 also had fewer congeners detected, consistent with its greater distance from sources of contamination. In general, the profiles for Sites 1, 2 and 4 were similar to those of both Aroclor 1254 and Clophen A50. The old-style transformer oil used in Tasmania closely resembles Aroclor 1254. As stated earlier, the profile for Lake Pedder (Site 5) is consistent with contamination by transformer oil.

It was only possible to obtain total PCB for 4 ulcerated animals, and congener profiles for 3. The total PCB level was not significantly different to levels in healthy animals. The congener profile for ulcerated animals showed a predominance of hexa- and heptachlorobiphenyls (about 80%), whereas healthy animals from the endemic area had almost equal levels of penta- and hexachlorobiphenyls (about 35% each), and about 20% heptachlorobiphenyls. Although it is tempting to assign some significance to these findings, the low sample number for ulcerated animals is problematic.

In conclusion, there is no evidence to support the hypothesis that the disease ulcerative mycosis of platypuses in Tasmania is directly associated with high levels of PCBs, DDT or Lindane in tailfat. In general, the levels of these toxicants are below those recorded for European and American otters and mustelids, in which deleterious effects have been reported, but are higher than those reported for most Australian animals (Bolton-Grob *et al.*

in press). Although the levels are generally low, the incidence of high levels of PCBs in some animals suggests that there are point sources of contamination in some areas that may pose a threat to individual animals.

Chapter 4

An investigation into some immune parameters for the platypus

4.1 Introduction

The epidemiology of the disease ulcerative mycosis of platypus (*Ornithorhynchus anatinus*) is poorly understood. As the putative causal agent is the fungus *Mucor amphibiorum* (Obendorf *et al.* 1993), it is possible that the disease occurs due to an underlying immunodeficiency of Tasmanian platypuses. Most fungal infections are opportunistic, requiring some form of immunosuppression in the host before disease can occur. Occasionally, a primary disease can result in secondary fungal infections. For example, diabetes in humans can act as a precursor for infections by *Candida albicans*, which is normally a commensal. The ability of such fungi to infect is often due to a combination of immune dysfunction and altered blood and mucus parameters, such as an increase in glucose concentration (Geerlings 1999, Darwazeh *et al.* 1991). Other conditions which directly affect the immune system, such as HIV/AIDS, result in colonisation by numerous, normally harmless, fungi (Threlkeld and Dismukes 1989).

In Australia, many fungal infections of introduced livestock are caused by continued exposure to environmental fungi, normally associated with particular weather patterns. Thus, horses in tropical Australia are prone to infection by *Hyphomyces* and *Basidiobolus* after the summer monsoon season (Miller and Campbell 1982), and sheep in New South Wales have been reported with zygomycosis caused by *Conidiobolus incongruus* after heavy autumn and winter rains resulted in good pasture growth and abundant decomposed plant material (Carrigan *et al.* 1992). These cases affected introduced species, with native species apparently unaffected. That is, the hosts were apparently not well adapted to the pathogens and disease resulted.

Truly pathogenic fungi are rare, and tend to be confined to geographical areas that have environments which support their restrictive requirements for growth. Although ulcerative mycosis is restricted to certain areas in Tasmania (Munday and Peel 1983, Obendorf *et al.* 1993), the fungus *M. amphibiorum* is found in cane toads in the Northern Territory, Queensland and New South Wales (Speare *et al.* 1994), and has been isolated from amphibians in Western Australia and Victoria (Creeper *et al.* 1998, Slocombe *et al.* 1995). It therefore seems that its environmental requirements are not fastidious. Certainly temperature variations do not kill the fungus. It can grow well at temperatures between 15°C and 35°C, and can survive freezing (Stewart 1998).

To determine whether the platypuses in the north of Tasmania are immunosupressed, it was necessary to first gather information on basic functioning of the platypus immune system. Monotremes in general, and platypus in particular, remain largely unstudied as far as immune function is concerned. Platypuses possess the major organs associated with immunity in mammals, but do not possess a truly mammalian lymphoid system. Instead of lymph nodes, the platypus possesses multiple lymph nodules, each corresponding to a lymphoid follicle as found in the cortex of the lymph nodes of other mammals (Whittington 1988). In monotremes, the lymphoid follicles are bathed by lymph, but the lymph has many alternative pathways, and does not necessarily contact all the follicles. The other monotreme, the echidna, has been shown to have a low anamnestic response, which may be explained by the lack of true lymph nodes. In other mammals, immune complexes and antigen transported by the lymph must pass through the lymph nodes. Because not all the lymphoid follicles are contacted by the lymph in the echidna, the presentation of antigen is not as effective as it is in other mammals, so that future presentation of antigen results in a poorer response. Possibly, such a poor response also occurs with platypus.

There is still some confusion regarding the thymus in platypus. Both wild and captive adult platypuses have been reported by McColl (1983) as possessing readily identifiable thymuses, but that author could not find the thymus in 5 juvenile captive platypuses. This may have been due to involution of thymus caused by chronic stress. Other authors have not been able to find a thymus in adult captive animals. The spleen of the platypus is similar to that of the other monotreme, the echidna. The capsule and trabeculae contain little smooth muscle, suggesting that the spleen is not a site of erythrocyte storage. Peyer's patches have been found in the small and large intestines of wild platypuses, but they are rare (Whittington 1988).

There are certain basic studies which can be undertaken to help understand immune function in mammals. The lymphocyte proliferation assay measures the ability of lymphocytes to proliferate in the presence of mitogens. There are several mitogens commonly used, such as phytohemagluttinin (PHA) and concanavilin A (ConA) for T cell proliferation, lipopolysaccharide (LPS) for B cell proliferation, and pokeweed mitogen (PWM) for B and T cell proliferation. These mitogens cross link cell surface receptors on lymphocytes, and produce non-specific proliferation. The assay is routinely used for humans (Rosetti *et al.* 1997, Finn *et al.* 2000), and has been developed for use with a wide range of animal species (Stone *et al.* 2000, De Marez *et al.* 1999, Quist *et al.* 1997).

Although the assay is not as specific as many that target particular cell surface markers that distinguish particular immune cell sub populations, it can give a general indication of the state of the B and T cell population.

The lymphocyte proliferation assay is a useful non specific assay, but does not reveal anything about the specific response of the platypus to *M. amphibiorum* infection. One of the hypotheses to explain the presence of ulcerative mycosis in Tasmanian platypuses, but not mainland populations, is that the putative causative agent, *Mucor amphibiorum*, has been introduced into the state. It has also been suggested that *M. amphibiorum* is not the only fungus capable of causing the disease. The closely related fungus *Mucor circinelloides* has been isolated from the ulcer of a platypus (Stewart *et al* .1999), and this fungus is also capable of forming sphaerule-like bodies *in vitro* (Stewart 1998). These bodies are the infectious stage of *M. amphibiorum*, and have been found in the ulcers of all ulcerated animals examined (Munday and Peel 1983, Obendorf *et al.* 1993, Connolly *et al.* 1998, Stewart *et al.* 1999).

If *M. amphibiorum* is a natural part of the flora of Tasmania, it would be expected that platypuses in all areas of the state would be routinely exposed to the fungus, and if *M. amphibiorum* is normally potentially pathogenic to platypuses, then animals exposed to the fungus, and which remained healthy, would presumably have mounted a humoral response, which could be determined through the use of various assays such as the ELISA. Such an assay would be useful not only for serologically screening platypuses in the endemic and non endemic area, but would also be potentially useful in determining whether other fungi, such as *M. circinelloides*, are implicated. However, there are several problems associated with the use of an ELISA for screening for antibodies against fungal pathogens.

In a previous study, rabbits were injected with formalin-killed, macerated isolates of *M. amphibiorum* from a Queensland cane toad and a Tasmanian platypus, and also with *M. circinelloides* isolated from a Tasmanian platypus. An ELISA was developed, but cross reactivity was so high that sera from all animals reacted equally with antigens produced from all 3 fungal isolates (Stewart 1996). If *M. amphibiorum* and *M. circinelloides* share only some common epitopes, then the high cross reactivity may have been due to the high dose given to the rabbits, and to the use of macerated material. In the wild, animals are more likely to be exposed to whole spores, and at a smaller dose. Also, the fungal material macerated included both spores and hyphae. Naturally occurring infections do not show evidence of hyphae (Munday and Peel 1983, Obendorf *et al* .1993). Thus, it was possible

that the cross reactivity seen was due to the hyphae, and not to the probable infectious stage, the spore. Using spores from either *M. amphibiorum* or *M. circinelloides* to act as the antigen in an ELISA demonstrated that cross reactivity was still as high as for macerated hyphae and spores. Apparently, *M. amphibiorum* and *M. circinelloides* share many epitopes, both in the hyphae and the spores.

Problems associated with cross reactivity are common when dealing with pathogens from closely related species. For example, ELISAs developed to detect prior exposure in fish to *Vibrio harveyii* have faltered due to cross reactivity with other environmental bacteria (pers comm Crosbie). Many bacterial species are highly cross reactive, due to common antigens such as lipopolysaccharide. The problem is particularly prevalent in fungal species, and much work has been done in raising monoclonal antibodies specific to epitopes from individual species. However, cross reactivity can still occur, particulary in the Mucoracaea. Monoclonal antibodies raised against water soluble somatic antigens and wall fraction antigens from *Rhizopus arrhizus* were found to be equally reactive with other members of the Mucoracaea, but not with fungi from other families (Jensen *et al.* 1996). The presence of antibodies directed against common epitopes complicates serological screening. For example, serum from healthy people contains antibodies directed against one or more components of the surface of the pathogenic fungus *Cryptococcus neoformans*, but these antibodies are also reactive with epitopes from other yeast cells (Keller *et al.* 1994).

As stated earlier, if the fungus *M. amphibiorum* has been introduced into Tasmania, animals outside the endemic area of the state presumably would not carry antibodies specific to *M. amphibiorum*, but may have antibodies to other closely related species of fungus. If animals on the mainland were routinely exposed to *M. amphibiorum*, and had co-evolved with the fungus, it could be expected that populations there would have antibody levels to this fungus that were significantly greater than those for unexposed Tasmanian platypuses, which would only carry antibodies to the ubiquitous environmental fungi which may share epitopes with *Mucor amphibiorum* . In such a situation an ELISA could still be useful for comparing mainland and Tasmanian platypus.

To overcome some of the problems associated with an ELISA, methods such as the Western blot can be employed. This assay involves isolating the proteins from the fungus, and then separating the proteins, based on size, by running an electric current through a gel to which the fungal proteins have been added. An electric current is then used to transfer the

separated proteins to nitrocellulose. The nitrocellulose is immersed in dilute serum from the animal of interest. If the serum contains antibodies specific to any of the proteins, they bind to them. The nitrocellulose can be stained, and the bands visualised. Not only does this method allow for visualisation of reactions, but can also detect differences between species. Some species contain unique antigenic proteins which are not apparent in an ELISA but which become visible in a Western blot. The Western blot has the disadvantage of requiring relatively large quantities of reagents.

Another hypothesis to explain the presence of ulcerative mycosis in Tasmanian platypuses is that there is a subpopulation of atopic platypuses in Tasmania. Atopy describes the condition in which there is an inappropriate immune response to innocuous environmental allergens. It is characterised by Type 1 hypersensitivity, in which IgE is directed against the allergens. The resultant release of mediators produced by IgE sensitised mast cells produces an acute inflammatory reaction (Roitt *et al.* 1996). As well as the common aliments associated with the condition, such as hay fever, asthma and rhinitis, the release of the mediators can also produce severe dermatitis and eczema (Sampson 1988, behrendt and Ring 1990). Although the predominant allergens assocaited with atopy are pollen and dustmites, some fungal species are also capable of producing atopic infections (Koyama *et al.* 2000, Nielsen 1984). The infections are often associated with severe itching (Behrendt and Ring 1990), which has also been noted in platypuses suffering from ulcerative mycosis. It is possible that the disease occurs in Tasmanian platypuses because of the presence of an atopic subpopulation. Because atopy is characterised by elevated IgE levels, presumably the screening of platypus sera for the immunoglobulin with an ELISA could reveal whether elevated levels were present in ulcerated animals, or in animals from both within and outside of endemic areas. Before developing such an ELISA, it was first necessary to identify and isolate platypus IgE, and then, because of the small quantity of native IgE available, produce recombinant IgE.

4.2 Materials and Methods

4.2.1 Lymphocyte proliferation assay

Platypuses were trapped from 5 areas in Tasmania. Two sites were in the non endemic area (Salmon Ponds and Lake Pedder), and 3 in the endemic area (Glengarry, Upper Blessington and Cressy) (Table 4.1).

All animals were captured in fyke nets. After capture, the animals were placed in hessian sacks for transport to the laboratory. At the laboratory, the animals were placed in a hessian sack from which the corner had been removed. The bill of the animal was allowed to protrude through the hole, and blood was taken from the dorsal venous sinus using a 22 or 25g needle. The blood collected was placed in an EDTA treated tube, and stored at ambient temperature before the proliferation assay was performed. The time between capture and bleeding never exceeded one hour. With the exception of one sample (E5), all assays were set up within 3 hours of blood collection. The sample E5 was incubated for 48 hours prior to stimulation. Four ulcerated platypuses were tested (Table 1). The female platypus (E4) from Upper Blessington was severely debilitated, with approximately 50% of the tail missing due to ulceration. The male platypus (E3) from the same area had a large ulcer (4cm diameter and 0.5cm deep) on the tail, but was otherwise in good condition. The Cressy ulcerated platypus (E2) had a small ulcer on the inside of the right hip (2cm long by 1cm wide). The Glengarry ulcerated platypus (E5) had ulcers on the tail, hips and back.

Peripheral blood mononuclear cells (PBMC) were isolated by mixing the blood 1:1 with Dulbecco's medium (DMEM). The DMEM was reconstituted in MilliQ water and supplemented with 3.7g/l sodium bicarbonate. The pH was adjusted to 7.4 with concentrated HCl. The solution was then filter sterilised under vacuum and stored at 4°C, following overnight incubation at 37°C to check for bacterial contamination. To 500ml of the medium was added 100ul of a 1.0M stock solution of HEPES, 500ul of a 0.05M stock solution of 2-B-mercaptoethanol, 1.5ml gentamicin 100 and 5ml of glutamine. The blood was then carefully layered onto twice the volume of Histopaque in a round-bottomed tube. The sample was centrifuged for 35 minutes at 400g. The cell layer was removed with a glass pipette and placed in a sterile v-bottomed Falcon tube with 10ml of DMEM. This was centrifuged for 10 minutes at 400g. The supernatant was discarded, the pellet of cells resuspended, and a further 10ml of DMEM added. After a further 10 minute, 400g centrifugation the pellet was resuspended in 0.5ml of DMEM with 10% fetal calf serum/ added. Cells were enumerated using a haemocytometer. Viability of cells was determined using trypan blue exclusion.

Cell density was adjusted to either $1x10^6$/ml, $2x10^6$/ml or $4x10^6$/ml using DMEM + 10% FCS, and the cells placed in 96 well, round bottomed plates. The mitogens Phytohemagglutinin (Sigma), Lipopolysaccharide (Sigma), Concanavilin A (Sigma) and Pokeweed Mitogen (Sigma) were reconstituted in DMEM, and added to the wells at the desired concentrations. The range of concentrations chosen were those that had previously

been shown to induce proliferation in PBMC from other mammalian species (Lahvis *et al.* 1993, Wilkinson *et al.* 1992, Senogle *et al.* 1978). The plates were incubated at 32C° in air with 10% CO_2. The cultures were pulsed 18 hours prior to harvesting with 1.0uCi 3H-Thymidine. A Skatron cell harvester was used to harvest the cells. Disks were dried overnight, and then added to 2ml of scintillation fluid and label incorporation was determined using a Beckman scintillation counter. Results are expressed as a stimulation index (SI), where SI = counts per minute stimulated cells/counts per minute unstimulated cells. All treatments were performed in triplicate wells. Control (unstimulated) cells were pulsed after the same incubation time as stimulated cells. Cell viability was determined by trypan blue exclusion.

Optimal cell densities, incubation times and mitogen concentrations were determined for use with platypus PBMC.

Data were analysed using the JMP 3.2.1 statistical package. Data were checked for normality and homogeneity of variances using the Shapiro-Wilk W test and Bartlett's test respectively, and a one-way analysis of variance (ANOVA) was performed at a significance level of $P<0.05$.

4.2.2 Western Blot

4.2.2.1 Preparation of rabbit anti-platypus serum

Blood was taken from a male platypus captured in the non-endemic area (Salmon Ponds), and the serum separated off. The serum was diluted 1:2 with saline, and a saturated ammonium sulphate solution (final concentration 45% v/v) added. The solution was stirred for 30 minutes at 4°C. The precipitate was deposited by centrifuging at 3000rpm for 30 minutes at 4°C. The precipitate was washed with 40% ammonium sulphate and re-centrifuged. The precipitate was redissolved in the same volume of PBS as the original serum. The solution was then centrifuged to remove any insoluble material. The resultant gamma globulin was reprecipitated using 40% ammonium sulphate. The precipitate was deposited by centrifuging, and washed with 40% ammonium sulphate. The precipitate was then redissolved in a minimum volume of PBS. The gamma globulin was dialysed against

seven changes (2L) of PBS at 4°C, and centrifuged to remove any precipitate. A 1:20 dilution was prepared, and the absorbance at 280nm measured using a spectrophotometer. The protein content was determined, assuming that a reading of 1 was equivalent to a gamma globulin content of 0.74mg/ml (fowl gamma globulin concentration = 0.74mg/ml at absorbance 1). Two rabbits were injected with 0.5ml of the gamma globulin mixed with an equal volume Freunds complete adjuvant, with the protein concentration being 12ug/ml. The rabbits were re incoculated six weeks later with the same volume of platypus gamma globulin in Freunds incomplete adjuvant. The process was repeated six weeks later, with the rabbits being bled after a further four weeks. An ELISA was performed to determine the response of the rabbits to the platypus gamma globulin.

4.2.2.2 Rabbit anti-*Mucor amphibiorum* and anti-*Mucor circinelloides* serum

This serum was produced as described previously (Stewart 1996). Briefly, one isolate of platypus-derived *M. amphibiorum*(Tas3), one cane toad-derived isolate (Q2), and one platypus-derived isolate of *M. circinelloides* (Tas 5) were grown to maturity, formalin-killed, and macerated. Rabbits were injected with the macerated material three times at four week intervals, with the final bleed taking place four weeks after the final injection.

4.2.2.3 *M. amphibiorum* and *M. circinelloides* isolates used in Western blots

Six isolates of *M. amphibiorum* and one of *M. circinelloides* were used in the Western blots (Table 4.3).

All isolates were grown on Sabouraud's agar, and incubated at 25°C for seven days. The cultures were checked for bacterial contamination, and mated against tester strains to confirm identity. Subcultures were then taken from each plate, and plated out onto fresh Sabouraud's agar. Six plates of each isolate were incubated at 25°C for five days, and checked for contamination. Cultures free of contamination were frozen at -80°C for two days. The cultures were removed from the freezer, and the fungal material scraped off with a scalpel, and weighed. The fungal material was placed in tubes with an equal volume of a 0.05M Tris and 0.15M NaCl solution, plus an additional 0.5ml per isolate. Each isolate was sonicated for seven minutes, continuous cycle, using a Bransden sonicator. Following sonication, an additional 1ml volume of Tris/NaCl solution was added. Each isolate was

then centrifuged at 2600g for 30 minutes. The supernatant was removed, and aliquoted into cryo tubes at 100ul per tube. The tubes were frozen at -80°C until required.

4.2.2.4 Gel electrophoresis

Four strength of resolving gels were attempted; 10, 15, 17 and 20%. The 10% gel comprised 5ml of polyacrylamide monomer (Sigma Acrylamide/Bis) with 3.75ml of running buffer and 6.25ml distilled water. The solution was degassed, and 100ul of 10% ammonium persulphate, 150ul of 10% SDS, and 10ul of TEMED (Sigma) added. Higher percentage gels were prepared by increasing the concentration of the polyacrylamide. Stacking gels for all resolving gels comprised 0.66ml polyacrylamide monomer, 1.25ml stacking buffer, 3ml distilled water, 50ul ammonium persulphate, 50ul SDS and 10ul Temed.

The fungal samples were reduced by adding an equal volume of reducing diluent (60mM Tris, 25% glycerol, 2%SDS, 14.4mM 2-mercaptoethanol, 0.1% bromophenol blue), and boiling for two minutes. The samples were loaded into the gels at 10ul per well, and electrophoresed in buffer (25mM Tris, 192mM glycine, 0.1% w/v SDS) until the dye front was within 1cm of the bottom of the gel (usually 90 minutes). Protein bands were visualised using a silver stain procedure modified from Harlow and Lane (1988). Briefly, gels were fixed for 30 minutes in a solution of 50% ethanol, 10% acetic acid and 40% distilled water. They were then placed in a 5% ethanol, 1% acetic acid, 94% distilled water solution for 15 minutes. The gels were washed three times in distilled water, placed in a solution of 0.2% sodium thiosulphate for one minute, washed three times, exposed to a 0.2% silver nitrate/0.15% formalin solution for 20 minutes, washed three times, and then developed in a solution of 6% sodium carbonate, 0.05% formalin and 0.2% sodium thiosulphate. Development was stopped with 1.5% sodium EDTA. Each gel was loaded with a molecular weight markers (Bio Rad).

4.2.2.5 Immunoblotting

Specific activity of the rabbit anti-*M. amphibiorum* and rabbit anti-*M. circinelloides* sera was assessed by Western blot. Fungal samples prepared as described previously were run on SDS-Page (15% resolving gel and 4% stacking gel) under reducing conditions. Separated proteins were electrotransferred onto 0.45um nitrocellulose membrane (MFS, USA) in transfer buffer (Tris 48mM, glycine 39mM, methanol 20%v/v) using a Semiphor

semi-dry transfer unit with a maximum current setting of 0.8 mA cm^{-2} and a voltage limit of 50v for 60 minutes. Success of protein transfer was confirmed using a temporary total protein stain (0.1% ponceau S w/v and 5% glacial acetic acid v/v, Sigma). The temporary stain was removed by rinsing the nitrocellulose in Tris buffered saline (20mM Tris, 500mM NaCl, pH 7.5). The molecular marker band was excised and permanently stained with Amido black (10% glacial acetic acid, 25% iso-proponol, 0.1% Amido black, 65% distilled water) for one minute, and then destained (10% glacial acetic acid, 25% iso-proponol, 65% distilled water) for 30 minutes (Gershoni and Palade 1982). Residual binding sites on the nitrocellulose membranes were blocked with 3% skimmed milk in Tris buffered saline (TBS) for one hour, washed with TBS, TBS+0.05% tween 20, and TBS again for five minutes per wash. The membranes were then incubated in the different anti sera diluted in blocking buffer (1% skimmed milk in TBS) for 90 minutes and the wash step repeated. The membranes were then incubated in goat anti-rabbit IgG (whole molecule) conjugated to horseradish peroxidase (Sigma) diluted 1:1000 in blocking buffer for 90 minutes. The wash step was repeated, and the reactive bands visualised with 3,3' diaminobenzidine tetrahydrochloride (DAB) peroxidase substrate (Sigma). All steps were carried out at room temperature. Pre bleeds and lanes containing only reducing buffer were included for each blot.

4.2.2.6 Immunoblotting using platypus sera

4.2.2.7 Platypus sera used in Western blots

Serum from 12 platypuses was collected, with four coming from the endemic area in Tasmania, (including an ulcerated animal), four from non endemic areas of Tasmania, two from Victoria and two from Queensland. Blood for both this assay, and the ELISA, was collected as described in the general materials and methods, and the serum separated using serum separation tubes. The serum was frozen at -80°C until required.

The immunoblotting procedure for use with platypus sera was optimised using sera from an ulcerated platypus that had been euthanased. Dilutions of 1:25, 1:50, 1:75, 1:100, 1:125 and 1:150 were tested against the six fungal isolates, with a 1:100 dilution being selected as optimal. Platypus sera from other animals was used at a dilution of 1:100.

When testing sera from other platypuses for reactivity with the fungal isolates, the procedure was the same as for immunoblotting using rabbit sera, with the following exception. The platypus sera was diluted 1:100 in 1% skimmed milk, and the nitrocellulose

membrane was incubated for 90 minutes in the solution. The membrane was washed three times, and then treated as per the rabbit sera protocol. When performing an assay, a replicate gel was cast and run, and transferred to nitrocellulose at the same time as the gel that was to be used for immunoblotting. This was performed due to the unreliability of the ponceau stain in staining low molecular weight bands. To ensure that transfer to the nitrocellulose membrane was successful, one of the membranes was stained with Amido black. If transfer was successful, the second membrane was the probed with the desired platypus serum.

4.2.3 ELISAs

4.2.3.1 Platypus anti-*M. amphibiorum* ELISA

The ELISA was developed and performed by Dr Richard Whittington (Elizabeth Macarthus Agricultural Institute 1999) Briefly, a culture of *M. amphibiorum* grown on Sabouraud's agar was disrupted by sonication, diluted to 10ug/ml in borate buffer (100mM boric acid, 25mM disodium tetraborate, 75 mM NaCl pH 8.4), and 100ul of this suspension added to each well of polystyrene microtitre plates and the plates incubated for 16 hours at 4°C. After washing five times in distilled water with 0.05% v/v tween 20, free binding sites were blocked by adding PBST and 1w/v ovalbumin to each well and incubating plates for 30 minutes at room temperature. After washing plates, 100ul of platypus serum diluted 1:200 in PBSTO was added to each well and plates incubated for 90 minutes at room temperature. After washing as above, 100ul of rabbit antiserum raised against platypus IgG and IgM diluted 1:3200 in PBSTO, was added to each well and the plates incubated for 90 minutes at room temperature. After washing, 100ul of swine anti-rabbit immunoglobulin-horseradish peroxidase conjugate diluted 1:1500 in PBSTO was added and the plates incubated for 90 minutes at room temperature. After washing, the chromogen was added and the plates incubated at room temperature on a shaker. The reaction was stopped after 20 minutes by the addition of 0.01% w/v sodium azide in 0.1M citric acid. Absorbance was read at 405nm.

4.2.3.2 Platypus sera used in the ELISA

Five sera samples were obtained from platypuses at the Melbourne Zoo in Victoria. A total of 17 sera were from Tasmanian animals. Nine sera samples were obtained from healthy animals in the endemic area of Tasmania, four samples from healthy animals in the non endemic area, three from ulcerated animals in the endemic area and one from an animal with a healed lesion on the foot.

4.2.3.3 IgE Elisa

Thirty serum samples were tested; two from Victoria, two from Queensland, 23 from healthy Tasmania platypuses, and three from ulcerated Tasmanian platypuses. The Tasmanian samples were collected by bleeding the platypus from the dorsal venous sinus of the bill, and placing the blood in serum separation tubes (Greiner Vacuette®). The tubes were spun at 400g for 5 minutes, the sera pipetted off and placed in eppendorf tubes before freezing at -80°C until required. The mainland samples were donated by the Healesville Sanctuary, Victoria, and by Professor Cliff Gallagher from Taronga Zoo. All plates used for the ELISAs were from the one batch of 96 well plates from TPP®.

4.2.3.4 Collection of platypus splenic material

Two female platypuses were captured at the Salmon Ponds, Tasmania. The animals were transported to the Kingston Veterinary Hospital, where they were anaesthetised with Isofluorane. A small section of spleen (approximately 2cm long) was removed from each animal, and immediately frozen in liquid nitrogen. The samples were sent to Dr Lars Hellman, Uppsalla University Sweden, where immunoglobulin classes were determined, and recombinant IgE produced.

4.2.3.5 Production of rabbit anti-platypus serum

Recombinant IgE was added to Montanide adjuvant to produce a final concentration of 250 mg/ml protein. Two rabbits were injected with 1ml of the solution. After six weeks, the rabbits were re-inoculated. Four weeks later, the final booster was given, and after a further 3 weeks the animals were bled, the serum removed, and frozen at -80°C until required.

4.2.3.6 Production of *M. amphibiorum* antigen

The *M. amphibiorum* antigen was produced by growing a culture of Tas1 on 5 plates of Sabouraud's agar, until the cultures covered the plates. The plates were frozen at -80°C, the fungus scraped off and placed in a glass tissue grinder and ground for 10 minutes. The macerated material and fluid was added to eppendorf tubes and centrifuged at 7000g for 5 minutes. The supernatant was removed and used fresh as the antigen.

4.2.3.7 Optimisation steps

4.2.3.7.1 Optimisation of goat anti-rabbit conjugate

Optimal reagent dilutions were determined after performing a series of checkerboard titrations (Crowther 1995)

The goat anti-rabbit conjugate (Bio-Rad) was optimised by adding the rabbit anti-platypus IgE to the first row of wells at an initial dilution of 1:20 in coating buffer (50mM sodium hydrogen carbonate pH 9.5), and serially diluting across the plate to a final dilution of 1:20480. The plate was incubated overnight at 4°C. Blocking buffer was added at 200ul per well, and the plate incubated for 60 minutes at room temperature. The plate was washed three times by hand with PBS, using a wash bottle. The goat anti-rabbit horseradish peroxidase conjugate was added to the top row of wells at an initial dilution of 1:200 in 0.25% casein ($w.v.^{-1}$), and serially diluted down the plate to a final dilution of 1:25600. After a 60 minute incubation at room temperature, the plate was washed three times as previously described, and 0.05% o-phenylenediamine (OPD) free base (Sigma) in citrate phosphate buffer (pH5) with 0.012% ($w.v.^{-1}$) H_2O_2 was added to all wells. After 15 minutes in the dark, the reaction was stopped with the addition of 50ul per well of 3M HCl. The optical density was read at 492nm using a microplate reader (Spectra Rainbow Therma; Tecan). The goat anti-rabbit horseradish peroxidase conjugate dilution was determined to be 1:2000 and was defined as the least amount required to yield a significant result (Arkoosh and Kaattari 1990). The last row of wells acted as controls, with no rabbit anti-platypus IgE added.

4.2.3.7.2 Optimisation of rabbit anti-platypus IgE

A similar procedure was used to determine the optimal rabbit anti-platypus IgE concentration. The plates were coated with recombinant IgE at an initial concentration of 5ug/ml, and doubly diluted across the plate to a final concentration of 4.8pg/ml. The plate was incubated and washed as for the first optimisation, before the addition of the rabbit anti-platypus IgE serially diluted down the plate at an initial dilution of 1:50. After incubating and washing, the goat anti-rabbit conjugate was added at a constant dilution of 1:2000. As in the first optimisation, the optimal dilution of rabbit anti-platypus IgE (1:6400) was defined as the least amount that would yield a significant result (Arkoosh and Kaattari 1990). The last row of wells acted as controls, with no antigen (recombinant platypus IgE) added.

4.2.3.7.3 Optimisation of the *M. amphibiorum* antigen

A similar procedure was used to determine the optimal concentration of *Mucor* antigen. The plates were coated with *Mucor* antigen at an initial dilution of 1:100, and serially diluted across the plates before overnight incubation at 4°C. The plates were washed before the addition of platypus sera from a heavily ulcerated animal at an initial dilution of 1:100, and serially diluted across the plates. The plates were incubated and washed, and rabbit anti-platypus IgG added to all wells at a dilution of 1:3200. After further incubation and washing, goat anti-rabbit at 1:2000 was added to all wells. The last row of wells acted as controls, with no antigen added.

4.2.3.7.4 ELISA for the detection of *M. amphibiorum*-specific platypus IgE

The wells were coated with the *Mucor* antigen at 1:800 and incubated overnight at 4°C. The plates were flicked to remove the antigen, and then blocked with 200ul of 0.5% casein ($w.v^{-1}$) in PBS (pH 7.2) for 1 hour at room temperature. Plates were then washed three times in PBS by hand, using a wash bottle. The plates were flicked dry, and 50ul of platypus serum in 0.25% casein ($w.v^{-1}$) was added to all wells but the blanks at a starting dilution of 1:400 and doubly diluted down to 1:51,200. The plates were incubated for 1 hour at room temperature, washed 3 times as previously described, and 50ul of rabbit anti-platypus IgE added to all wells at a constant 1:6400 dilution. The plates were incubated for 1 hour at room temperature, washed as previously described, and 50ul of goat anti-rabbit sera (Bio Rad) added at 1:2000 to all wells. Plates were incubated for I hour at room temperature, washed as previously described, flicked dry and OPD + H_2O_2 added at 50ul per well. The reaction was stopped after 15 minutes with 3M HCL at 50ul per well, and the plates read at 492nm.

Each serum sample was replicated using two wells. Each plate had two rows of blank wells in which all reagents except the samples were present. Two rows on each plate were also run with serum from the same heavily ulcerated platypus. The optical density of each sample was calculated by subtracting the optical density of the blank wells from that of the sample wells. A control plate was run with serum from the heavily ulcerated animal, and a mean OD calculated for this sample. On test plates, if the OD of the heavily ulcerated animal serum was $\geq \pm$ 10% of the mean of the control plate, the plate was discarded.

The data were analysed with a one-way analysis of variance (ANOVA) using the JMP 3.2.1 statistical package. The Shapiro-Wilk W test and Bartlett's test were used to test assumptions of normality and homogeneity of variances respectively. Significant

differences were highlighted as multiple comparisons of means (Tukey-Kramer HSD test) were performed. A significance level of $P<0.05$ was adopted.

4.3 Results

4.3.1 Lymphocyte proliferation

Stimulation indices for platypus lymphocytes exposed to PHA were optimal at five days incubation, and at cell densities of $1x10^6$ and $2x10^6$ per ml. Optimal mitogen concentration was between 12ug/ml and 25ug/ml. Peak stimulation of approximately eight was achieved in this assay with $1x10^6$ cells/ml (Figures 4.1, 4.2, 4.3).

Cells exposed to the mitogen ConA responded well at cell densities of $2x10^6$, and for incubation times of four, five, six and seven days. Optimal stimulation was achieved after 6 days incubation at a concentration of 12ug/ml of ConA (Figure 4.4).

The mitogen ConA induced the greatest proliferation in platypus PBMC, followed by PHA, LPS and PWM (Figure 4.5, Tables 4.4, 4.5, 4.6, 4.7).

Optimum cell density for PHA was $1x10^6$ cells/ml (Figure 4.6), and $2x10^6$ cells/ml for Con A (Figure 4.7).

One ulcerated platypus responded well to ConA, LPS and PHA (Figure 4.8).

The mean response of healthy platypus PBMC to all mitogens was greater than that in ulcerated animals (Table 4.8), and the response was significantly greater for PHA and PWM (Figure 4.10).

Mean counts per minute (cpm) for unstimulated platypus lymphocytes were generally below 500, regardless of cell density or incubation time (Table 4.9).

There was no significant difference between mean counts per minute of unstimulated PBMC from healthy animals (mean 451, SD 237, range 189-866) or ulcerated animals (mean 507, SD 182, range 338-700). Calculation based on PBMC at $2x10^6$/ml.

4.3.2 Western blot

Isolates of *M. amphibiorum* possessed low molecular weight (LMW) proteins between 6.2kDa and 18.4kDa (Figure 4.11), and proteins of high molecular weight (HMW) above 43kDa which reacted with sera from rabbits inoculated with both *M. circinelloides* (Figure 4.11) and *M. amphibiorum*(Figure 4.12).Sera from rabbits inoculated with *M. circinelloides* reacted with HMW and LMW proteins from *M. amphibiorum* , but with only HMW proteins from *M. circinelloides* (Figure 4.11). proteins.

Serum from a heavily ulcerated platypus reacted with both HMW and LMW bands when *M. amphibiorum* was used as the antigen, but only with HMW bands when *M. circinelloides* was the antigen Figure 4.13). The reactive bands were the same as those seen when serum from rabbits injected with either *M. amphibiorum* or *M. circinelloides* reacted with either *M. circinelloides* or *M. amphibiorum* antigens (Figures 4.11, 4.12). Healthy platypuses from the endemic area, non endemic areas, Victoria and Queensland all possessed antibodies that only reacted with the HMW proteins of both *M. amphibiorum* and *M. circinelloides* (Figure 4.14).

4.3.3 ELISAs

4.3.3.1 Platypus anti-*M. amphibiorum* ELISA

The optical densities achieved for Tasmanian healthy platypuses from endemic or non endemic areas were not significantly different, but did differ significantly from both ulcerated platypuses and healthy Victorian animals. The reading for the platypus with the healed lesion was similar to those for the ulcerated animals (Figure 4.10).

4.3.3.2 ELISA for the detection of *M. amphibiorum*-specific platypus IgE

The optical densities recorded for ulcerated platypuses were not significantly different from healthy Tasmanian platypuses, or Victorian or Queensland platypuses (Table 4.10).

Id code	Location	Sex	Weight kg	Capture month	Ulcerated
NE1	Lake Pedder	Male	1.8	Jan	No
NE2	Lake Pedder	Female	1.1	Jan	No
NE3	Lake Pedder	Male	2.1	Jan	No
NE4	Salmon Ponds	Female	1.1	June	No
NE5	Salmon Ponds	Male	1.5	June	No
NE6	Salmon Ponds	Male	1.2	June	No
E1	Cressy	Female	.7	June	No
E2	Cressy	Male	2.3	June	Yes
E3	Upper B'ton	Male	2	July	Yes
E4	Upper B'ton	Female	.7	July	Yes
E5	Glengarry	Male	1.5	June	Yes
E6	Glengarry	Female	.8	April	No

Table 4.1: Details of platypuses used for the lymphocyte proliferation assay. Animals with the prefix (NE) are from non-endemic areas, those with the prefix (E) are from endemic areas.

Days incubation	Cell Density $1x10^6$/ml	Cell Density $2x10^6$/ml	Cell Density $4x10^6$/ml
3	PHA	PHA	PHA
4	PHA	PHA ConA	PHA
5	PHA	PHA ConA	PHA
6		PHA ConA LPS PWM	
7	PHA ConA PWM	PHA ConA	
8		ConA PHA LPS PWM	

Table 4.2: The mitogens used for different incubation times, and for different concentrations of platypus PBMC.

Isolate	Species	Host	Location
Q1	*M. amphibiorum*	Cane toad	Queensland
Q2	*M. amphibiorum*	Cane toad	Queensland
W1	*M. amphibiorum*	Green tree frog	West Australia
Tas1	*M. amphibiorum*	Platypus	Tasmania
Tas2	*M. amphibiorum*	Platypus	Tasmania
Tas3	*M. amphibiorum*	Platypus	Tasmania
Tas5	*M. circinelloides*	Platypus	Tasmania

Table 4.3: Details of fungal isolates used in the Western blot

ID	ug/ml	max. cpm	max. SI	Days inc.
NE1	6	6905	12.7	7
NE2	6	3759	4.3	7
NE3	6	595	3.1	7
E1	12	6274	14.7	6
E2	*12*	*3015*	*6.7*	*6*
E3	*12*	*132*	*0.4*	*8*
E4	*12*	*290*	*0.4*	*8*
E6	6	2533	6.9	6

Table 4.4: The maximum response of platypus PBMC to the mitogen ConA. Italics indicate ulcerated animals.

ID	ug/ml	max. cpm	max. SI	Days inc.
NE1	6	2353	4.3	7
NE2	6	4225	4.9	7
NE3	25	1246	6.6	7
NE4	25	1173	7.3	5
NE5	25	1429	3.5	5
NE6	12	4220	5.6	5
E1	12	1557	3.6	6
E2	*12*	*1356*	*3*	*6*
E3	*12*	*518*	*1.5*	*8*
E4	*12*	*1599*	*2.3*	*8*
E5	*12*	*865*	*4.5*	*5*

Table 4.5: The maximum response of platypus PBMC to the mitogen PHA. Italics indicate ulcerated animals.

ID	ug/ml	max. cpm	max. SI	Days inc.
NE1	15	2316	4.3	7
E1	25	1025	2.4	6
E2	*25*	*1340*	*3*	*6*
E3	*10*	*800*	*2.4*	*8*
E4	*10*	*1285*	*1.8*	*8*

Table 4.6: The maximum response of platypus PBMC to the mitogen LPS. Italics indicate ulcerated animals.

ID	ug/ml	max. cpm	max. SI	Days inc.
NE1	6	1034	1.9	7
NE2	6	1818	2.1	7
NE3	6	325	1.7	7
E1	12	785	1.8	6
E2	*12*	*639*	*1.4*	*6*
E3	*2*	*149*	*0.4*	*8*
E4	*2*	*118*	*0.2*	*8*

Table 4.7: The maximum response of platypus PBMC to the mitogen PWM. Italics indicate ulcerated animals.

Mitogen	Healthy platypus Stimulation indices mean and range		Ulcerated platypus Stimulation indices mean and range	
PHA	**5.1**	3.6-7.3	**2.8**	1.5-4.5
ConA	**8.3**	3.1-14.7	**2.5**	0.4-6.7
LPS	**3.3**	2.4-4.3	**2.4**	1.8-3
PWM	**1.8**	1.8-2.1	**0.7**	0.2-1.4

Table 4.8: The mean stimulation index and range of responses of PBMC from healthy and ulcerated platypus to four mitogens.

Id	Cell density	3 day inc.	4 day inc.	5 day inc.	6 day inc.	7 day inc.	8 day inc.
NE1	$2x10^6$					542	
NE2	$2x10^6$					866	
NE3	$1x10^6$					189	
NE4	$1x10^6$	133	236	161			
NE5	$2x10^6$	195	270	411			
NE6	$4x10^6$	209	1064	755			
E1	$2x10^6$				427		
E2	*$2x10^6$*				*484*		
E3	*$2x10^6$*						*338*
E4	*$2x10^6$*						*700*
E5	*$1x10^6$*	*121*	*1086*	*192*			
E6	$1x10^6$		434	494	363	369	

Table 4.9: The counts per minute for unstimulated platypus PBMC from healthy and ulcerated (italicised) animals, incubated for different times and densities.

Sera from:	n=	optical density	S.D.	Range
Vic. platypus	2	0.0180	0.0021	0.0165 - 0.0195
Q'land platypus	2	0.0245	0.0014	0.0235 - 0.0255
Tas. platypus (healthy)	23	0.0165	0.011	0.003 - 0.0450
Tas. platypus (ulcerated)	3	0.0170	0.0099	0.006 - 0.0255

Table 4.10: The optical densities recorded for platypuses tested in an ELISA for the detection of IgE specific for *M. amphibiorum* antigens

Fig 4.1

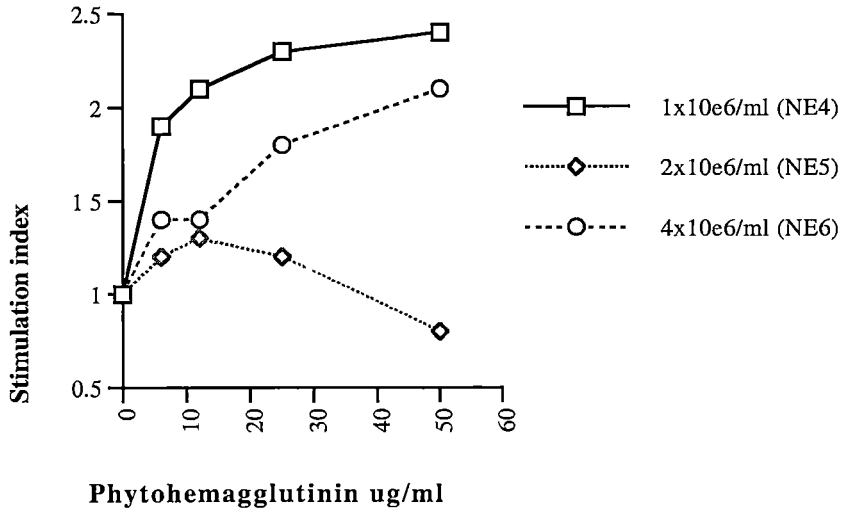


Fig 4.2

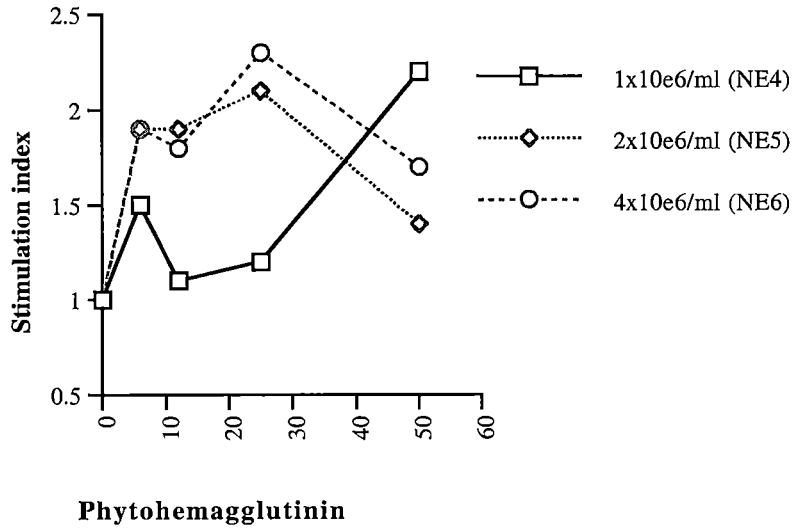
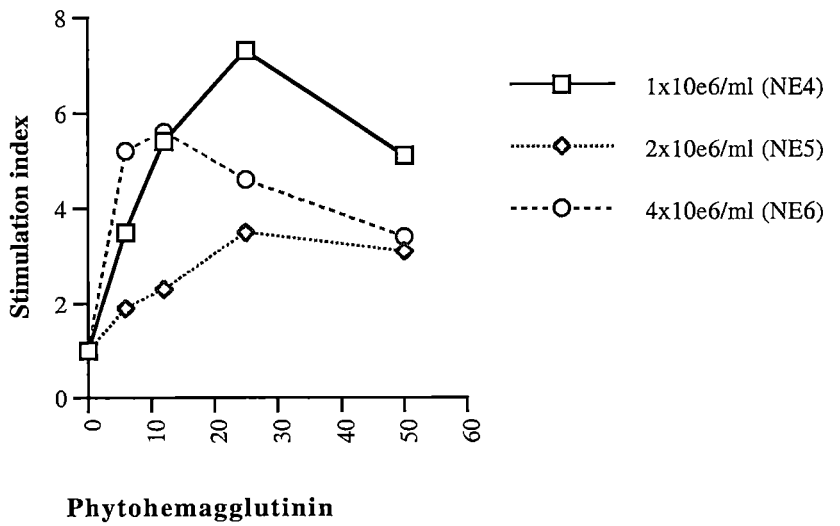


Fig 4.3



Figures 4.1, 4.2, 4.3: The effect of different cell densities and incubation times (3 day, 4 day and 5 day) on the proliferation of platypus PBMC exposed to the mitogen PHA

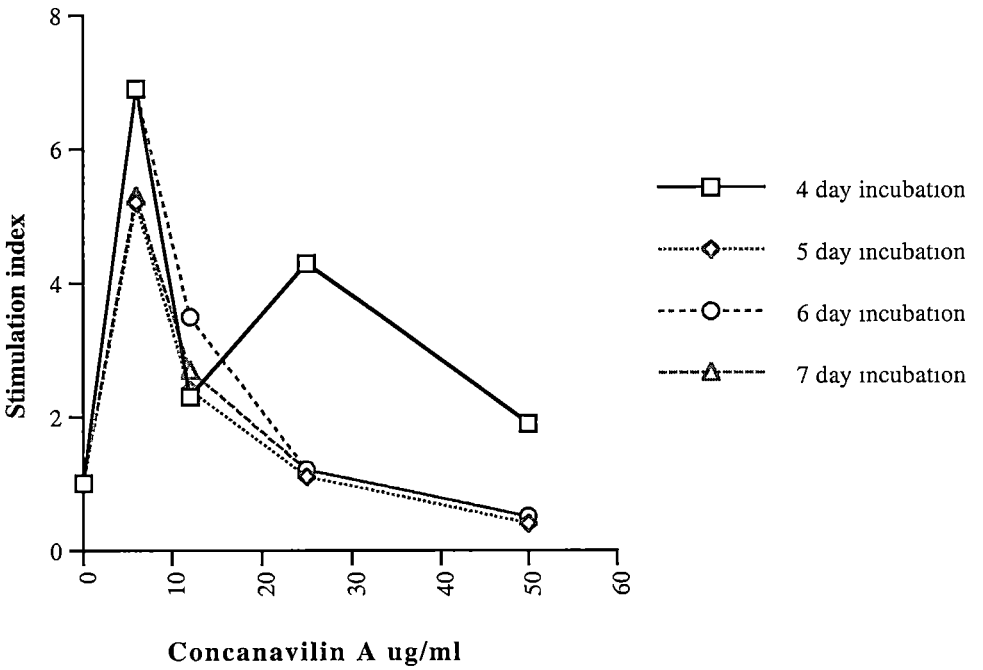


Figure 4.4: The effect of different incubation times on the proliferation of platypus PBMC from one animal (E6) exposed to the mitogen Con A.

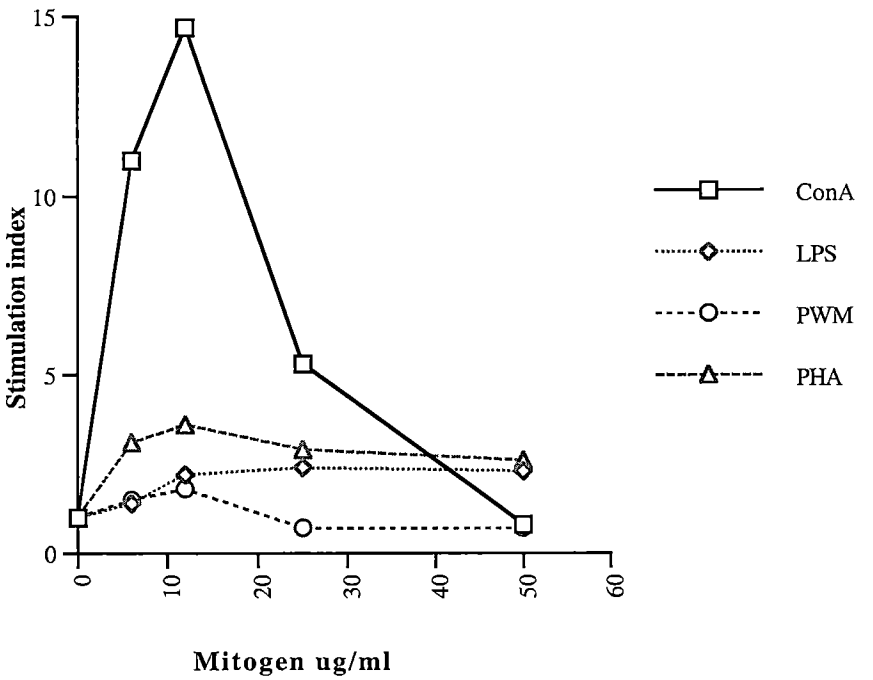


Figure 4.5: The effect of the mitogens ConA, LPS, PWM and PHA on the proliferation of PBMC from platypus E1 at a cell density of 2x10e6 and incubated for 6 days

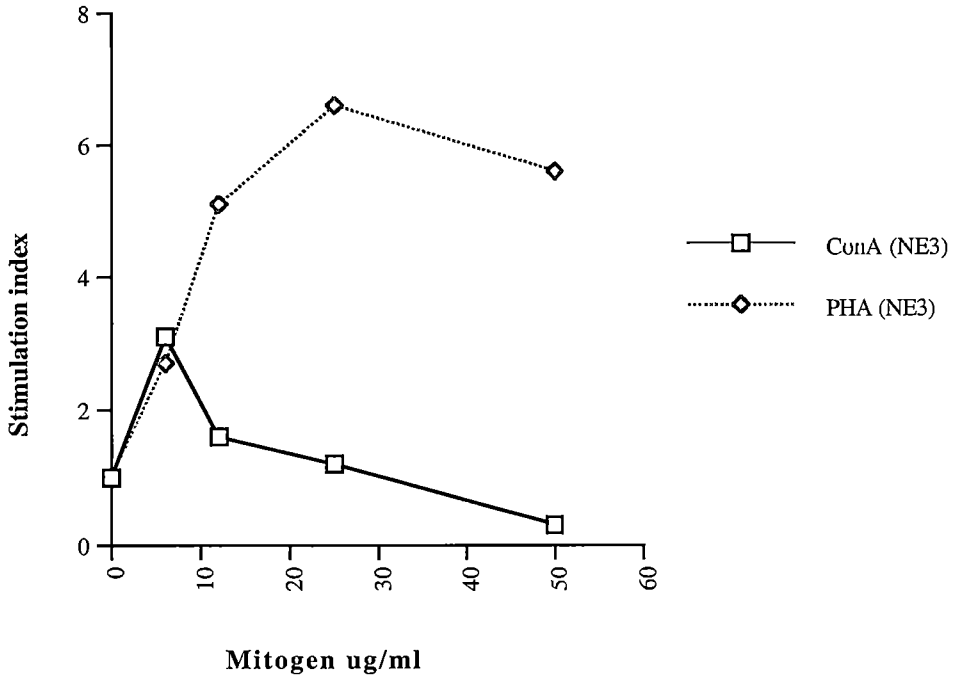


Figure 4.6: The effect of the mitogens PHA and ConA on the proliferation of platypusPBMC at a cell density of 1x10e6 and incubated for 7 days.

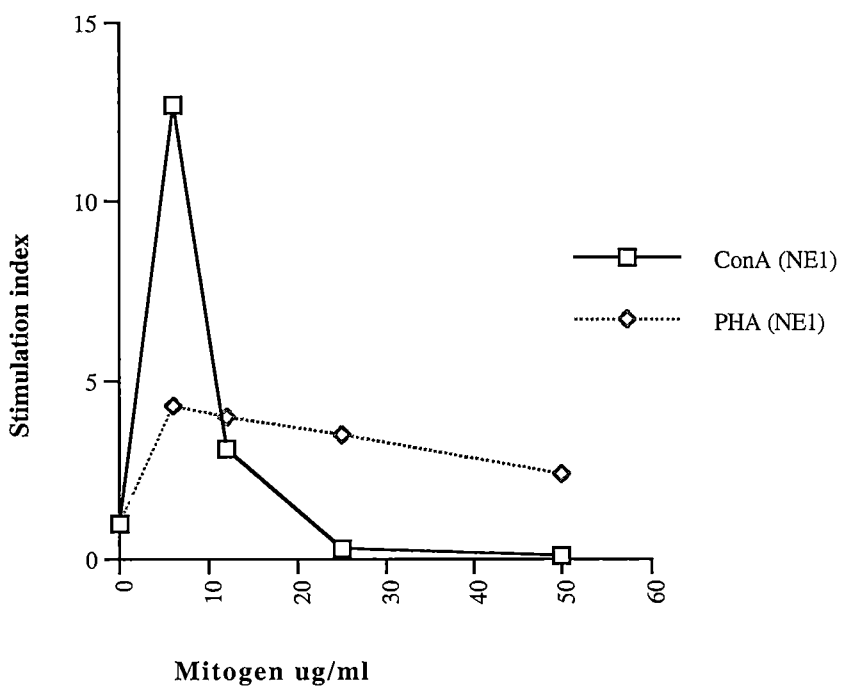


Figure 4.7: The effect of the mitogens PHA and ConA on the proliferation of platypus PBMC at a cell density of 2x10e6 and incubated for 7 days.

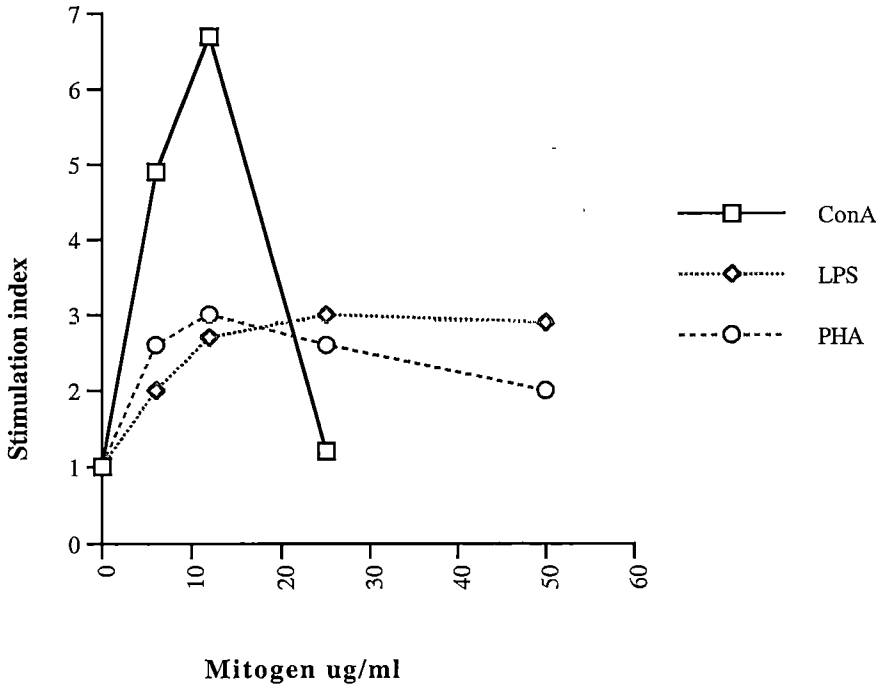


Figure 4.8: The effect of the mitogens ConA, LPS and PHA on the proliferation of PBMC from an ulcerated platypus (E2). Cells were incubated for 6 days at a density of 2x10e6.

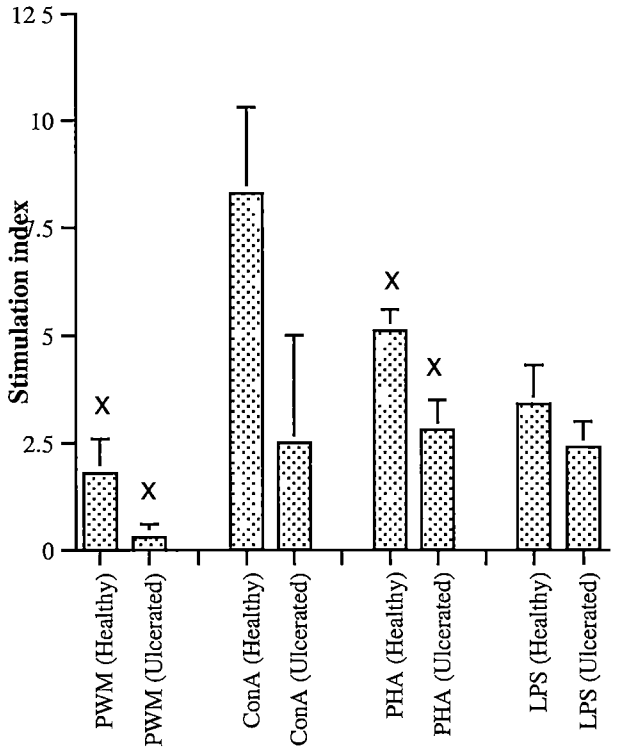


Figure 4.9: The effect of ulceration on the stimulation index of platypus PBMC exposed to four different mitogens. The symbol "x" denotes a significant difference between the stimulation index for the healthy and ulcerated animals for that mitogen.

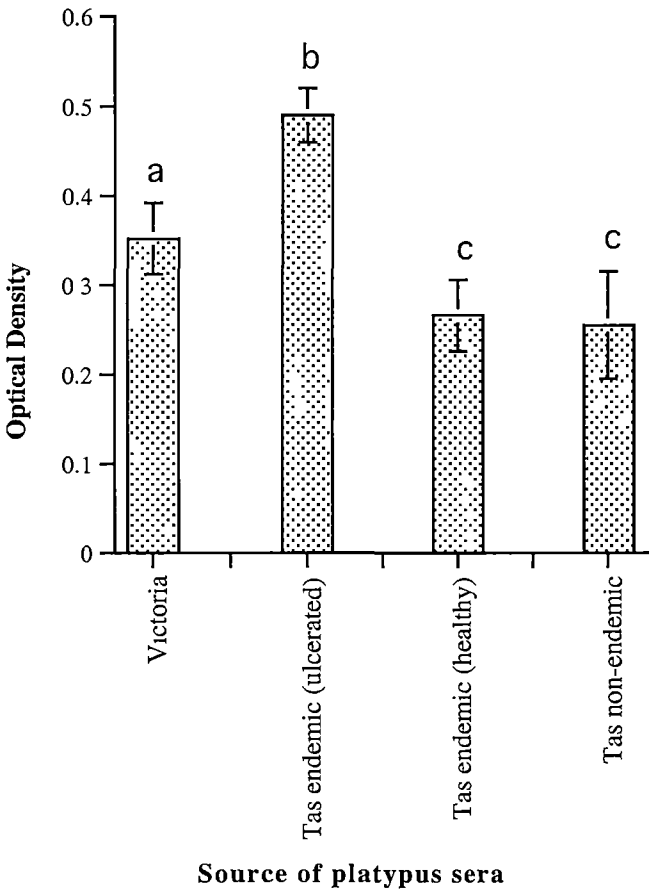


Figure 4.10: The optical densities recorded in an ELISA using platypus sera from various sources against *Mucor amphibiorum* antigen. Different superscripts indicate significantly different results at $p<0.05$

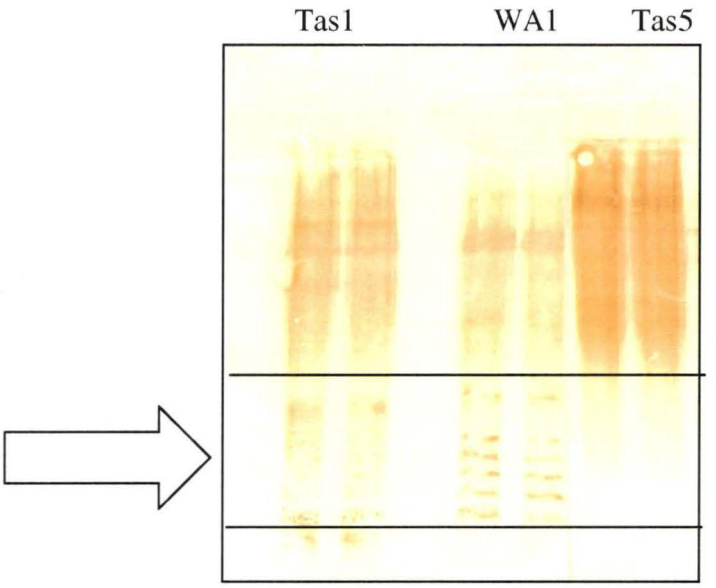


Figure 4.11: Western blot showing the response of sera from a rabbit injected with a killed culture of *M. circinelloides* (Tas5) to two duplicate isolates of *M. amphibiorum* (Tas1, WA1) and to a duplicate isolate of *M. circinelloides* (Tas5). The area indicated by the arrow shows banding of low molecular weight proteins detected in cultures of *M. amphibiorum*, but not in a culture of *M. circinelloides*.

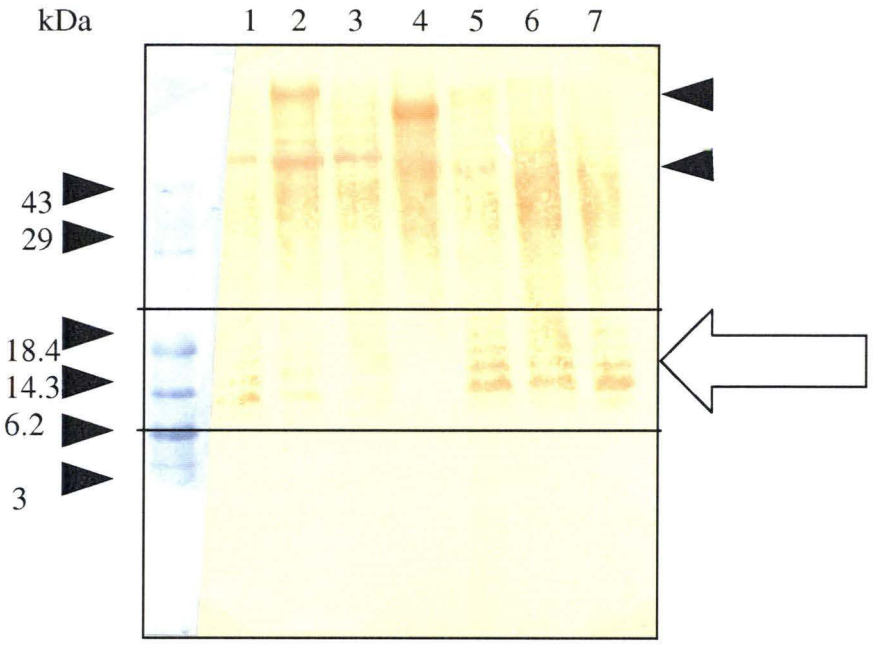


Figure 4.12: Western blot showing the response of serum from a rabbit injected with a killed culture of *M. amphibiorum* to six isolates of *M. amphibiorum* and one isolate of *M. circinelloides*. Lanes 1,2,3,5,6,and 7 denote respectively Tas1, Tas2, Tas3, Q1, Q2 and WA1. Lane 4 denotes the *M. circinelloides* isolate Tas5. The large arrow indicates the area in which the sera was reactive with low molecular weight proteins of *M. amphibiorum*, but not *M. circinelloides*, cultures. The small arrows on the right indicate the high molecular weight proteins

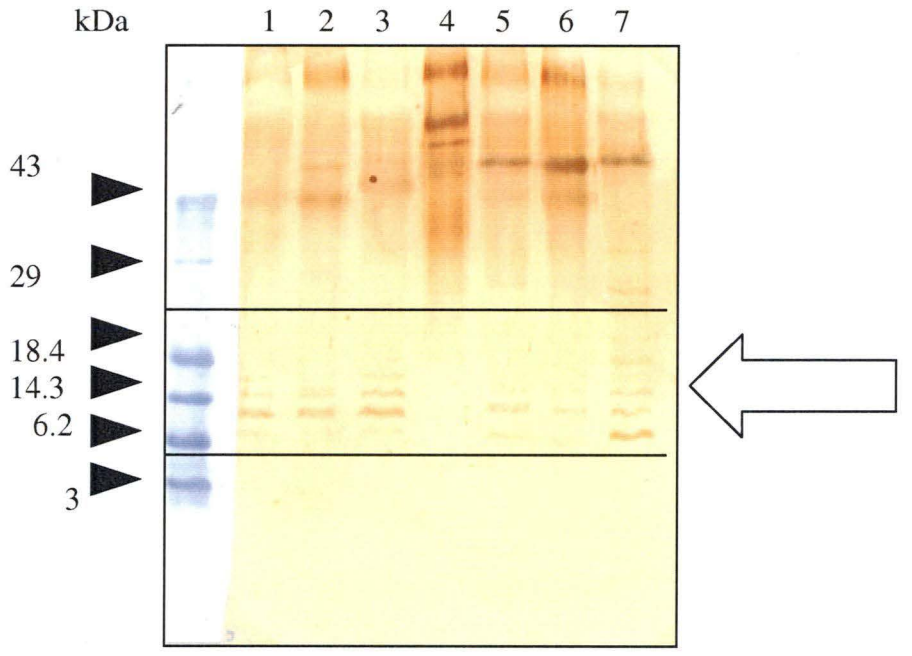


Figure 4.13: Western blot showing the response of serum from an ulcerated platypus to different isolates of *M. amphibiorum* and one isolate of *M. circinelloides*. Lanes 1, 2,3,5,6 and 7 denote respectively *M. amphibiorum* cultures Tas1, Tas2, Tas3, Q1, Q2 and WA1. Lane 4 denotes the *M. circinelloides* culture Tas5. The large arrow indicates the area in which the serum was reactive with low molecular weight proteins of *M. amphibiorum*, but not *M.circinelloides*, cultures.

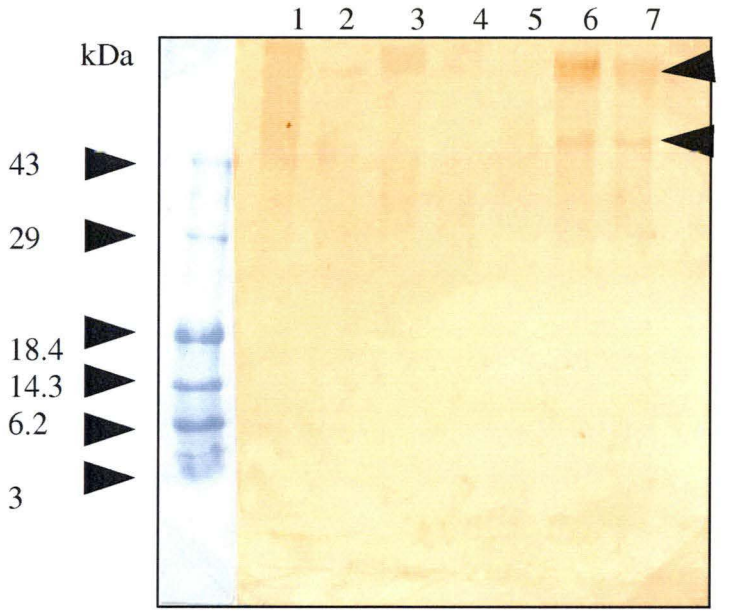


Figure 4.14: Western blot showing the response of serum from a healthy platypus in the endemic area for ulcerative mycosis to six isolates of *M. amphibiorum* and one isolate of *M. circinelloides*. Lanes 1,2,3,5,6 and 7 denote respectively Tas1, Tas2, Tas3, Q1, Q2 and WA1. Lane 4 denotes the *M. circinelloides* isolate Tas5. The arrows to the right of Lane 7 indicate HMW proteins. A similar response was noted in sera from platypuses from the non endemic areas, Queensland and Victoria.

4.4 Discussion

The lymphocyte stimulating properties of mitogenic lectins are recognised in many different species. The response of individuals within a species to these mitogens can provide valuable information on the functional aspects of the immune system. The lymphocyte proliferation assay is now routinely used in both human and animal health. The common mitogens, PHA, ConA, LPS and PWM have been found to work in a large number of species. Dogs, cats, mice, humans and fish all respond well to all or some of these mitogens. Despite the wide spread use of this assay, it has never been applied to a monotreme.

Developing a successful assay for use with the platypus was complicated by several factors. Ideally, when developing such an assay, all the parameters would be tested on lymphocytes from one blood sample taken from one animal at one time, thus ensuring that differences in response are due to the parameters being tested, and not to individual variation between animals. This was not possible with the platypus. The maximum blood sample that can be obtained from a platypus is between 0.5 and 2ml. Taking blood from a platypus is complicated by the limited number of sites available for extraction. Some researchers use a cardiac puncture, but this procedure poses unacceptable risks to the animals. The only other available site is the venous sinus that traverses the upper bill of the platypus. The sinus contains little blood, especially when the animal is stressed, and is slow to refill following bleeding. Unstressed platypuses held in captivity and anaesthetised provide only between 2 and 3ml of blood. The problem could be overcome to a limited extent if an individual animal could be reliably re-captured several times. Unfortunately, reliable re-capture is not possible. Also, parameters can change over time in individual animals, further complicating the assay. Holding animals in captivity would have allowed for regular testing, but logistically this was not possible. Platypuses do not lend themselves to captivity, due to their propensity for suffering stress (Whittington 1991, 1993).

The platypuses tested responded relatively well to two of the mitogens used, PHA and ConA, which are both T cell mitogens. The B cell mitogen LPS had little effect on platypus lymphocytes. PWM, which is both a B and T cell mitogen, similarly had little effect. Such a lack of response to PWM is not uncommon (pers. comm. Woods). A small proportion of the normal human population has a low or absent response to PWM. It is likely that this is the case with other mammals as well. Responses to LPS are often low for many species. The harbour seal has responses similar to that achieved for the platypus (SI two to three), with a maximum response in one animal of eight (de Swart 1995), while bottlenose dolphins didn't respond at all to LPS (Lahvis *et al.* 1993). Bandicoots also do not respond

to this mitogen (pers. comm. Bettiol), and a low response has also been observed in koalas (Wilkinson *et al.* 1992). The low response to B-cell mitogens may be explained by either a lack of appropriate receptors on platypus B-cells, or by low numbers of B-cells present in the blood.

The concentration of mitogen required for maximum proliferation can differ markedly between species, and between laboratories. ConA at a concentration as low as 2.5ug/ml has been shown to be effective in bottlenose dolphins (Lahvis *et al.* 1993), whereas 50ug/ml is required for chicken lymphocytes (Gogal *et al.* 1996). Koalas respond well to concentrations between one and six ug/ml (Wilkinson *et al.* 1992). In humans ConA gives maximum results at eight ug/ml. In the platypus, the maximum response was achieved at either six or 12ug/ml. Similarly with PHA, platypus lymphocytes responded best to concentrations between six and 25 ug/ml. This is within the range reported for other species, from 0.5ug/ml for humans (Smith *et al.* 1978) to 12.5 for cattle (Senogles *et al.* 1978).

The length of incubation is also important in the proliferation assay. In many species, for example mice (Kaldjian *et al.* 1992), the opossum (Infante *et al.* 1991) and chickens (Gogal *et al.* 1996), maximum proliferation is achieved within three days. With other species, longer incubation times are required, although six days appears to be the maximum time required for other species (Lahvis *et al.* 1993). Platypus cells showed no significant response at three days. At four days, there was a marked response in cells exposed to ConA, but it appears that five days is the minimum time required for cells exposed to PHA to respond. Incubations of up to seven days still returned high stimulation indices for both these mitogens. The slow response of platypus lymphocytes to mitogens may reflect in part the low body temperature of the animal (32°C). It is likely that the lower temperature in comparison to other mammals results in a slower metabolic rate within the lymphocytes. It is recognised in humans that an elevated temperature results in not only a more rapid response to certain mitogens (Smith *et al.* 1978) but also in an increased SI (Huang *et al.* 1996). Platypus lymphocytes were also incubated at 37C°, but there was no significant response to any of the mitogens tested (data not shown), and it was likely that this temperature was too high.

The cell densities required for maximum proliferation were within the range of other species tested. Although the data is limited, it suggests that PHA works best with 1x10e6 cells/ml, and ConA at 2x10e6 cells/ml. Cell densities greater than 2x10e6 resulted in greater cpm, but as the cpm for the control cells also increased, there was no increase in SI.

Comparison of SI with other species is complicated by the different methods used by different experimenters. Many other assays reported in the literature use fresh PBMC, pulsed on day one, as the control cpm. There is some evidence that there are short lived suppressor cells in human PBMC. If human PBMC are incubated for 24 hours before stimulation, there is an increase in proliferation (Kalmar and Gergely 1981). Thus cells pulsed on day one may have an artificially low cpm in comparison to cells incubated for several days, resulting in a high SI. Also, when doing a time trial, a cpm for control cells should be calculated for each day's incubation, as baseline cpm may increase or decrease over time.

The cpm for platypus PBMC was considerably lower than that for other species. Bottle nose dolphins can return cpms of 160,000 when PBMC are stimulated with ConA. However, the SI could not be calculated for this species, as the control cpm was not given (Lahvis *et al.* 1993). In contrast, harbour seals achieve cpms of about 50,000 for ConA, with a SI of 97. Interestingly, although PHA and ConA are both T cell mitogens, they may activate different populations of lymphocytes (Shifrine *et al.*1978). In the case of harbour seals, in a sample of five animals, the animal with the highest SI for ConA had the lowest for PHA, and the animal with the lowest for ConA had the highest for PHA (deSwart 1995). Such a trend was not noted in platypuses.

Responses to mitogens can vary within species. Work with *Monodelphis domestica* has, in one study, shown that ConA is most mitogenic, followed by PHA and then PWM, which was inconsistently mitogenic (Infante *et al.* 1991). These findings are in accord with the platypus' response. However, another study found that PWM was strongly mitogenic, and moreover the SI for ConA was significantly higher than that achieved in the other study (Brozek *et al.* 1992). Similarly, de Swart (1995) recorded maximum cpms for ConA and PHA of 50,000 and 5,000 respectively in harbour seals, whereas DiMolfetto-Landon *et al.* (1995) recorded maximum cpms of 150,000 and 20,000. The range of the response can also vary greatly between individuals in a species. Healthy seals in the study by deSwart returned cpms for ConA between 18,000 and 58,000. The five harbour seals sampled by DiMolfetto-Landon *et al.* (1995) showed an even greater range of responses to ConA, from a low of 30,000 to a high of 150,000. Proliferation can be affected not only by the general health of an animal, but may also be affected by season (Wilkinson *et al.* 1992). Dietary factors can also contribute to suppressed proliferation (Calder *et al.* 1995).

Baseline cpms also vary markedly between species. Both platypus and harbour seals generally have baseline cpms of under 500 (deSwart 1995)), whereas mice can have baseline cpms of 6,600 (Kaldjian *et al.* 1992), and cattle can have readings as high as 40,000 (Senogles *et al.* 1978). It is important to measure baseline cpm for different incubation times, as well as for different cell densities. Baseline cpms that are considerably higher than the average may indicate an immune dysfunction, even if the SI appears normal. For example, in humans with atopic dermatitis, increased spontaneous uptake of tritiated thymidine by lymphocytes has been reported, resulting in increased baseline cpm (Lachapelle *et al.* 1980). Viral infections can also result in spontaneous proliferation of lymphocytes (Stone *et al.* 2000, Quist *et al.* 1997). Two animals returned high cpms after two days incubation. One of these animals was ulcerated, but as the blood was stored for 48 hours prior to stimulation, it cannot be said with certainty that the increase in cpm was due to the disease. This animal had a low baseline cpm after three days incubation, with the high cpm occurring after four days. This was mirrored in another animal that was caught in Lake Pedder. The assay on this animal was run at 4x10e6 cells/ml. It is possible that the high cell density resulted in a higher concentration of accessory cells leading to the high reading. That the ulcerated animal's cpm dropped to a low level the following day suggests that this was an artefact.

The proliferation of platypus lymphocytes may be increased by using homologous sera in place of FCS. It has been noted in a range of marsupials that the use of FCS results in very poor lymphocyte survival in comparison to autologous sera. It was not possible to test this because of the difficulty in obtaining reasonable quantities of platypus serum.

The significant difference in responses to PHA, but not ConA, in healthy and ulcerated animals was interesting. In the case of ConA, the two badly ulcerated animals from Upper Blessington had indices of only 0.4, whereas the slightly ulcerated animal from Cressy had an SI of 6.7. Although not significant, there was a trend for decreasing response with increasing severity of disease with this mitogen. The differences in response of healthy or ulcerated animals to PHA was significant, but a trend of decreasing response with increasing severity of ulceration could not be seen. The two severely ulcerated Upper Blessington animals showed lower responses (0.4 and 0.2) than the lightly ulcerated Cressy one (1.4), but the heavily ulcerated Glengarry animal had a SI (4.5) higher than some healthy animals. Although lymphocytes from ulcerated animals were, in some cases, incubated for longer than lymphocytes from healthy animals, this is unlikely to have affected the results (pers. comm. G. Woods). Although it is possible that cells were dying,

if this were the case it would be expected that the cpm for unstimulated cells would have been decreasing over the incubation period. This was not observed.

Many mycoses actually result in an increase in lymphocyte proliferation. For example, fish suffering from Ulcer Disease Syndrome have a four fold increase in proliferative response to LPS, and a 10 fold increase in response to ConA (Faisal and Hargis 1992). Similarly, mannoproteins in *Candida albicans* are powerful proliferative agents (Bromuro *et al.* 1994). Even in diseases such as paracoccidioidomycosis, where lymphocytes from infected individuals proliferate poorly in response to antigens specific to the fungus responsible, proliferation rates are higher than those for healthy individuals when other stimuli are used (Mendesgiannini *et al.* 1997). None of the ulcerated platypuses showed increased response to any of the mitogens, and in fact two of the ulcerated animals had SI below 1 for PWM and ConA. It is possible that a sub set of T cells are responsible for this poor response. In other mycoses Th2 cells have been shown to be important in mounting an effective resistance to fungi. It is possible that increasing severity of disease causes a concomitant decrease in proliferation of a T cell sub set activated by ConA; that is, the disease is having a direct effect on cell function. In the case of PHA, where severity of disease is not associated with decreased proliferation, it is possible that another sub set of T cells is activated; that is, animals with a good PHA response may have a normal sub set of PHA-activated T cells, leading to a more likely recovery from infection than those with a low PHA response. These results may explain why some animals make a full recovery from the disease, whilst others die.

In conclusion, platypus lymphocytes responded to the T cell mitogens ConA and PHA, with ConA being more effective. The B cell mitogen LPS did stimulate cells, but SI were low. The B and T cell mitogen PWM was the least efficient mitogen used. Although the SI were low in comparison to some species, they were on a par with others. The long incubation period required for the lymphocytes probably reflects the low body temperature of the platypus in comparison to other mammalian species. Ulcerated animals had a significantly lowered response to PHA and PWM, and a lowered response to ConA and LPS. Some Tasmanian platypuses may have impaired an impaired T cell response to PHA activated cells, which may be an important sub set involved in fighting mycoses. This impaired response, while not directly responsible for infection with *M. amphibiorum*, may prevent the animals from fully recovering.

The reaction of sera from rabbits inoculated with *M. circinelloides* was interesting. Although in an ELISA cross reactivity is so high between *M. amphibiorum* and *M. circinelloides* that they cannot be separated, in a blot there are LMW bands that appear unique to *M. amphibiorum*, even though in silver stained gels *M. circinelloides* appears to have protein bands in the same molecular weight range. This discrepancy could be explained several ways. The most likely explanation is that *M. circinelloides* possesses these proteins, but in such low quantities that they are not detected by antibodies in either anti-*amphibiorum* or anti-*circinelloides* sera. That these proteins are only present in very small quantities is supported by the lack of difference in an ELISA. If these low molecular weight proteins were responsible for much of the antibody activity, and *M. amphibiorum* possessed significantly higher quantities of these proteins, then an ELISA would record a higher reading for *M. amphibiorum*.It may also be that *M. circinelloides* does not possess these proteins. The ability of anti-*circinelloides* sera to react with the LMW proteins may indicate the presence of epitopes common to both the lwm proteins in *M. amphibiorum* and to the HMW proteins in *M. circinelloides.*

The identical response of the rabbit sera to proteins from isolates sourced from Queensland, West Australia and Tasmania suggests that these isolates share common epitopes, and that they cannot be separated on the basis of of a Western blot.

No reaction with LMW proteins was observed in any healthy platypuses, but all animals did respond to the HMW proteins. These proteins are common to both *M. amphibiorum* and *M. circinelloides*, and may possibly occur in other *Mucor* species.The presence of antibodies in all animals tested to these proteins suggests that *M. circinelloides* is responsible for the activity. It appears likely that antibodies to the LMW proteins found in *M. amphibiorum* are only produced in response to an active infection. While some animals that recover from the disease presumaby possess antibodies to these proteins, other animals that have not had an active infection will not.

Although sera from mainland platypuses did not react with LMW proteins from *M. amphibiorum*, it is clear from the ELISA results that these platypuses produced significant quantities of antibody in response to the HMW proteins common to both *M. amphibiorum* and *M. circinelloides.* The significantly higher response of mainland platypuses in comparison to Tasmanian healthy platypuses from both endemic and non endemic areas may be explained in several ways. Firstly, mainland platypuses may be exposed to greater

numbers of *M. circinelloides* spores, leading to greater antibody production. The temperature on the mainland is generally higher than in Tasmania, and may be more conducive to the growth and sporulation of *M. circinelloides*.Secondly, mainland platypuses may mount a more effective response to the fungus. Tasmanian platypuses may be quite capable of destroying the *M. circinelloides* spores probably through phagocytic cells, but may not be presenting antigen efficiently to B cells, whereas mainland platypuses may not only destroy the fungus, but also efficiently process and present antigen, leading to increased antibody activity. It may also be that mainland platypuses are regularly exposed to *M. amphibiorum*, and have higher titres than unexposed Tasmanian platypuses. However, the evidence does not support this. Now that the disease is endemic in certain parts of Tasmania, and has been for several years, it would be expected that healthy animals in the endemic area would be equally exposed to *M. amphibiorum* as their Victorian counterparts. Additionally, platypuses in non endemic areas would not be exposed to the fungus. In this scenario, Victorian and healthy Tasmanian endemic platypuses, being equally exposed to the fungus, would have equivalent antibody levels, and both would have higher levels than the non exposed platypuses in Tasmania's non endemic areas. The data do not support this, with Tasmanian ELISA results being similar for healthy animals from endemic and non endemic areas, and with both being significantly lower than those for Victorian platypuses.

It seems likely that platypuses only mount a significant humoral response following an active infection. The ELISA result for the platypus from Upper Blessington which had a healed lesion on its foot was identical to that from one badly ulcerated animal, and not significantly different to the results for the other ulcerated animals. The lesion had obviously been healed for some time, and a biopsy revealed only one fragmented non viable sphaerule-like body in the scar tissue. Of course, it cannot be stated at present whether the antibody is protective or not. In many mycoses, both protective and non protective antibodies are produced, and in some cases antibodies are produced that are disease-enhancing (Pirofski and Casadevall 1996, Casson *et al.* 1998).

The ELISA for the detection of IgE specific to *M. amphibiorum* did not detect any significant differences between ulcerated or healthy platypuses from Tasmania, or platypuses from Queensland or Victoria. Based on these results, it seems unlikely that there is an atopic subpopulation of platypuses in Tasmania. As stated earlier, atopic individuals have been reported as having greatly increased IgE levels.

The ELISA developed for the detection of IgE specific to *M. amphibiorum* was considered a more useful assay than an ELISA for the detection of total IgE. Such an ELISA would not reveal whether any IgE present was directed against *M. amphibiorum* antigens, or antigens from some other source. Other research has shown that the ELISA used in this research can detect antigen-specific IgE (Souza-Atta *et al.* 1999), and that in many allergic responses the levels of IgE can typically be around 9 times the levels found in healthy individuals (Saini *et al.* 1998). The high variable range of values exhibited by platypuses may suggest some cross reactivity with IgG, which has been noted in other research (Souza-Atta *et al.* 1999). The sensitivity of the ELISA may be improved by removing IgG from the sera prior to testing. Removal via protein G column would probably be unsuitable, as there are often IgG autoantibodies directed against IgE when IgE levels are high (Shakib *et al.* 1995), thus stripping out IgG would also strip out some IgE. A more appropriate method may be to use reagents that block IgG binding sites, thus reducing the reaction of the IgG with *M. amphibiorum* antigens. Such a method has been successfully used for the detection of IgE specific for isolates of*Leishmania chagasi* (Souza-Atta *et al.* 1999).

In conclusion, Tasmanian platypuses have a normal, but low response to the mitogens tested, and the results are comparable to recent responses reported for mainland platypuses. There was no difference between the responses for animals from endemic or non endemic areas. Some ulcerated animals recorded lowered responses for some of the mitogens, but whether the lower response caused the disease, or vice versa, could not be determined. The relatively high response of one ulcerated animal suggests that a lowered response is not required for the disease to occur. The significantly higher optical density recorded for Victorian platypuses in an ELISA for the detection of *M. amphibiorum* specific antibodies was interesting. Given the cross reactivity with *M. circinelloides*, it is possible that Victorian platypuses are routinely exposed to higher levels of *M. circinelloides* exposure. Alternatively, Victorian platypuses may be more effective at processing antigen than their Tasmanian counterparts. The Western blot developed was useful for visualising bands of LMW protein in *M. amphibiorum*, but not *M. circinelloides*, cultures. But as sera from rabbits inoculated with *M. circinelloides* could also detect these bands, it seems likely that these bands are not specific to *M. amphibiorum*.. They are either present in the same proteins in *M. circinelloides*, or occur as common epitopes on other proteins. It does not seem likely that a serolgical assay for the detection of prior exposure to *M. amphibiorum* is possible, as the data suggest that an active, or recently healed infection, is required for an antibody response to be measurable. It also seems unlikely that there is an atopic sub population of Tasmanian platypuses. In terms of immunological response, it seems likely

that platypuses are similar to other mammals. They possess the major organs associated with immunity in mammals, and also possess two IgG classes, two IgA classes and one IgE class. To date, no classic IgM has been identified, but it is likely that one of the IgA classes fulfils this role (pers com Lars Hellman).

Chapter 5

The weights, sex ratios and capture rates for healthy and ulcerated platypuses in Tasmania

5.1 Introduction

The disease ulcerative mycosis of platypus has been present in some Tasmanian platypus populations since at least 1982, when the condition was first reported (Munday and Peel 1983). From the limited data available, infection rates of around 36% seem typical in newly affected populations (Connolly *et al.* 1998). Males are apparently more likely than females to be ulcerated, with 43% of males, and 23% of females found to be ulcerated at Brumby's Creek in a 1994 study (Connolly *et al.* 1998).

Previous studies of the condition have concentrated on short-term trapping, usually for less than one year (Connolly *et al.* 1998). Another study concerned with the condition collected data on only moribund and dead platypuses (Obendorf *et al.* 1993). Apart from these two studies, the only available information is anecdotal, relying on reports from environmental groups, fishermen and other interested parties. There are many important questions that are still unanswered concerning the disease. Whether the prevalence of the disease abates during the year, possibly due to winter mortalities, is not known. During winter, platypuses are swimming in water at temperatures as low as 4°C. Given the nature and size of the ulcers, it is possible that such temperatures are low enough to induce hypothermia in badly ulcerated animals. Also, the ulcers predominantly occur on the tail, the reserve for platypus fat, so it may also be that affected platypuses eventually succumb to malnutrition. Because of the limited research carried out to date, and the secretive nature of the platypus, it was not known prior to this study whether the disease is invariably fatal, or whether some animals make a full recovery.

Some captured platypuses with ulceration have been emaciated, but whether the emaciation is a result of the condition, or vice versa, is not known (Obendorf *et al.* 1993). There have been no studies to compare body weights between healthy and ulcerated animals, and animals from endemic and non endemic areas.

To understand the impact of the disease ulcerative mycosis on platypus populations, it is necessary first to gather background information on the size of populations in selected endemic areas, the apparent sex ratio, the general condition of both healthy and ulcerated platypuses, and the presence of permanent versus transient animals in an area. This information could then be compared with information on platypuses from other, non endemic sites to determine whether there are certain basic differences between endemic and

non endemic populations. It would also be useful to know whether infection rates in endemic areas are stable, or whether there are seasonal or yearly fluctuations.

The aims of the present study were severalfold. By trapping Brumby's Creek and nearby waterways it was hoped that a comparison could be made between infection rates for 1994 when the Connolly *et al.* (1998) study was performed, and for rates for the years following. Also, it was decided to follow the course of the disease in an area following the initial report of the disease. Glengarry had its first reported case of the disease in 1995 (pers comm B. Munday), and it was planned to trap in the area during the period 1997-2000. A site at the base of Ben Lomond was also selected, as the river system in the area is generally pristine with relatively fast-flowing water. Outside of the endemic area, trapping was performed in one lake (Lake Pedder) and in one river (Salmon Ponds), with the aim of determining any possible differences in weights, sex ratios or general health of platypuses both within and outside of the endemic area. It was also envisaged that ulcerated platypuses would be recaptured, allowing for detailed analysis of weight, health and development of ulcers.

5.2 Material and methods

5.2.1 Trapping

Platypuses were trapped over a four year period, from 1997 to 2000. In the endemic area, platypuses were trapped at four different sites: Glengarry, Cressy, Upper Blessington and Connorville (Figure 1). In non endemic areas, platypuses were trapped at two sites: Lake Pedder and the Salmon Ponds. One week of trapping was also performed at King Island, another non-endemic area. Generally, trapping was conducted for a five day period. Table one records when each area was successfully trapped. Weeks in which trapping occurred, but no animals were caught, are not recorded. Springfield, in the north east of the state, was trapped for three days in 2000 following reports of numerous platypuses in the area.

Trapping was undertaken using both fyke nets and unweighted gill nets. In small streams, fyke nets were exclusively used. The nets were placed such that the wing of the net was staked against one bank, and the cod end of the net run onto the other bank, and staked in place. The first hoop of the net was positioned such that the majority of the hoop was submerged. If the current was strong, rocks were placed on the bottom of the hoop, and

along the bottom of the wing of the net. The gill nets were set in the late afternoon, and checked early in the morning.

Gill nets were used in larger streams, and occasionally in farm dams. The gill nets were attended at all times, and animals removed immediately after capture.

Captured animals were removed from fyke nets by firmly grasping the animal by the tail. After removal from the net, animals were placed in dry, individual hessian sacks. Usually, animals were brought to the laboratory for sampling. At the laboratory, animals were placed, in their sacks, in individual plastic bins lined with sacking. The bins were placed in a quiet area, in the dark, until the animal was needed.

5.2.1.2 Trapping sites

Brumby's Creek (Cressy)
The stream is located in agricultural land used primarily for grazing and cropping. The stream varies greatly in size and depth, depending on the release of water from a hydro electric power station that feeds the stream. There are many small rivulets and ditches that fill when water is released from the power station. These are often visited by platypuses from the main stream. Fyke nets were used in these areas, and gill nets in the stream proper. The bed of the stream is soil, and water plants are common in most parts of the stream. Sheep and cattle have access to the stream at many locations.

"Connorville" (Cressy)
The site here is similar to that at Cressy, but the waterway is smaller, and is naturally fed from the central plateau. The water depth did not exceed 1.5m in the areas trapped. The stream bed was clay, with numerous water plants present along the length of the stream. Both sheep and cattle graze along the banks.

"North Camelford" (Upper Blessington)
This property is located at the foothills of Ben Lomond. The stream running through the property is fed directly from the mountain, and is considered pristine. The stream has a rocky bottom, numerous riffles and several pools up to 2m deep. The surrounding land is given over to cropping and grazing of sheep and cattle.

Glengarry

The Glengarry area combines agricultural land devoted to sheep and cattle with pockets of woodland. The stream that runs through the area is small. In some areas it flows through flat pasture, and in others it has banks up to 2m high. The stream bed varies from muddy to stony bottom. Water plants are common in some parts, and absent in others. There are several farm dams in the area that were trapped, as well as the stream. The area was divided into three sampling sites (Figure 5.2)

Salmon Ponds

The Plenty river flows past the Salmon Ponds, a trout and salmon hatchery. The river is up to 10m wide in the trapping area. There are numerous riffles and deep pools (2m), and the bottom is a combination of rock and coarse river sand. There are several small canals used to feed the hatchery that were also trapped. Cattle are grazed near the river, and opium poppies are grown on the land opposite the hatchery.

Lake Pedder

This lake is located in the south west, and is a hydro electric dam. There is no agricultural activity in the area. The shoreline of the lake is rocky, with the forest coming to within several metres of the water.

5.2.3 Sampling

All animals were weighed using a spring balance (Jet Aer Corp., USA) and sexed, and blood samples taken. Blood was taken by first placing the animal in a hessian sack from which the corner had been removed. The hole was large enough to allow only the bill of the animal to protrude. The animals was held with thumbs pressed firmly along the shoulders and the back of the neck, and with fingers holding the body. Depending on the size of the platypus, either a 21 or 25 g needle was used to draw blood from the dorsal venous sinus of the bill. The plasma was separated and frozen at -80°C until required.

A sterile swab was used to gather material from the nasal passages of 12 platypuses; 5 from the non endemic area, and 7 from the endemic area. The swabs were streaked onto Sabouraud's agar, and the plates incubated for 48 hours at 25°C. Any growth with a gross morphology similar to either *Mucor amphibiorum* or *Mucor circinelloides* was removed

with a sterile needle and transferred to fresh agar. Identification of cultures was based on the key of Schipper (1978).

If the animal was ulcerated, a sterile swab was rubbed over the ulcer, and then streaked onto Sabouraud's agar and incubated as previously described.

One ulcerated and one animal that had recovered from ulceration were biopsied. The animals were restrained in a bag, with the affected limb protruding. The area was anaesthetised with 1% Xylocaine (Astra) prior to biopsying. The sections of tissue were fixed in 10% formol-saline, embedded in paraffin, sectioned at 4um and stained with haematoxylin and eosin.

Each animal was fitted with a PIT tag, using the Trovan Passive Transponder System. The tag was inserted below the skin, between the shoulder blades of the animal.

A small piece of skin was taken from the rear foot webbing of both trapped and accidentally killed animals. In the case of trapped animals, the foot was protruded through the open end of the sack while the animal's head and body were restrained in the sack. A sterile scalpel was used to remove the skin sample, which was stored in alcohol prior to analysis. Forty three samples from throughout Tasmania were forwarded to Dr Shiro Akiyama for microsatellite variation analysis (see appendix 1), and compared with samples collected from throughout mainland Australia.

Animals were returned to the point of capture and released as soon as possible after sampling (always before dark on the same day).

New scales (Salter Australia) were required approximately 18 months into the study. To negate possible differences between old and new scales, all weights used in the results are for animals caught in the first 18 months. No animals were caught in the non endemic area after the second scales were purchased.

5.2.4 Statistical analyses

Data were analysed with a one-way analysis of variance (ANOVA) using the JMP 3.2.1 statistical package. ANOVA assumptions of data normality and homogeneity of variances were tested using the Shapiro-Wilk W test and Bartlett's test respectively. Significant

differences were highlighted as multiple comparisons of means (Tukey-Kramer HSD test) were carried out. For all tests a significance level of $P<0.05$ was adopted.

5.3 Results

In total, 78 platypuses were captured in the study period. Seventy were captured in the six main study sites (Table 5.1), six in King Island, one at Springfield and one at Karoola. At Glengarry, 21 animals were captured at Site 1, nine at Site 2 and five at Site three. All ulcerated animals captured at Glengarry were from Site 1.

5.3.1 Platypus weights

There was no difference in mean weights of male platypuses from endemic and non endemic sites (endemic 1.9kg, SD 0.2, range 1.3-2.3kg, n=9; non endemic 1.9kg, SD 0.4 range 1.2-2.3kg, n=10). Female platypuses were lighter than males, but females' weights were not statistically different between endemic and non endemic sites (endemic 1.0kg, SD 0.3, range 0.7-1.2kg n=3; non endemic 1.2kg, SD 0.4, range 0.7-1.7kg, n=7). Female platypuses from King Island were significantly lighter than mainland Tasmanian females (mean 0.56kg, SD 0.08, range 0.5-0.7).

5.3.2 Effect of ulceration on weight

There was no effect of ulceration on the weight of male or female platypuses (Figure 5.3)

5.3.3 Sex ratios

5.3.3.1 Ratio of healthy versus ulcerated for male and female platypuses

A greater percentage of male platypuses than female platypuses were ulcerated (Figure 5.4).

5.3.3.2 Sex ratios for endemic and non endemic sites

Males comprised 67% of the captures in endemic areas, and 57% of captures in non endemic areas.

5.3.4 Capture rates

5.3.4.1 Monthly capture of platypuses

Over the four year study period, platypuses were captured in all months except August (Figures 5.5, 5.6, 5.7, 5.8). Ulcerated platypuses were captured in all years except 1999.

5.3.4.2 Capture rates per week of trapping in endemic areas

There was little difference between years for numbers of healthy animals caught per week of trapping. The most successful year was 1998, and the least 1999 (Figure 5.9). There was a greater difference for ulcerated platypuses, with 1997 being most successful, and 1999 the least successful (Figure 5.10). The mean weekly capture rate for the four years of trapping was 2.2.

5.3.4.3 Capture rates per week of trapping in non endemic areas

Trapping was only performed in 1997 and 1998 in non endemic areas, with 3.5 animals caught per week of trapping.

5.3.5 Recapture rates

Platypuses were recaptured at all sites except Connorville in the endemic area, and Lake Pedder in the non endemic area (Table 5.2).

5.3.5.1 Recapture of ulcerated platypuses

Of 12 ulcerated platypuses captured, 3 were recaptured. All were males, one from Nth. Camelford, one from Cressy and one from Glengarry. The Glengarry platypus was first captured 9/2/97, and recaptured approximately 3km away on 16/11/1997 The Nth. Camelford platypus was first caught on 27/2/1997, and recaught on 14/7/1998. The Cressy platypus was first caught on 17/6/1998, and recaught on 26/5/1999. The Glengarry platypus had a lesion, which was biopsied, on the right front foot at first capture. Sphaerule-like bodies were seen in histological sections of the biopsy. On recapture the ulcer had totally resolved. The Nth. Camelford platypus had one ulcer approximately 4cm in diameter on the dorsal aspect of the tail which appeared to be resolving on first capture. On recapture, the ulcer was approximately 6cm in diameter. The animal had not lost weight, and appeared to be otherwise healthy. The Cressy platypus possessed a small ulcer on the inside of the left thigh which had resolved by the time of recapture.

5.3.6 Location of ulcers on affected animals

Affected animals had ulcers on the feet, tail, hip, thigh, back and neck (Table 3). No ulcers or lesions were seen on the head, bill, or underside of the body.

5.3.7 Results of nasal swabs

Of the 5 nasal swabs taken from platypuses at the Salmon Ponds, four were positive for *M. circinelloides*. Seven swabs were taken in the endemic area, with five being positive for *M. circinelloides*.

5.3.8 DNA analysis

The research by Dr Shiro Akiyama involved a comparison of platypuses sourced from Queensland, New South Wales, Victoria and Tasmania. Primers were developed to detect microsatellites in platypus genomic DNA. Microsatellites are segments of DNA with tandem repeats of short sequence motifs, usually 1 - 4 base pairs of nucleotides, and are highly interspersed in the genomes of all eucaryotes. Many of them consist of non-functional intergenic sequences, whilst others are associated with specific genetic disorders. They generally evolve faster than coding regions, making them useful for phylogenetic research. Six microsatellites were detected and analysed, with allele size, frequency and heterozygosity being compared between Tasmanian and mainland populations. Significant differences were noted between Tasmanian and mainland platypuses in some alleles. Mitochondrial DNA analysis also showed about a 2% difference between mainland and Tasmanian platypuses. Full details of methods and results are in Akiyama (1998).

	1997	1998	1999	2000
Glengarry	11 (4)	14	6	4
"Camelford"	3 (2)	3 (2)	-	-
"Connorville"	2 (1)	-	-	1 (1)
"Brumby's Cr"	-	3 (1)	2	-
Lake Pedder	-	5	-	-
Salmon Ponds	13	3	-	-

Table 5.1: Yearly captures of platypuses at the six sample sites. Numerals in brackets denote ulcerated platypuses.

	1997	1998	1999	2000
Glengarry	11 (2)	14 (3)	6 (4)	4
"Camelford"	3	3 (1)	-	-
"Connorville"	2	-	-	1
"Brumby's Cr"	-	3	2 (1)	-
Lake Pedder	-	5	-	-
Salmon Ponds	13 (1)	3	-	-

Table 5.2: Yearly captures of platypuses at the six sample sites. Numerals in brackets denote numbers of recaptures

Location	Foot (front)	Foot (rear)	Tail	Hip	Thigh	Back	Neck
G'garry	X						
G'garry			X	X			
G'garry			X	X	X	X	
G'garry			X				
C'ford	X						
C'ford			X				
C'ford			X				
C'ford			X	X			
C'ville			X	X		X	
C'ville			X	X		X	
Cressy					X		
S'field							X

Table 5.3: The location of ulcers on the 12 affected platypuses captured at 5 endemic sites

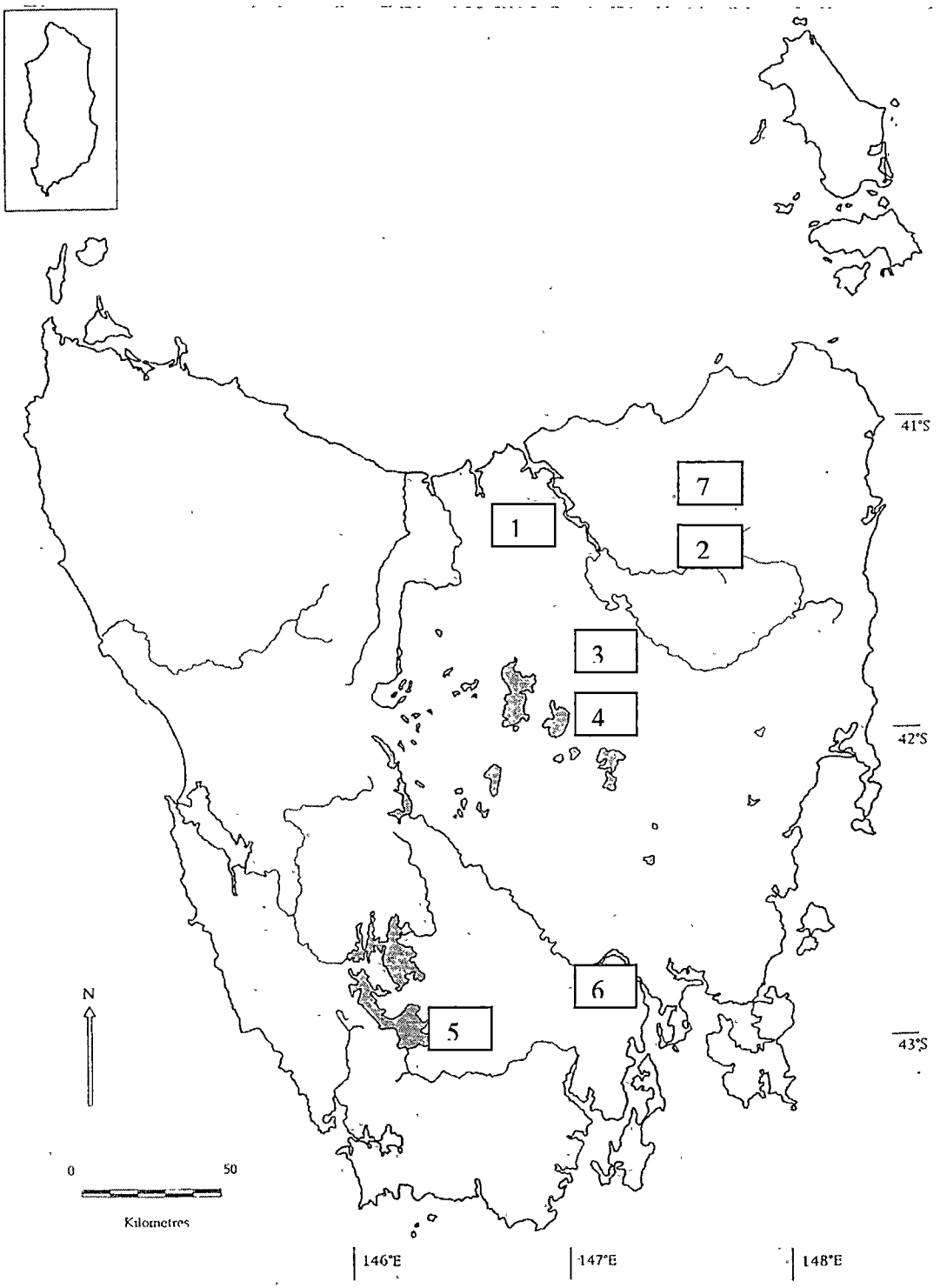


Figure 5.1: Map of Tasmania showing the six main sites at which platypuses were trapped. Site 1 Glengarry, Site 2 "North Camelford", Upper Blessington, Site 3 "Brumby's Creek" Cressy, Site 4 "Connorville" Cressy, Site 5 Lake Pedder, Site 6 Salmon Ponds. Site 7 is Springfield, the area at which one ulcerated platypus was caught in 2000

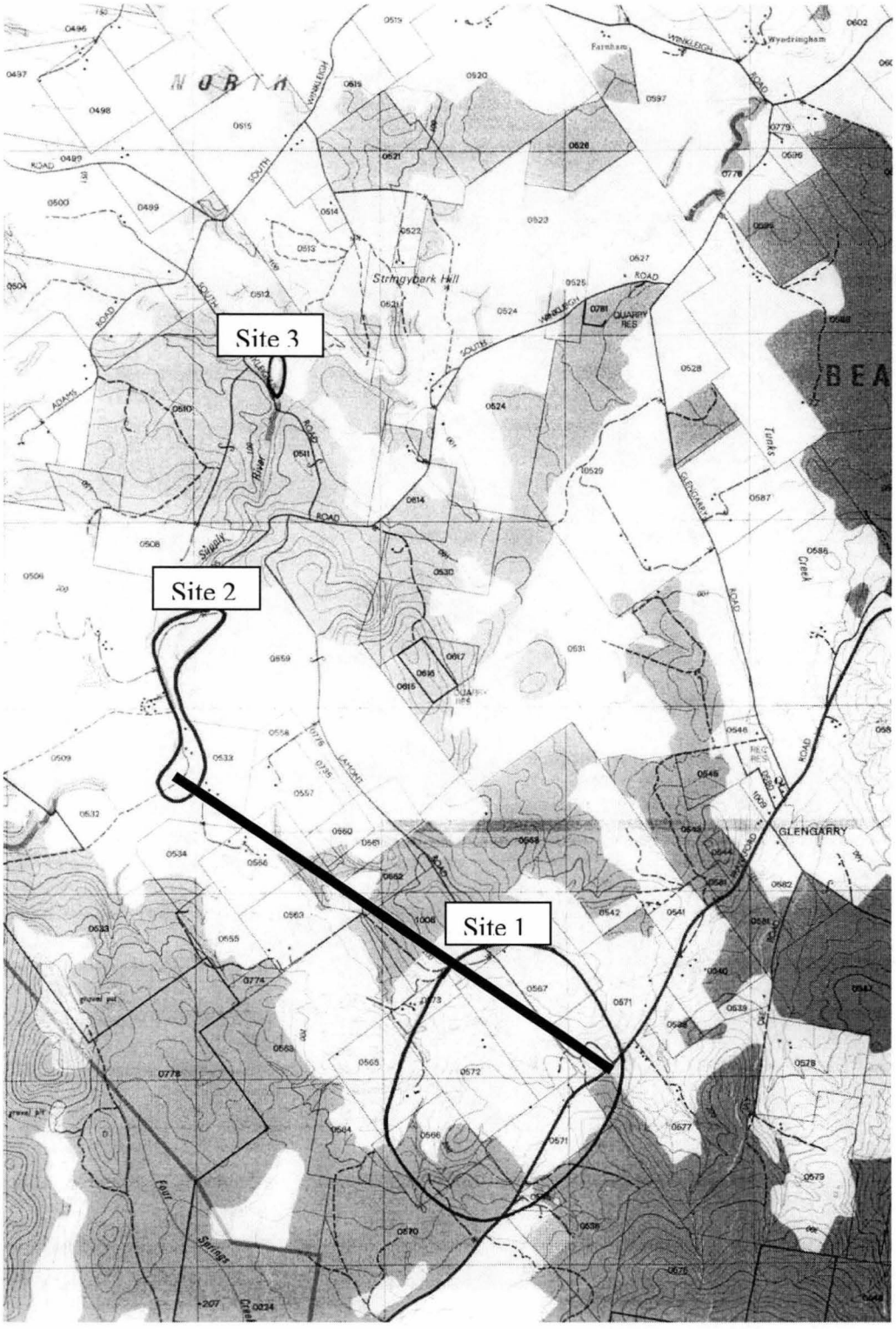


Figure 5.2: The Glengarry area showing the three different sampling sites. All ulcerated animals were trapped at Site 1. The line from Site 1 to Site 2 indicates the distance travelled (approx. 3km) by an ulcerated animal first trapped at Site 1, and subsequently trapped at Site 2

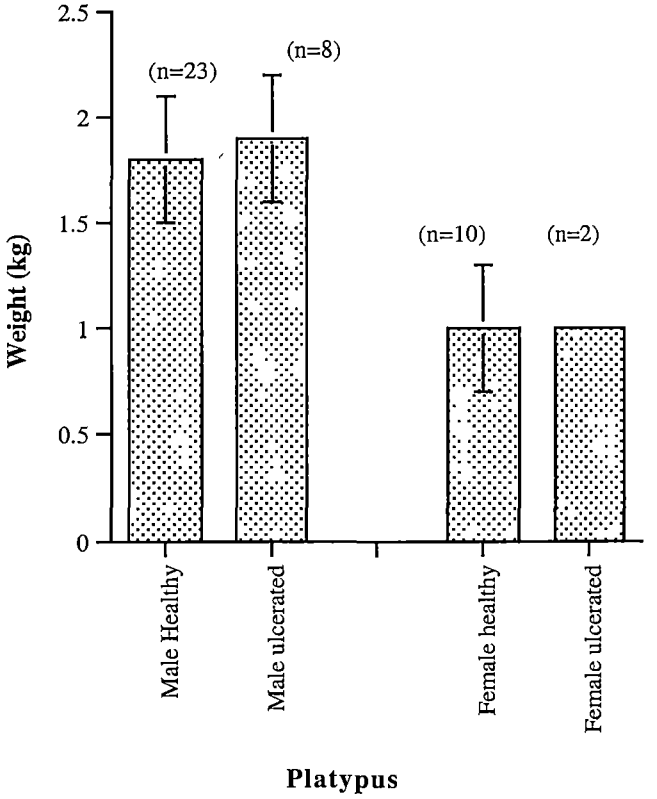


Figure 5.3: The mean weights of healthy male and female platypuses from endemic and non endemic sites, and of ulcerated platypuses from endemic sites

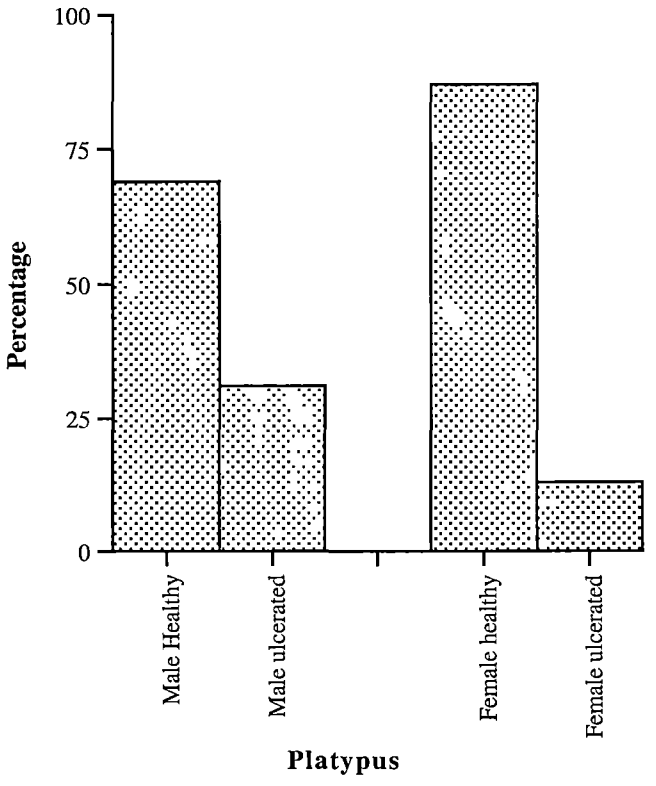


Figure 5.4: The percentage of healthy versus ulcerated male and female platypuses from the endemic area

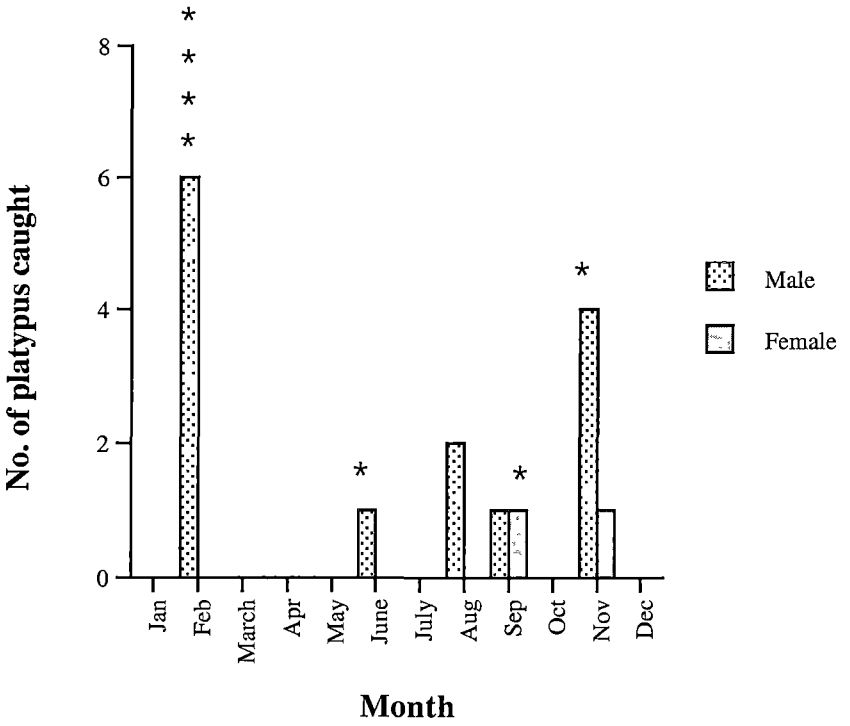


Figure 5.5: Monthly captures of platypuses in the endemic area for the year 1997. The symbol (*) denotes an ulcerated platypus.

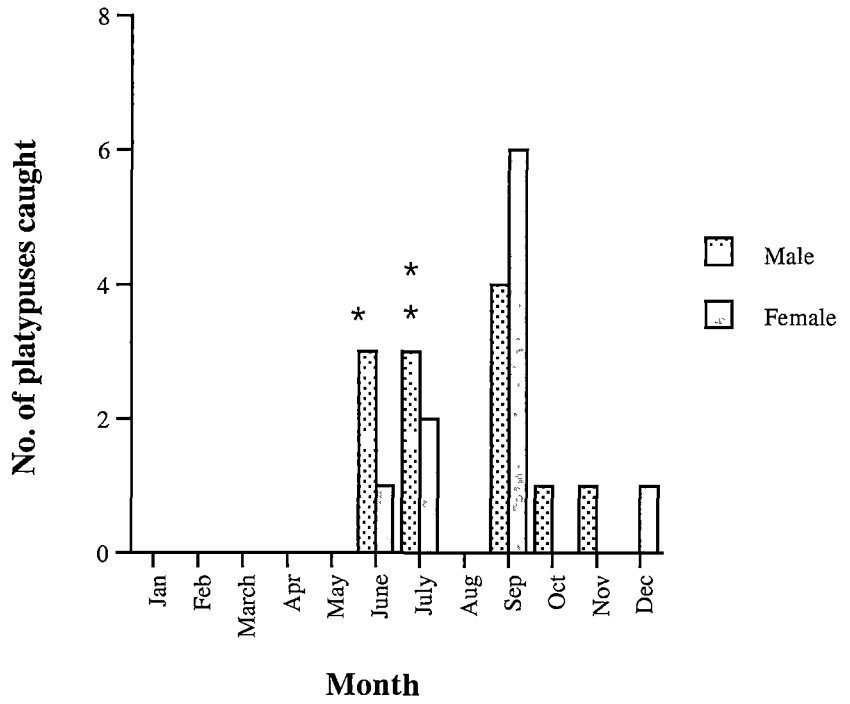


Figure 5.6: Monthly captures of platypuses in the endemic area for the year 1998. The symbol (*) denotes an ulcerated platypus.

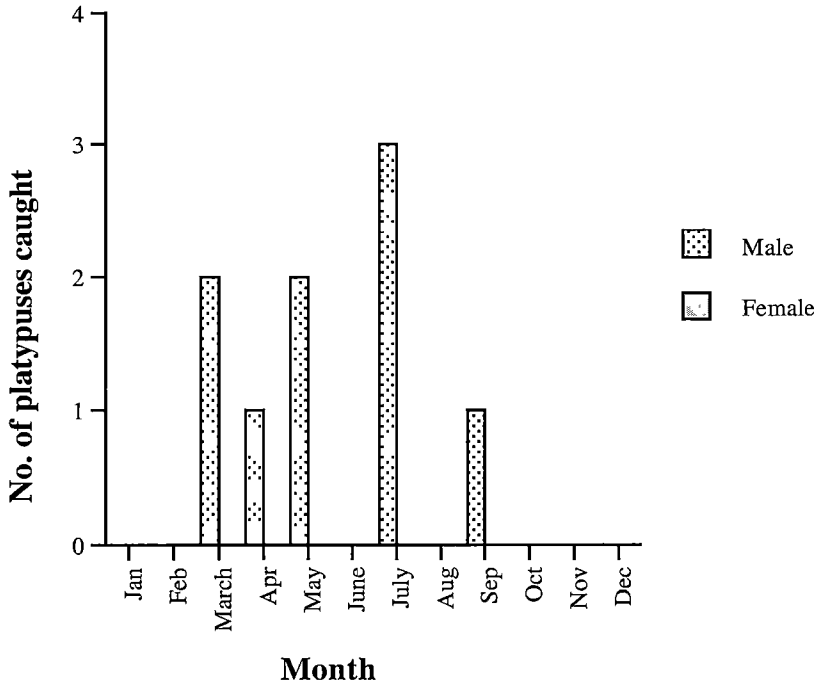


Figure 5.7: Monthly captures of platypuses in the endemic area for the year 1999

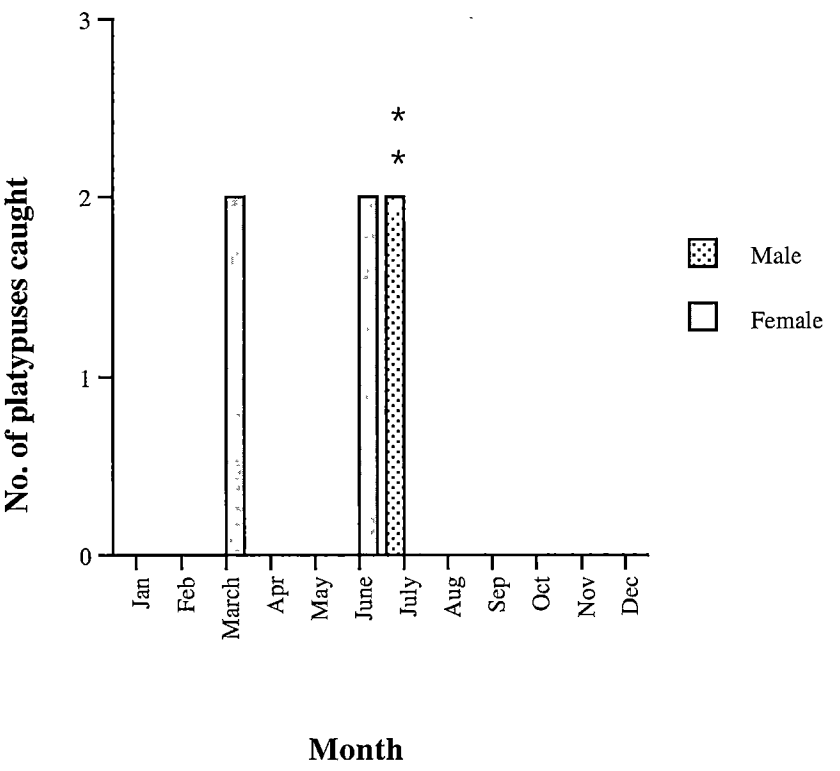


Figure 5.8: Monthly captures of platypuses in the endemic area for the year 2000. The symbol (*) denotes an ulcerated platypus.

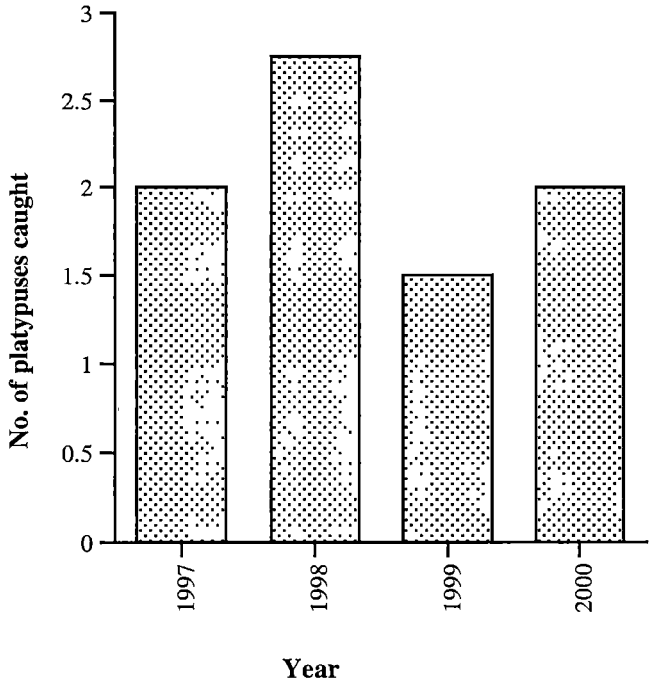


Figure 5.9: The mean number of healthy platypuses captured per week of trapping in the endemic area

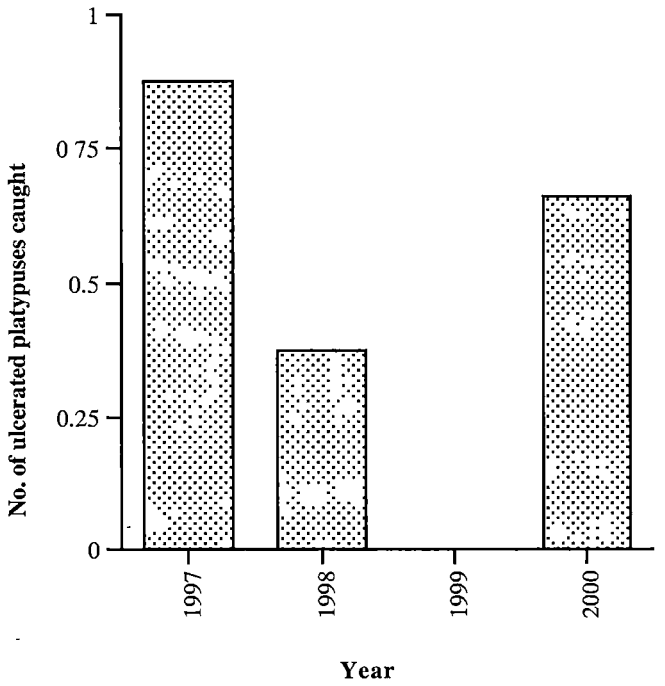


Figure 5.10: The mean number of ulcerated platypuses caught per week of trapping in the endemic area

5.4 Discussion

There was no significant difference in weights between platypuses from endemic and non endemic sites, although the weights of platypuses from the endemic sites were below those previously recorded. Connolly and Obendorf (1998) reported a mean weight for male platypuses in the north of the state of 2.4kg, and 1.4kg for females. It seems likely that the discrepancy is due to the type of scales used in the different studies. In this study, female platypuses are on average 53% of the weight of the males, which accords with the females in the Connolly and Obendorf study being 57% of the weight of the males. Also, the study of Connolly and Obendorf (1998) trapped animals primarily at Brumby's creek. In general, the animals there are larger than those found at Glengarry, where the majority of the animals in the endemic area were captured in this study. This probably reflects food availability in the Brumby's Creek area. As the waterway is fed from a hydroelectric dam, water levels can fluctuate frequently and markedly. As the water rises, many small drains and ditches fill with water, providing fresh prey for the platypuses. At Glengarry, the waterways are smaller, and not prone to frequent changes in level. Also, the area has few runnels or ditches that flood, with the streams generally keeping within their banks even after heavy rains. The presence of platypuses in farm dams in the area on a regular basis suggests that the streams themselves do not provide enough food for the entire population. Platypuses apparently regularly inhabit the farm dams for up to several weeks, only leaving when food becomes scarce. Conditions on King Island are even more rigorous for platypuses, with only a few small waterways habitable. It is possible that the smaller size of the female platypuses is directly related to a lack of suitable habitat and abundant food in comparison to mainland Tasmanian platypuses, although there is some evidence of inbreeding (Akiyama 1998) which could explain this observation.

The recovery of *M. circinelloides* from the nasal passages of platypuses in both the endemic and non endemic sites further supports the hypothesis that the route of infection of *M. amphibiorum* is via inhalation of spores. It is likely that the burrows of the platypus are the infective reservoirs in which *M. amphibiorum* grows. The fungus does not grow in strong direct sunlight, and is not common in the wider environment. It has not been isolated from water or soil, or from frog or platypus faeces in Tasmania (Connolly *et al.*. 1998), and has only once been isolated from soil in Queensland, despite intensive investigation (Speare *et al.*. 1994). The burrow provides ideal growing conditions for the fungus, with constant temperature and humidity, and no sunlight. Although *M. amphibiorum* was not isolated from the nasal passages of any of the animals, this was not surprising. It is likely that platypuses are only exposed to *M. amphibiorum* in the burrow; and not while foraging for

food. Also, not all burrows will be infected, and not all animals entering an infected burrow would inhale a significant number of spores. Repeated diving by platypuses may also clear spores from the nares.The fungus *M. circinelloides* is ubiquitous, and platypuses would be exposed to spores from this species both whilst diving and foraging, and when moving through undergrowth. Also, it is possible that spores of *M. amphibiorum* were present in the nares of the platypuses, but in such low numbers that they were overgrown by the spores of *M. circinelloides*, which has a faster growth rate than *M. amphibiorum*

Surprisingly, ulceration did not seem to affect the weights of animals. In fact, ulcerated male animals had a mean weight slightly higher than non ulcerated animals. A possible explanation for this may be that larger males have a bigger territory than smaller males, and therefore visit more burrows than other animals. If the disease is spread via inhalation of spores in shared burrows, the more burrows a platypus visits, the more likely it is to be exposed to the spores. Such a scenario may also explain the higher infection rates in males. Male platypuses can regularly travel up to 10km in a single overnight period (Serena *et al.*. 1998), with some males travelling up to 15km a night from one catchment to another (Gardner and Serena 1995). Also, in one study, it appeared that males shared burrows with other males, and females with females (Serena 1994). Thus, an infected male is more likely to deposit spores in a burrow used by other males than in burrows used by females. Alternatively, smaller males with the disease may succumb more rapidly to the disease, resulting in a greater proportion of large animals.

The larger territories of the males may also explain the greater number of males caught. Of the 72 individual animals caught in mainland Tasmania, 45 (or 64%) were males . There was no significant difference between male/female ratios at the endemic and non endemic sites, and the ratios were comparable with those described by Connolly and Obendorf (1998), with 23 (or 64%) of 36 animals caught being males. At the Salmon Ponds, 63% of animals captured were males, and males comprised 60% of captures at Lake Pedder. If ulcerative mycosis is causing significantly more mortalities in males rather than females, it is not reflected in capture rates. There was no discernible monthly pattern for capture rates, suggesting that platypuses in both endemic and non endemic areas are active throughout the year. Ulcerated platypuses were captured in all seasons, suggesting that neither hot conditions associated with summer, nor cold conditions associated with winter, are killing the majority of affected animals.

The low capture rate per week of trapping demonstrates the difficulties inherent in studying a cryptic animal such as the platypus. At most sites, a minimum of 6 fyke nets, and up to 10, were set, with nets being occasionally moved to new areas of rivers if no animals were being caught. Recapture of platypuses was also rare, with 84% of animals only being caught once, which is in accord with the 78% recorded by Connolly and Obendorf (1998). Although it has been reported that netting has no effect on future recapture of individual animals (Playford *et al.*. 1998), the rapid decline in captures at the Salmon Ponds, from 13 in 1997, to 3 in 1998, and none in the following years, suggests that animals do learn to avoid nets. This area was trapped by the author, and by two other groups during the study period. Animals in areas being retrapped have been observed swimming around or over fyke and gill nets; further supporting the hypothesis that platypuses are capable of remembering trapping events, and thus avoiding future captures. The inability to consistently recapture animals makes study of individual, ulcerated animals problematic.

The captures per week of trapping were higher in the non endemic areas (3.5) than the endemic areas (2.2). Whether this is a reflection of more suitable habitats in the non endemic areas, or a result of decreased populations in the endemic area due to ulcerative mycosis could not be determined. It would not seem unreasonable to conclude that mortalities due to the disease result in reduced population densities in comparison to unaffected sites. In fact, if the figure of 35% of animals being affected in endemic areas is applied, a reduction in population due to disease seems correct. A 35% reduction of the 3.5 tcaptures per week in non endemic areas equates to 2.3 captures per week, a figure close to that observed in endemic areas.

Although recapture of platypuses was rare, the successful recapture of three ulcerated animals provided valuable information on the course of the disease. The male animal recaptured at Nth. Camelford was still in good health 18 months after first capture. Although the ulcer had appeared to be resolving on first capture, it had grown by second capture. It's possible that the severity of ulceration is influenced by season. First capture was in February, when food is abundant. Perhaps during such times the ulcer starts to resolve, but as winter approaches, and food becomes scarcer and water colder, the ulcer starts to grow again. The recapture of a healthy, previously ulcerated Cressy animal demonstrated that some platypuses can recover from an active ulcer. The ability of some animals to fully recover from the disease was further supported by the recapture of a previously ulcerated platypus at Glengarry, and by the capture of a platypus at Nth.

Camelford that had a healed lesion on the front foot. A biopsy from both these animals revealed the presence of sphaerule-like bodies.

Although the exact number of platypuses that die from ulcerative mycosis is not known, many do succumb to the disease and are found dead on riverbanks. The survival of some affected platypuses for at least 18 months after infection has important implications not only for the spread of the disease, but also for the continuation of the disease in platypus populations. If, as has been hypothesised, the disease is in part due to inherent susceptibility caused by genetic differences between Tasmanian and mainland platypuses, then the survival of ulcerated platypuses for long periods will ensure a susceptible population is always present. If the disease rapidly killed affected animals, or debilitated them, then these animals would be incapable of breeding, resulting in animals carrying the genes associated with susceptibility dying out. Because ulcerated animals are still active over at least two breeding seasons the genes for susceptibility will continue to be passed on. Because males are predominantly affected, the survival of ulcerated animals will result in shared burrows in affected animals' areas being constantly re-infected with *M. amphibiorum*. Although ulcerated animals are still capable of breeding, it is likely that increased mortality in these animals will eventually result in infection rates dropping in affected areas. This seems to be the case at Brumby's Creek, where only one animal with a resolving ulcer was trapped during the study period, although ulcerated animals were observed. If the infection rate reported by Connolly *et al.*. (1998) was constant, more ulcerated animals would have been expected. Anecdotal evidence from fishermen and farmers in the area also suggests the infection rate has dropped.

The location of ulcers on the body of affected platypuses differed slightly from that previously reported. Connolly *et al.*. (1998) report hindlimbs as being the most commonly affected (38%), followed by the tail (19%). In this study the tail was the most commonly affected (67%), followed by the hip area (42%). The study by Connolly *et al.*. (1998) also reported ulceration on the head and the bill, which wasn't reported in this study. Such ulceration was however noted by Obendorf *et al.*. (1993). That study examined moribund and dead platypuses, and perhaps in more severe cases the ulceration spreads to the bill and head. Unfortunately, neither study reports whether animals with ulcers on the head/bill also had ulcers on other parts of the body, although the original work by Munday and Peel (1983) describes a platypus with severe ulceration on the tail and the bill.

There was no evidence to suggest that local conditions at the various sites were responsible for the presence of ulcerative mycosis. Previous studies by Connolly *et al.*. (1998) found no differences in water quality between sites in which the disease occurred, and those in which it didn't. Presence or absence of livestock did not seemingly influence infection rates, and neither did water flow or water level. It seems likely that the disease is not influenced by the environmental conditions normally found in Tasmania.

The collaborative work with Dr Shiro Akiyama demonstrated the genetic differences that exist between Tasmanian and mainland Australian platypuses. The differences are great enough for the Tasmanian platypuses to be classed as a subspecies. The differences may be due to the relatively long period of isolation that the Tasmanian platypuses have experienced. Tasmania has been cut off from mainland Australia for at least 12,000 years, due to rising water levels. It is likely that the period of isolation is even longer, as the land bridge between Tasmania and the mainland may not have had an adequate supply of fresh water to ensure easy movement by platypuses. It has also been suggested that the Tasmanian platypus represent a southern cold-climate adapted gene pool that has evolved over the past million years (Akiyama 1998). If this is indeed the case then significant differences in the genome would be expected.

Although the genetic differences between mainland Australian platypus and Tasmanian platypus populations cannot be directly linked to the disease ulcerative mycosis, it is not unreasonable to assume that some Tasmanian platypuses are inherently susceptible to the disease. Such inherent susceptibility has been noted in human populations. For example, point mutations in the genes coding for mannose-binding lectin are associated with increased susceptibility to mycotic infections (Turner *et al.* 1993, Lipscombe *et al.*. 1992). Further, some of these point mutations are associated with different racial groups (Ten *et al.*. 1999). The disease coccidioidomycosis is 5 -20 times more likely to occur in the disseminated form in blacks and Filipinos than in Caucasians (Flynn *et al.* 1979, Pappagianis 1980, Pappagianis *et al.* 1979), but this susceptibility is not related to impaired cell-mediated immunity. There is some evidence that the major histocompatibility complex antigen phenotypes may influence susceptibility (Calich *et al.* 1985, Cox *et al.* 1988, Kirkland and Fierer 1983). Thus, susceptibility to the disease ulcerative mycosis may not necessarily be directly associated with impaired immunity.

If it is accepted that there is a susceptible population of platypuses in Tasmania of about 30%, then the incidence and prevalence of the disease in the areas investigated can be

explained as follows. The Cressy, Connorville, and perhaps Upper Blessington sites, have a high prevalence of the susceptible genotype. Therefore, there is continued occurrence of ulcerated platypuses because there are always a few susceptible but naive individuals in the population. Site 1 at Glengarry has platypuses with an average prevalence of the susceptible genotype. When the disease first reached the area about 30% of animals were affected. Some animals died, whilst others recovered, leading to a population free of lesions. The disease will re-occur as more naive animals migrate into, or are born in the area. Site 2 at Glengarry has a low prevalence of the susceptible genotype. This site is on the boundary of the known epidemic zone, and at least one mildly affected animal is known to have travelled into the area. The nine animals trapped in the area were all healthy. Site 3 at Glengarry may lie outside the epidemic zone. Even if the population has a high prevalence of the susceptible genotype, the relative resistance of the population in Site 2 may provide a buffer against the introduction of the disease.

In conclusion, platypuses from the endemic area do not appear to differ from their counterparts in the non endemic areas as far as weight and sex ratios are concerned. Male platypuses were generally larger than females, and were more likely to be both initially and subsequently trapped than females. In endemic sites, males were also more likely to be ulcerated than females. It seems likely that the disease is predominantly spread by males, who have a larger territory and are more likely to share burrows with other males. The collaborative work with Dr Shiro Akiyama demonstrated genetic differences between Tasmanian and mainland Australian platypuses. Tasmania may possess a sub-population of platypuses susceptible to infection. Further, the genotype for susceptibility may not occur uniformly throughout the state, with some areas, such as Site 2 at Glengarry, apparently having a low prevalence of the susceptible genotype.

General Discussion

General Discussion

The research undertaken into the epidemiology of the disease ulcerative mycosis of platypus supports the hypothesis that the fungus responsible for the condition has been introduced into Tasmania. Further, many Tasmanian platypuses are inherently susceptible to the isolate of *M. amphibiorum* that has become established in the state. If this is indeed the case, it has serious implications for mainland Tasmanian platypuses outside of the known endemic area. Alternatively, that the disease is caused by a mutated native isolate of *M. amphibiorum* is also supported by the data. Mortality rates in ulcerated animals may be in part influenced by impaired function in a T cell sub set activated by PHA.

The immunological assays performed did not point to any evidence of immunosuppression in healthy Tasmanian platypuses, either within or outside the endemic area. Cellular response, as measured by the lymphocyte proliferation assay, was uniform for such animals from throughout the state. Although the lymphocyte proliferation stimulation indices were lower for Tasmanian platypuses than for many eutherians, they were consistent with the responses reported for some marsupials (Infante *et al.*(1991), and are on a par with recent indices reported in mainland Australian populations of platypuses (pers. comm. Young). The low response in platypuses in comparison to many other species probably reflects the low core body temperature of the animal.

A significantly lowered response was noted in ulcerated animals to the mitogens PHA and PWM, with a lowered response to ConA and LPS also evident. The trend of decreasing proliferation in response to ConA with increasing severity of ulceration suggests that the disease can alter immune function, perhaps suppressing T cells activated by ConA. The presence of severely ulcerated animals with relatively high responses to PHA, and mildly ulcerated animals with low responses, may indicate that different sub sets of T cells are stimulated by ConA and PHA. Such a phenomenon has been noted in other species (Shifrine *et al.* 1978). The sub set stimulated by PHA may be protective in the long term; that is, animals with a good response to PHA may fully recover, whilst those with a poor response may succumb. This would in part explain why some animals make a full recovery. Responses to PWM were also significantly lowered in ulcerated animals. This is both a B and T cell mitogen, and may stimulate the same T cell sub set as PHA.

That some mitogen-treated cells actually had a lower response than untreated (control) cells may also indicate that the mitogens were retarding cell division instead of promoting

it. Of course, it is possible that the disease had resulted in damage to the lymphocytes, and that the mitogens, instead of promoting division, had actually become toxic.

Previous work had demonstrated a high degree of immunological cross reactivity between *M. amphibiorum* and *M. circinelloides* (Stewart 1996). Sera from rabbits injected with killed cultures of both species reacted equally well with antigens from either *M. amphibiorum* or *M. circinelloides*. To visualise the areas of cross reactivity a Western blot was developed. This clearly showed that both species of fungus share common antigenic proteins of relatively high molecular weight. Interestingly, there were several bands of low molecular weight proteins present in *M. amphibiorum* that stained equally well with sera from rabbits injected with either *M. amphibiorum* or *M. circinelloides* . Although these bands did not stain in blots using *M. circinelloides* as the antigen, using sera from rabbits injected with either *M. amphibiorum* or *M. circinelloides*, it seems likely that it does possess these proteins, but in such small amounts that they do not stain in a blot.

In a study by Connolly *et al.* (1999), the results from an ELISA using sonicated *M. amphibiorum* as the antigen showed that sera from Tasmanian platypuses with ulcers had high optical densities (OD) in comparison to sera from healthy Tasmanian animals, but the ODs for healthy animals were also quite high. This suggests that platypuses already had antibodies to an antigen found in *M. amphibiorum.* This was most likely the high molecular weight protein common to *M. amphibiorum* and *M. circinelloides* seen in the Western blot.

The inability of a Western blot to detect LMW antibodies to *M. amphibiorum* in any animals other than those with active or recently healed ulcers suggests that an active infection is required for a detectable antibody response to be mounted. The Western blot also showed that *M. amphibiorum* shares many antigenic determinants with *M. circinelloides.* This high level of cross reactivity means that an ELISA based on polyclonal antibodies will not be able to detect the difference between antibodies produced by normal, environmental exposure to *M .circinelloides* (and probably many other *Mucor* species), and those produced by exposure to *M.amphibiorum.*

Although the ELISA developed by Whittington was unsuitable for screening platytpus for previous exposure to *M. amphibiorum*, because only affected animals gave a significant response (i.e. it only confirms that the animals are ulcerated), it did prove useful in this study in comparing sera from Tasmanian and Victorian platypuses. The Victorian platypuses had a significantly higher OD than Tasmanian platypuses, raising the possibility that Victorian platypuses are more efficient at processing antigens from *M.*

circinelloides, and raising antibodies to them. The heightened OD does not seem to have been caused by exposure to *M. amphibiorum*, as a Western blot for these sera showed bands associated with *M. circinelloides*, but not with low molecular weight bands associated with *M. amphibiorum*. Obviously both Tasmanian and Victorian platypuses are equally efficient at killing *M. circinelloides* spores, as neither group has been unequivocally reported as suffering from infection from this fungus. Of course, it is also possible that the Victorian platypuses were exposed to higher levels of *M. circinelloides* than their Tasmanian counterparts, resulting in increased antibody activity. The Victorian animals were kept at a zoo, and may have been exposed to unusually high levels of *M. circinelloides* in the nesting boxes although this seems unlikely, given the nature of the materials used in their construction.

It is possible that Victorian platypuses are more efficient at processing *M. circinelloides* antigens, and raising antibodies to them, than Tasmanian platypuses. This possible reduced ability of Tasmanian platypuses is inconsequential when dealing with *M. circinelloides*, but may be important when the platypus is faced with the pathogenic *M. amphibiorum*.

The work does not support the theory that *M. circinelloides* is also implicated in the disease. Although this species is capable of forming sphaerule-like bodies *in vitro* (Stewart 1998), it was incapable of causing disease in cane toads, with no organs from toads challenged with this fungus showing the presence of slb. The DNA sequencing work supports the hypothesis that the fungus is an environmental contaminant, with a high degree of variability between isolates in this species. The eventual domination of two cultures of *M. amphibiorum* by *M. circinelloides* suggests that occasionally this fungus contaminates cultures of *M. amphibiorum*, and can occassionally overgrow the *M. amphibiorum* when conditions are suitable.

The DNA sequencing of *M. amphibiorum* showed little variation between (+) mating strains of the species, with greater variation occurring between (+) and (-) mating strains. The Tasmanian isolates were identical, but whether this means that they all originated from a single point source outside Tasmania could not be determined. In many fungal species, there is great variation in the ITS sequences, and it is occassionally possible to relate a sequence to a geographic location (Bryan *et al.* 1994, Rohel *et al.* 1997). Pathogenicity and/or virulence are also sometimes reflected in ITS sequences (Xue *et al.* 1992). It is tempting to state that the Tasmanian isolates represent a more pathogenic group than the other two (+) mating types, which together form a second, less pathogenic group which in turn is more pathogenic than the group to which the (-) mating type belongs. This may

indeed be the case, but a much larger sample size will be required to resolve this point. Further work is also required to determine the range of *M. amphibiorum* in Australia. The fungus has only recently been described in Australia, and it is not known whether the isolation of the fungus from amphibians in Queensland, Victoria and West Australia has occurred because researchers have been actively seeking it, or whether it has only recently been introduced into those states. It is possible that the fungus has been brought to Australia from Hawaii, when cane toads were brought to Australia to control pests in cane fields (Barton 1997). At present, close to one percent of cane toads are infected with the fungus (Speare *et al.* 1994). If the fungus is found in Hawaii, then it is likely that many of the toads brought to Australia were infected. Isolating the fungus from Hawaii and sequencing the ITS regions may provide valuable information on the origins of this fungus in Australia.

The low level of variation in ITS sequences of isolates from geographically distant areas makes identification of origin of the isolates difficult. It was hoped that isolates separated by great distances would display greater variation in sequences, but the lack of variation between isolates from Western Australia and Queensland suggests that such variation may not occur. However, the presence of identical sequences in isolates from such widely separated areas may also support the introduction of the fungus to Australia in cane toads, and its spread from that state to others via infected amphibians in fruit consignments. Western Australia is separated from Queensland not only by a vast distance, but also by desert, and has been for thousands of years. During this time of isolation some genetic drift could be expected if the fungus was truly endemic throughout Australia.

The pathogenicity trials provided valuable information on the different isolates of *M. amphibiorum*, and grouped the isolates in the same way as the ITS sequencing. The Tasmanian isolates formed one group, which, although no more virulent than the other isolates, were capable of producing more infectious forms than the mainland isolates. All isolates were capable of infecting cane toads, but the Tasmanian isolates were more capable of producing large numbers of the infective stage of the fungus (sphaerule-like bodies) *in vivo*. The Queensland (-) mating type infected the toads, but by the end of the trial very few sphaerule-like bodies (slb) had survived. The other two mainland isolates produced more slb than the (-) mating type in the livers of toads, but the numbers were significantly lower than for the Tasmanian isolates. In the other organs examined (heart and lung) the case was not quite so clear, probably because the trial did not run long enough for significant differences to become clear in these organs.

The ability of the Tasmanian isolates to rapidly produce slb is not reflected in their growth *in vitro*. When growing *in vitro*, the Tasmanian isolates had the same rate of growth as the Queensland isolate Q2, and a slower rate of growth than Q1. The spores of the isolates were all of a similar size, therefore ruling out the possibility that large spore size was preventing the successful clearing of the fungus from the toads. The (+) mating types also produced a larger proportion of slb with daughter cells than the (-) mating type. As in other species, (-) mating types may be less able to produce long term infections than (+) mating types. Both types are equally capable of infecting the host, but the host can easily clear the (-) mating type from affected organs, whereas (+) mating types reproduce at such a rapid rate that the host immune system is overwhelmed. Although the (+) mating types were capable of rapidly dividing in the host, an initial number of spores is required for the infection to establish. Exposure to small numbers of spores resulted in no long term infection.

The results of the sampling for the xenobiotics PCB, DDT and Lindane provided valuable information on the state of many Tasmanian waterways. Although the levels of these xenobiotics in the tail fat lipid of platypuses are generally lower than those regularly found in many European mammals, the presence of some animals with very high levels, especially of PCBs, is of concern. It does not seem that the xenobiotics are involved in the disease ulcerative mycosis, but further work is required to determine whether the levels found represent a risk to platypus populations. Platypuses may be highly susceptible to some xenobiotics. Neonatal platypuses are very poorly developed, and are totally incapable of metabolising xenobiotics. Any xenobiotics present in the mother's milk can act directly on target organs. Eutherian neonates are more developed when they begin to suckle, and they are capable of metabolising xenobiotics as efficiently as an adult (Bolton-Grob *et al.*, in press). Very little work has been done in Australia on terrestrial animals and xenobiotics. The little work that has been done has concentrated on ruminants such as kangaroos. Most xenobiotics eventually find their way into an aquatic environment, so a more suitable way to determine contamination in an area is to sample aquatic organisms. The platypus is relatively high in the food chain, and as such represents an excellent bio-indicator for pollution. Although Tasmania prides itself on having a "clean and green" image, the presence of moderately high levels of PCBs in Lake Pedder, a large lake in a wilderness area, suggests that the image is based primarily on ignorance.

The research undertaken supports several hypotheses that would explain the presence of the disease ulcerative mycosis in Tasmanian, but not mainland Australian, platypus populations. The fungus responsible, *M. amphibiorum*, may have been introduced into the state, exposing a naive population to a new pathogen. Secondly, *M. amphibiorum* may be

native to Tasmania, and a native isolate has mutated to become pathogenic to platypuses. Both of these events may be associated with a genetic predisposition to infection in the platypus.

Many fungal species regularly enter Tasmania. They can be found on vegetables, and may be transported to the state both in and on livestock. Potted plants brought into the state carry a variety of fungi, and it is likely that some fungal species are carried to the state by migrating birds. The most likely method of introduction of *M. amphibiorum* into the state is via amphibians, especially green tree frogs. These amphibians are often found in bunches of bananas imported into Tasmania from Queensland. Most of these amphibians are removed prior to shipment, and the majority of those that escape detection are destroyed by Tasmanian customs inspectors. However, some animals do reach supermarkets. Not all of these animals are infected with the fungus. It has been estimated that at least several hundred frogs find their way into Tasmania each year. If the infection rate for the frogs is the same as for cane toads (about one percent), then around five infected frogs find their way into the state each year. In many cases, the fungus probably dies. Fungus in a frog discarded into rubbish and buried at a tip site is unlikely to grow. Even if it did, it wouldn't pose a threat unless the fungus was exposed and carried to other areas. Other frogs that may be released by people into the wild also are unlikely to spread the fungus. The fungus can be killed by exposure to direct sunshine, so many frogs that died in the wild would also not spread the fungus. However, very occassionally environmental conditions will be suitable, and the fungus will be able to spread from the frog to the surrounding environment.

Once in the environment, it is still unlikely that the fungus would establish itself. Even if it did, it may not pose a threat to wildlife. As shown in the pathogenicity trials, exposure to low levels of spores did not result in long lasting disease in cane toads. It is possible that Tasmania actually harbours quite a number of different strains of *M. amphibiorum* that exist purely as saprobic fungi. Some time prior to 1982 an isolate of *M. amphibiorum* may have became established in the environment near Campbelltown in the north of Tasmania. The river at that time was low, due to drought conditions (Munday and Peel 1983). As a consequence, water flow was slow. The combination of a moist, warm environment in the reed beds was ideal for the growth of *M. amphibiorum.* A platypus could have inhaled spores from this isolate. Unlike other isolates of *M. amphibiorum*, this one was capable of very rapid production and division of sphaerule-like bodies. In mainland platypuses, such isolates pose no threat, but in Tasmania the combination of exposure to a new fungus, combined with inherent susceptibility due to genetic drift, resulted in the disease becoming established. Obviously the Tasmanian platypuses'

immune system functions well in most circumstances, as they exhibit no signs of opportunistic diseases, and are relatively disease-free with the exception of the blood-borne parasites trypanosomes (Munday *et al.* 1998).

Although only (+) mating types have been found in Tasmania, it is likely that (-) mating types find their way into Tasmania, but do not establish. In cane toads, they are unable to produce long-lasting infection. This has been noted in other species, where (+) mating types are more pathogenic (Michailides and Spotts 1986). Also, (-) mating types are sometimes less robust, and can die when conditions are unsuitable.

The second hypothesis, that a native *M. amphibiorum* isolate has mutated, is also supported by the research. Both the ITS data and the pathogenicity data group the Tasmanian isolates together. Such grouping could occur both via the introduction of the species, and by the mutation of a native species. Both hypotheses would explain the appearance of the disease in one location, and the slow spread of the disease to new areas.

The research into the genetics of platypuses showed that Tasmanian platypuses are distinct from their mainland counterparts, and it is possible that one of the differences is an increased susceptibility to infection by *M. amphibiorum.* As stated previously, Tasmania has been isolated from mainland Australia for at least 12,000 years (Hope 1973). Although the Bassian land bridge disappeared at that time, isolating Tasmanian platypuses from the mainland, it is likely that Tasmanian platypuses had been effectively cut off many years prior to this. It has been suggested that the Tasmanian platypus represent a southern cold-climate adapted gene pool that has evolved over the last million years, maintaining some integrity even during periods of contact through its adaptations to high latitude temperatures and photoperiods (Akiyama 1998). In such a scenario, it is quite possible that such isolation has resulted in increased susceptibility to either an introduced or native-mutated isolate of *M. amphibiorum.* Even very small genetic differences, such as those exhibited by different human racial groups, can result in increased susceptibilities to different fungal diseases. For example, blacks and Filipinos are up to 20 times more likely to suffer disseminated coccidioidomycosis than Caucasians (Pappagianis 1980). Such susceptibility does not seem to be due to a defect in cellular immune response, but does seem to be related to features of the major histocompatibility complex antigen phenotypes and blood groups.

Whether platypus have been infected by an introduced or mutated isolate of *M. amphibiorum,* the disease has spread slowly to others in the area. Burrows provide an excellent environment for the fungus. They are warm, moist, and not exposed to direct

sunlight. An ulcerated platypus entering a burrow would leave sphaerule-like bodies on the walls and floor of the burrow. These would grow into the hyphal form of the fungus, producing many spores. Another animal entering the burrow would disrupt the spore-bodies, releasing spores into the air. These would be inhaled by the platypus. Most would be destroyed by phagocytes in the lungs, but some survive in the phagocytes, where they are carried to other parts of the body. The platypus has a rete system encircling the pelvic girdle. Spores not destroyed by the immune system would be trapped in the fine network of blood vessels, where they would form slb, and eventually result in ulceration. Occasionally spores would become trapped in other areas, such as beneath the skin of the feet.

As male platypuses have larger territories than females, and tend to share burrows with other males (Serena 1994), they are more likely to contract the disease (which the data supports), and spread it to new burrows. It is likely that the disease will eventually affect all mainland Tasmanian platypuses. There are few waterways in Tasmania that are not connected to other rivers and streams, and platypuses are quite capable of travelling long distances overland to new rivers (Serena *et al.* 1998). Once established, it will not be possible to eradicate the disease. Although there are likely to be inherently immune animals in the population, it is not likely that these animals will outbreed susceptible animals. Ulcerated animals can still be active, and presumably can still breed. Although some animals are inherently susceptible, it is also possible that environmental conditions can result in additional stressors that produce disease in animals that normally would be capable of successfully fighting the infection.

In conclusion, approximately 30% of Tasmanian platypuses seem to be susceptible to infection by *M. amphibiorum.* This susceptibility can be in part explained by the isolation of Tasmanian platypuses from mainland populations for a considerable time, resulting in genetic drift. Further, some strains of *M. amphibiorum* are more capable of rapidly producing the infectious propagules (slb) than others. One of these strains has either been introduced into Tasmania, or a native strain has mutated. Infection alters some immune parameters, and recovery from disease may rely on the healthy functioning of a T cell subset, with animals with impaired function succumbing.

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