

ATTACHMENT 6: Quarantine guidelines and protocols for amphibians

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[Lynch M.](#) Amphibian quarantine protocols Melbourne Zoo. Attachment 6. *In*: Speare R and Steering Committee of Getting the Jump on Amphibian Disease. Developing management strategies to control amphibian diseases: Decreasing the risks due to communicable diseases. School of Public Health and Tropical Medicine, James Cook University: Townsville. 2001: 157-161.

Draft guidelines for international translocation of amphibians with respect to infectious diseases

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Introduction

The aim of these guidelines is to propose measures that will significantly lower the risk of disease introduction during the international translocation of amphibians. They are based on those proposed by the IUCN Species Survival Commission's Veterinary Specialist Group (Cunningham *et al.*, in press) which are intended for captive breeding programmes covered by the IUCN Species Survival Commission.

1. International amphibian translocations

Amphibians are translocated internationally mainly for commercial reasons or as deliberate or unintentional introductions, with a smaller movement within conservation programs. The magnitude of these movements is significant. For example 180, 000 amphibians of at least 21 European species listed in appendices I and II of the Berne convention were imported into the UK alone, between 1981 and 1990 (Cunningham & Langton, 1997).

Commercial activities centre on the pet, food and laboratory animal trade. For example, farm-reared bullfrogs (*Rana catesbeiana*) are transported internationally as either live animals, or as frozen, skinned products. This trade supports employment in the countries of origin, as well as in the restaurants of Europe, Asia, North and South America and elsewhere. Dwarf clawed frogs (*Hymenochirus curtipes*) of African origin have been introduced widely throughout the USA to stock ornamental ponds, and a wide range of tropical and temperate species are moved globally as part of the pet or amateur hobbyist trade. These trades are a particular concern for disease introduction since outdoor enclosures allow contact between exotic and native species and because exotic pet species are often released into inappropriate areas accidentally or deliberately. Scientific use of amphibians has also created a commercial industry. For example, the laboratory use of African *Xenopus* species supports a significant movement of these species globally each year.

Deliberate international translocations of amphibians involve attempts at biocontrol, e.g. the introduction of the cane toad, *Bufo marinus*, into Australia, or release of exotic species into the wild for aesthetic reasons, e.g. the release of exotic European ranid frogs into the UK. Unintentional introductions also occur, such as the transport of amphibians during movement of foodstuffs such as bananas both within Australia and internationally.

Conservation programs also may result in international translocation of amphibians. However, this movement is on a far smaller scale than commercial activities for the pet and food trade and disease testing and quarantine procedures are often pre-requisites for translocation.

2. Infectious disease threats to amphibians

Wildlife populations are under threat from a range of emerging infectious diseases (Cunningham 1996, Daszak *et al.* 2000). Although increasing at a rapid rate, knowledge of the identity and epizootiology of infectious diseases that affect amphibians is relatively poor. However, two diseases, amphibian chytridiomycosis and ranavirus disease, have recently emerged as major threats to the survival of wild amphibians on a global scale. These have the potential to cause significant mortality if introduced into naive populations. It has been proposed that the spread of both diseases may have been directly influenced by the activities of humans (Daszak *et al.* 1999). Although further investigations are required before such hypotheses are substantiated or refuted, it would be wise and prudent to ensure measures are taken to minimise the threats of introduced disease when working with, and in particular when translocating, amphibians, regardless of the purpose of the work.

3. IUCN SSC VSG guidelines for screening of amphibians in IUCN translocation programmes

The following section is taken directly from the IUCN guidelines for disease screening of amphibians in translocation programs (Cunningham *et al.*, in press). These guidelines covering all amphibian translocations conducted under the auspices of the IUCN. Because these translocations represent direct conservation intervention and often involve endangered species, they are particularly stringent. A discussion of potential modification of these for other forms of amphibian translocation is given in section 4, below.

3.1 General

As with all animals, when considering the translocation of amphibians, both the source and destination of the animals must be taken into account. The longer an animal is maintained in captivity, for example, the greater the chance it will have an altered complement of symbiotic and parasitic flora and fauna to that found in its natural habitat. The ultimate goal of screening animals prior to translocation is to prevent the co-introduction of alien organisms and to maximise the chance success rate of the project. As it is impossible (through lack of knowledge, funds, *etc.*), and probably impractical even if possible, to ensure this is done to a final conclusion, pragmatic alternatives have to be taken.

3.2 Quarantine period and general screening to determine suitability for release

Animals to be translocated should be quarantined, either prior to shipment, in a holding area on arrival, or preferably both, prior to release. The time of this quarantine period is arbitrary given the lack of knowledge of amphibian diseases, but should certainly be no less than 30 days.

During this holding period, every animal should be examined for obvious signs of ill-health. The presence of ill-health (presence of lesions, poor body condition, *etc.*) automatically renders an animal unfit for release on welfare grounds.

Animals should be examined for subclinical presence of parasites (taken here to include eukaryotic organisms, prokaryotic organisms and viruses). The presence of parasites does not necessarily rule out animals for release, provided the parasites present are naturally enzootic to

the area of release. If there is a large number of animals, it may not be necessary to examine each animal for evidence of parasites, provided a statistically meaningful number are examined from each batch within parasite-transmission contact (defined as contact close enough for the transmission of a specific parasite to occur between hosts and for a long enough time period, *e.g.* pre-patent period for certain nematodes, to enable such transmission to be detected). The statistically significant sample size can be calculated using the following formula, taken from DiGiacomo & Koepsell (1986): $n = \log(1-C)/\log(1-P)$ Where n = number of animals to be sampled, P = prevalence of infection and C = desired probability of finding at least one infected animal.

Any animal that dies during the pre-release quarantine period must be necropsied and examined for evidence of disease, including specific histopathological examinations and culture for iridoviruses and cutaneous chytrids.

Measures should be taken, within reason, to prevent the release of animals into an area where disease to which they are not immune is enzootic. There should, therefore, be some knowledge of the parasite status of animals in general, and amphibians in particular, at the release site, for example by conducting necropsies on animals found dead or killed (such as those hunted/fished) in the area. If animals of the same species are already present at the site of release then, if possible, a statistically meaningful number should be examined to enable a reasonably accurate picture to be gained of the endemic parasite flora and fauna. The presence or absence of ranaviruses and cutaneous chytrid fungi, in particular, must be determined prior to the release of the translocated animals.

Given the dangers of potentially catastrophic epizootic ranavirus disease or cutaneous chytridiomycosis, animals harbouring these organisms must not be used for translocation. Sites where evidence of ranavirus disease or cutaneous chytridiomycosis are found must not be used for the release of amphibians. There are many different types of amphibian ranavirus and this may also be the case for amphibian chytrid fungi. Therefore, even where evidence of such a parasite is found in both translocated animals and release sites, it is strongly recommended that caution be erred upon and no release be conducted.

Finally, it should be remembered that the alteration of the exposure to parasites following the release of translocated animals can have unforeseeable consequences, including harmful effects on genera, orders or classes other than those of the target animals (Cunningham, 1996; Daszak *et al.*, 2000).

3.3 Minimum screening required

These procedures should be carried out as indicated above for live animals destined for translocation and, where possible, during necropsy of animals that have died during the translocation period, or those collected from target release sites.

No immunisations are currently available for ranavirus disease, cutaneous chytridiomycosis or other significant infectious diseases of amphibians.

A) Cutaneous chytridiomycosis. Diagnosis is by identification of characteristic intracellular flask-shaped sporangia and septate thalli within the superficial epidermis (Berger *et al.*, 1998; Daszak *et al.*, 1999; Pessier *et al.*, 1999). The most reliable technique is histology, either of a toe-clip taken from a live animal, or of toe-clips and ventral skin (from the pelvic “drink” patch) taken from a necropsied animal. Full protocols for examination and histology are given in a web-published article (Berger *et al.*, 1999), available at the “Amphibian diseases

home page” run by R. Speare of the James Cook University, Australia (<http://www.jcu.edu.au/dept/PHTM/frogs/ampdis.htm>). Wright’s- or Diff-Quik- (Difco Laboratories, Detroit, Michigan, USA) stained smears of skin scrapings (Pessier *et al.*, 1999) or impression (touch) smears of ventral pelvic (“drink”) patch skin stained with Wright’s or Diff-Quik are also potentially useful, however smears are less reliable than histologic analysis. Research is currently underway to develop ELISA and other antibody-based tests and PCR-based tests; polyclonal antibodies against chytrids (not *Batrachochytrium dendrobatidis* specific) are available from the Australian Animal Health Laboratory (Geelong Australia) together with an immunoperoxidase protocol.

B) **Ranaviruses.** Animals exhibiting lesions or clinical signs consistent with the range observed in ranavirus disease of anurans and urodeles (Cunningham *et al.*, 1996; Bollinger *et al.*, 1999) should be necropsied and viral presence determined by culture in commercially available cell lines. Due to differential culture characteristics of various ranaviruses, a range of cell lines, including fish and amphibian cells, should be used. Cell lines in which ranaviruses have been successfully cultured include fathead minnow (FHM) epithelial cells (European Collection of Animal Cell Cultures No. 88102401), *Rana pipiens* embryo fibroblast cell line (ICR-2A, ECACC), epithelioma papulosum cyprini cells (EPC cells, Life Technologies, Grand Island, New York, USA), Chinook Salmon Epithelial (CHSE) cells and Vero cells. Culture should be conducted at between 25 and 27 °C as this appears to be the optimum range for ranavirus growth. Ranaviruses do not grow at temperatures above 30°C. The cytopathic effect (CPE) produced by ranaviruses depends on the virus species and the cell culture used, but typical ranavirus CPE in cell monolayers consists of discrete, progressive plaques of rounded-up and sloughing cells. Details (cells, temperature and procedures) for the isolation of ranaviruses can be found in the Office International des Epizooties (OIE) "*Diseases Manual for Aquatic Animal Diseases*".

Virus can be identified directly in tissues or in cell cultures by electron microscopy with the examination of ultra-thin sections and the examination of negative-stained particulate samples (Eaton *et al.* 1991, Hyatt *et al.* 1991). Unfortunately, no general serological test has yet been evaluated for the detection of antibodies within susceptible animals. Specific antibody detection assays exist for *Bufo marinus* and these assays can be adapted to a general competitive ELISA but the sensitivity and specificity of the latter is not known. A large number of ranaviruses have now been examined (Hyatt *et al.* 2000) and the data show that the OIE accepted EHNV antigen-capture ELISA (Hyatt *et al.* 2000) can be used to detect all known ranaviruses. Further details of these ELISAs are available from Dr Alex Hyatt, CSIRO Australian Animal Health Laboratory, Geelong, Victoria, Australia. Ranaviruses can also be detected (*in-vivo* and *in-vitro*) by PCR (Gould *et al.* 1995, Kattenbelt *et al.* 2000). PCR assays can also be used, the primers and methodology are described in the OIE "*Diseases Manual for Aquatic Animal Diseases*". It should be noted that PCR products should be sequenced to confirm the identity of the virus.

C) **Erythrocytic iridoviruses**

These can be identified by light microscopy of blood cells on air-dried, Giemsa-stained blood smears, with follow-up electron microscopy if intracellular inclusions are found.

D) Enteric and pulmonary helminths

The presence of helminth eggs or larvae can be detected using standard methods for light microscopical examination of wet faecal smears.

E) Enteric protozoa

Enteric protozoa can be detected using light microscopy of wet faecal smears. It should be remembered that a range of commensal, and possibly also symbiotic, protozoa may be found using this technique, in addition to parasitic organisms.

4. Potential modifications to IUCN guidelines

The quarantine and screening procedures outlined in section 3 would introduce a substantial economic hindrance to the commercial import and export of amphibians. Quarantine periods are usually charged to the importer by Customs (in the UK, USA and Australia) on a per diem rate. It is likely that a 30-day quarantine period would render the importation of amphibians into these countries for the pet trade or food trade effectively unprofitable. It is for individual governments to debate the relative pros and cons of measures likely to result in the cessation of international trade in amphibians. However, a growing amount of evidence now exists that chytridiomycosis is present in the following forms of amphibian translocation:

- 1) the pet trade (Daszak *et al.*, in prep a.)
- 2) the food trade (Mazzoni *et al.*, in prep b.)
- 3) the laboratory animal trade (Reed *et al.*, 2000)
- 4) the trade in exotic amphibians for ornamental ponds (Daszak *et al.*, 1999)
- 5) within captive breeding programmes in zoos (Pessier *et al.*, 1999).
- 6) introduced species, e.g. the bullfrog and the cane toad (Berger *et al.*, 1998; Daszak *et al.*, in prep b.).

Chytridiomycosis, ranaviral disease or both are already present in a number of countries. In some of these, they are associated with significant mortality and population declines. It could be argued that the economic cost associated with quarantine and screening of amphibians imported into these countries may be difficult to justify for a disease already present and significantly impacting on the native population. It is unknown if strains of these pathogens from one geographic region are more virulent in amphibians from another, suggesting that a significant risk would still be present. Furthermore, other programs to control epizootic disease in wild amphibians are likely to fail if infected animals continue to be imported. For these reasons, and due to the lack of effective treatment for chytridiomycosis, the association of this disease with mass mortality events and the volume of commercial trade in amphibians, the authors and co-signees of this document suggest that all amphibian translocations should be subjected to stringent disease monitoring.

In the real world, compromise situations may be sought. To prevent economic loss, the following modifications to section 3 may be considered. Note that these modifications will increase the risk of disease entry. A reduction of the quarantine period may not be effective in preventing translocation of chytridiomycosis, due to the two-three week period between initial infection and onset of noticeable clinical signs. An alternative may be the necropsy and histologic examination for chytridiomycosis or culture and/or assay for ranaviruses of a significant portion of imported individuals (10%) while the others remain on

a shortened quarantine. The future development of more efficient lab or field-based tests may alleviate the need for such compromise.

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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°C. 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°C but those kept at 27°C 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 be 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 –23⁰ 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

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