ATTACHMENT 6: Quarantine guidelines and protocols for amphibians

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Amphibian quarantine protocols: Melbourne Zoo

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Introduction

Currently Melbourne Zoo maintains and displays amphibians for conservation education purposes and for investigations into appropriate husbandry protocols for priority endangered species. Animals entering the collection are sourced from other zoo's and aquaria and occasionally from free-ranging populations. No species in the current collection are intended for release to the wild but it is possible that Melbourne Zoo will be involved in captive breeding-release programs in the future. Quarantine protocols for Melbourne Zoo's amphibians have been in operation for many years and are designed to reduce the risk of introducing pathogens to the collection, the spread of pathogens within the collection and the release of pathogens from the facility into receiving collections or free-ranging populations.

This report will document existing protocols and provide additional information about diagnostic techniques, disinfection and therapeutic agents that relate to the containment of *Batrachochytrium dendrobatidis* (fungus causing the disease chytridiomycosis) and other pathogens. It is intended as an information source for both veterinary and keeping staff. It is also intended that this document be adjusted as knowledge on amphibian pathogens increases.

1. Prevention of pathogen introduction

(i) Source of animals

Animals should ideally be sourced from reputable collections that adhere to similar amphibian quarantine protocols that are followed at Melbourne Zoo. Institutions that routinely submit dead specimens for post mortem examination and have an awareness of amphibian disease issues should be able to build up an understanding of the disease issues in their collections. Animals sourced from collections that can not provide an adequate disease history should undergo treatment for, or be submitted to, specific diagnostic procedures for *Batrachochytrium* during their quarantine period (See Sections 1.iii and 1.iv)

Animals that are received by public donation (eg. banana box frogs) will not be admitted to the collection unless identified as priority species. Non-priority species will be housed in the veterinary area until their transfer to the Amphibian Research Centre (ARC). All donated frogs that are displaying symptoms of infectious or unknown disease will be euthanised.

Donated frogs kept because they are a priority species and tadpoles and frogs collected from the wild should undergo treatment for, or be submitted to, specific diagnostic procedures for *Batrachochytrium* during their quarantine period (See Sections 1.iii and 1.iv). Currently there

is no evidence that egg masses carry infectious disease but if being collected from the wild they should be kept under quarantine until 60 days post-metamorphosis.

(ii) Length of quarantine period

The length of quarantine should be no less than 60 days and if suitable for the species of amphibian, the environmental temperature should be kept between 17 and 23^0C. This time period and temperature range has been determined by studies of the *Batrachochytrium* in *Mixophyes fasciolatus* frogs. All animals died within 60 days of infection if kept between 17 and 23^0C but those kept at 27^0C survived for over 2 months and the organism could still be recovered after this time (Lee Berger, pers com). Currently there is a lack of information about species variability in regards to susceptibility to chytridiomycosis and the possibility of carrier states so the use of prophylactic treatments and diagnostic procedures will be used for selected groups. In regards to pathogens other than chytrid fungus, there are many gaps in our knowledge of significant amphibian diseases and their epidemiology. A 60 day quarantine period combined with thorough pathological investigations minimises the risks of introducing diseases to the collection.

(iii) Treatment protocols

Unless specific pathogens amenable to treatment (eg. gastrointestinal parasitism) are identified during the quarantine period the only disease we would currently consider for prophylactic treatment is *Batrachochytrium*. The ARC in cooperation with the Australian Animal Health Laboratories (AAHL) have recently conducted a series of treatment trials for *Batrachochytrium* in *Mixophyes fasciolatus* tadpoles using itraconazole, fluconazole, benzalkonium chloride and methylene blue (Marantelli et al, 2000). None of the agents was identified as being a reliable treatment and itraconazole was found to be toxic at low doses. If tadpoles are entering the collection are considered to be risk sources in regards to *Batrachochytrium* (Section 1.i) then appropriate diagnostic techniques should be applied to a subset of individuals (Section 1.iv).

The most significant experimental treatment trial for chytridiomycosis in frogs was conducted at the National Zoo Pathology Department (Nichols and Lamirande, 2000). Three groups of juvenile frogs of the species *Dendrobates tinctoris* were experimentally infected and then two groups bathed for 5 minutes daily for 8 and 11 days in itraconazole. The drug was prepared from a 1% solution (Sporanox, Janssen Pharmaceutica) diluted to a final concentration of 0.01% using 0.6% saline. The control group died within 35 days while no frogs died in either of the treated groups. No histological evidence of *Batrachochytrium* was found in the treated frogs. For frogs considered as risk sources for *Batrachochytrium* this treatment will be administered during the quarantine period.

(iv) Diagnostic techniques

Current diagnosis of *Batrachochytrium* relies on microscopic identification of sporangia in frog skin or tadpole mouthparts. The fungus does not appear to be pathogenic to tadpoles but causes a hyperkeratosis in frogs and can be identified in wet mounts of shedding skin (particularly from the digits and the ventral surface of the thighs and inguinal area. Identification of sporangia in wet mounts is aided by staining with a drop of blue `Parker' pen ink. The chytrid can also be diagnosed in frogs by routine histology of skin or toe clips

preserved in formalin or ethanol. Tadpoles need to be sacrificed for histological examination of their keratinised mouthparts. As stated in Section 1.iii no effective prophylactic treatment has been identified for *Batrachochytrium* in tadpoles so if groups are considered to be a risk source for this disease a subset of individuals will be examined histologically during the quarantine period. In the case of tadpoles from highly endangered species where each individual has a high conservation value, consideration will be given to allowing metamorphosis and treating with itraconazole bathing.

Diagnosis of infection using wet preps or histology is difficult without appropriate training. Contact Lee Berger for advice on Lee.Berger@li.csiro.au and view histopathology sections on Rick Speare's web site at www.jcu.edu/school/phtm/PHTM/frogs/histo/chhisto.htm. Alex Hyatt from AAHL is currently working on developing more sensitive tests for detection of chytrid fungus.

Another group of pathogens capable of causing disease epidemics in amphibians are Ranaviruses of the family Iridoviridae. Ranaviruses have been detected in Australia but no epidemics in captive or free-ranging individuals have been reported. Ranaviruses are known to be able to persist in carrier states. Histopathological changes in frogs infected with pathogenic Ranaviruses include hepatic, renal and splenic necrosis. Haemosiderin deposition in the liver is often seen. A range of diagnostic assays including ELISA and PCR exist for detecting these viruses in living and dead animals. If Ranavirus infection is suspected on the basis of routine histological examination contact Alex Hyatt on alex.hyatt@li.csiro.au.

A veterinarian will examine all amphibians arriving at Melbourne Zoo and if possible, a faecal sample for endoparasite examination should be obtained during the quarantine period. All animals that die should be presented for post mortem examination. Toe pads and skin from the inguinal area should be included in the standard range of tissues submitted for histopathological examination.

(v) Husbandry protocols during quarantine period

Quarantine should be performed on an `all in, all out' basis. Quarantine frogs or tadpoles should be housed in a separate room to other amphibians. If new individuals are added to the room during the quarantine period a new 60 day quarantine starts for all individuals in the room.

Quarantine animals should be serviced after collection animals. Automated systems of watering and drainage are encouraged to reduce keeper contact with enclosures. Dedicated equipment should be kept in the quarantine area. Disposable gloves should be worn when servicing quarantine animals and keepers should wash their hands in a chlorhexidine solution and pass through a foot-bath containing `Virkon' disinfectant when leaving the quarantine area. `Virkon' foot-baths need to be changed every 7 days (earlier if pink colour is lost). Enclosures and equipment should be disinfected with a bleach solution (Na hypochlorite at 200 mg/L) when animals leave the quarantine area. Surfaces should be in contact with the disinfectant solution for 15 minutes.

2. Prevention of pathogen spread in collection

All keepers adhere to the same routine of enclosure cleaning so that any disease outbreak in an enclosure is more easily tracked to its point of origin. This routine should start with the most valuable animals and move in one direction only. Disposable gloves should be worn and discarded during enclosure servicing when moving between enclosures of species considered valuable. Filtration equipment should not be shared between groups if dealing with valuable animals. Automated systems of watering and drainage are encouraged to reduce keeper contact with enclosures.

If housing amphibians intended for release to the wild then these animals must be housed in a separate room that is in no way connected to rooms housing collection or quarantine animals. Ideally, these animals should be serviced by a keeper who has no contact with collection or quarantine amphibians. If this is not practical, these animals should be serviced before collection amphibians. Disposable gloves changed between enclosures and dedicated equipment for enclosures. Keepers should wash their hands in a chlorhexidine solution and pass through a foot-bath when entering and leaving the area.

3. Prevention of pathogen spread outside facility

(i) Release of animals to the wild

It is of paramount importance to avoid the introduction of novel pathogens into areas where animals are being released. Taronga Zoo has instituted a protocol intended to minimise introducing disease to the wild with the release of captive-bred Green and gold bell frogs (*Litoria aurea*). Fifteen tadpoles in each release group are sacrificed once/week for 3 weeks. Five of these animals are frozen and 10 are submitted for histopathological examination. If Melbourne Zoo participates in captive breeding-release programs the exact numbers of tadpoles that will sacrificed for screening will depend on the sensitivity of available diagnostic techniques and the clutch size of the species. Pre-release protocols plus strict adherence to husbandry protocols and ongoing disease investigation while animals are in captivity are essential for minimising disease risks. Pre-release protocols are likely to be expanded upon as more sensitive tests for chytrid fungus detection come on line.

(ii) Treatment of waste water

To avoid introducing pathogens into local environments waste water drained from all amphibian enclosures should be collected and treated with a bleach solution (Na hypochlorite at 200 mg/L) for 15 minutes before discharging into sewerage system.

4. Summary of quarantine protocols

- All animals entering zoo examined by veterinarian
- Quarantine period 60 days
- Optimal temperature range in quarantine 17 –23^0 C
- Some tadpoles may be sacrificed for exam if considered risk animals for *Batrachochytrium*

- Some frogs to undergo itraconazole baths if considered risk animals for *Batrachochytrium*
- Quarantine on all in – all out basis
- Potential release animals serviced first
- Quarantine animals serviced after collection animals
- Gloves to be worn when servicing quarantine animals and dedicated equipment used
- Virkon footbath to be used in quarantine area
- Equipment and enclosures disinfected with bleach at end quarantine period
- Move in one direction when servicing quarantine and collection animals
- Gloves to be worn when moving between valuable species in collection
- All tadpoles and frogs that die submitted for PM exam
- Animals for release kept in separate facility
- Waste water from enclosures to be treated with bleach before release

References

Marantelli G, Berger L and McInnes K, 2000. Investigations into the treatment of *Batrachochytrium* in frogs and tadpoles. In: Proceedings of workshop on amphibian disease, Cairns, August 2000. Pg. 27

Nichols DK and Lamirande EW, 2000. Treatment of cutaneous chytridiomycosis in blue-and-yellow poison dart frogs (*Dendrobates tinctorius*) In: Proceedings of workshop on amphibian disease, Cairns, August 2000. Pg. 51.