ATTACHMENT 6: Quarantine guidelines and protocols for amphibians

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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 amhpibians 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 coverng 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 ^{o}C as this appears to be the optimum range for ranavirus growth. Ranaviruses do not grow at temperatures above 30^{o}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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