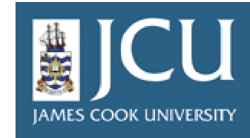

A REPORT FOR THE AUSTRALIAN GOVERNMENT DEPARTMENT OF SUSTAINABILITY, ENVIRONMENT, WATER, POPULATION AND COMMUNITIES

Guidelines for minimising disease risks associated with captive breeding, raising and restocking programs for Australian frogs

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Guidelines for minimising disease risks associated with captive breeding, raising and restocking programs for Australian frogs

1. Who should use this document?

- The guidelines are intended for use nationally by conservation agencies, zoos, scientific research staff, industry organisations (e.g., the pet industry), students, frog keepers, wildlife rescue and carer groups, frog interest groups/societies and other key interest groups who regularly deal with or are likely to engage in captive breeding, raising and restocking programs for Australian frogs.

- The guidelines outline the expectations of the Commonwealth of Australia represented by and acting through the Department of Sustainability, Environment, Water, Population and Communities (DSEWPaC) regarding precautionary procedures to be employed when working with frogs in Australia. The guidelines were developed in collaboration with recognised experts in the fields of wildlife health, husbandry, research and conservation. The intention is to promote implementation of specific guidelines by all individuals engaged in captive breeding, raising and restocking programs for Australian frogs.

- DSEWPaC recognises that some variation from the guidelines may be appropriate for particular research and other activities. Such variation should accompany any licence applications or renewals submitted to the relevant regulatory bodies for independent consideration. Variations should follow a risk analysis process which broadly involves hazard identification, risk assessment, risk management and risk communication.

Where activities occur in the wild or outside of a captive setting, these guidelines should be used in conjunction with the "**Hygiene protocols for the control of diseases in Australian frogs**", which can be found here:
http://www.environment.gov.au/biodiversity/invasive/projects/index.html#threat-10-11 .

2. Objectives

The objectives of these Guidelines are to:

- **Help improve the capacity for captive breeding, raising and restocking programs to contribute to Australian amphibian conservation**
- **Recommend best-practice procedures** for personnel, researchers, consultants and other frog enthusiasts or individuals who are involved in captive breeding, raising and restocking programs
- **Suggest workable strategies** for those regularly working or considering working with frogs in captive breeding, raising and restocking programs
- **Provide background information** and guidance to people who provide advice or supervise frog related activities
- **Inform regulatory, animal care and ethics committees** for their consideration when granting permit approvals

3. Introduction

Amphibians have declined globally. In the first global amphibian assessment, at least 43% of amphibian species with sufficient data were found to have declined in recent decades, 34 species have become extinct and a further 88 are possibly extinct (Stuart et al. 2004). In 2010, approximately 30% of amphibians were threatened globally (http://www.iucnredlist.org/documents/summarystatistics/2010_4RL_Stats_Table_1.pdf).

Conventional causes of biodiversity loss, including habitat destruction and invasive species, are playing a role in these declines. However, emergent diseases are strongly implicated in many recent rapid declines and extinctions. These factors are now acting globally and, most disturbingly, in protected and near pristine areas. While habitat conservation and mitigation of threats *in situ* are essential, for many taxa the requirement for some sort of *ex situ* intervention is mounting.

In response to the global threat posed by emerging infectious diseases, the World Organisation for Animal Health (OIE) has listed both chytridiomycosis and ranavirus as "notifiable" diseases to help control their spread. Similarly, numerous conferences and reports have been assembled to produce standards in managing diseases in wild and captive amphibian populations. Together, these measures prompted the development of the Commonwealth-funded "**Hygiene protocols for the control of diseases in Australian frogs"**, which can be accessed here: http://www.environment.gov.au/biodiversity/invasive/projects/index.html#threat-10-11.

While the hygiene protocols provide general information about strategies to minimise the transmission and spread of pathogens, particularly during field work activities, there remains a need to develop guidelines specific for captive breeding, raising and restocking programs for Australian frogs. This document fulfils this role.

3.1. History of captive breeding, raising and restocking programs for amphibians

In response to the limited resources available for practitioners involved in captive breeding, raising and restocking programs there have been a series of meetings organised by the IUCN (World Conservation Union), WAZA (World Association of Zoos & Aquariums) and CBSG (Conservation Breeding Specialist Group, of the IUCN Species Survival Commission) to discuss how the captive breeding community can and should respond to the amphibian decline crisis. A number of documents have been produced from these meetings and workshops that provide a strong lead globally and regionally to take action. These documents should be consulted in conjunction with the current guidelines by workers involved in captive breeding, raising and restocking programs in Australia. The more recent of these include:

- Draft **Guidelines and Procedures for Management of *Ex Situ* Populations of Amphibians for Conservation** (DAPTF 2005)**,** produced by the IUCN Declining Amphibian Population Taskforce *Ex Situ* Conservation Advisory Group
- The **Amphibian Conservation Action Plan** (ACAP) (Gascon et al. 2007), initiated during the Amphibian Conservation Summit in Washington D.C., USA

- The **Amphibian *ex situ* Conservation Planning Workshop Final Report** (Zippel et al. 2006)**,** arising from a workshop in Panama organised by CBSG/WAZA
- The **Amphibian Ark** project (http://www.amphibianark.org/)**,** initiated at the annual meeting of WAZA pulling together the global zoo community, CBSG and the IUCN Amphibian Specialist Group to fulfil the *ex situ* components of the ACAP
- The **Amphibian Population Management Guidelines** (Schad 2007), produced by the Amphibian Ark project
- The **Amphibian Conservation Resource Manual** (Grow and Poole 2007), produced by the Association of Zoos & Aquariums
- The **Amphibian Husbandry Resource Guide** (Poole and Grow 2008)**,** produced by the Association of Zoos & Aquariums
- **A Manual for Control of Infectious Diseases in Amphibian Survival Assurance Colonies and Reintroduction Programs** (Pessier and Mendelson 2010)**,** produced from a workshop coordinated by the IUCN/SSC Conservation Breeding Specialist Group.

The current Guidelines are adapted from many of the principles and discussions presented in these documents and in particular the most recent publication by Pessier and Mendelson (2010; which can be accessed here: http://www.cbsg.org/cbsg/workshopreports/26/amphibian_disease_manual.pdf#search=%22amphibian%22).

3.2. History of captive breeding, raising and restocking programs for amphibians in Australia

To date no regional planning has occurred in Australasia for *ex situ* amphibian management for conservation. Several documents, however, have set the agenda, including:

- A **National Conservation Action Plan,** produced by the Department of Environment and Heritage in 1996 (Tyler 1997); http://www.environment.gov.au/biodiversity/threatened/publications/action/frogs/index.html).
- A conference on the "**Declines & Disappearances of Australian Frogs**" (Campbell 1999); http://www.environment.gov.au/biodiversity/threatened/publications/frogs.html).
- The recent IUCN **Global Amphibian Assessment** (Stuart et al. 2004); and see http://www.iucnredlist.org/initiatives/amphibians
- A **Threat Abatement Plan** (TAP) for infection of amphibians with chytrid fungus resulting in chytridiomycosis. The TAP identified captive breeding and restocking as an important threat abatement action. It can be accessed here:
    - **TAP:** http://www.environment.gov.au/biodiversity/threatened/publications/tap/chytrid.html
    - **TAP Background document:** http://www.environment.gov.au/biodiversity/threatened/publications/tap/pubs/chytrid-background.pdf

  While specifically addressing chytridiomycosis, the TAP is relevant to a range of amphibian conservation issues and suggests multiple actions that are relevant to captive breeding, raising and restocking programs.

It is clear from these documents and broader consultation with amphibian conservation biologists that the need for various kinds of *ex situ* intervention has increased significantly in recent years.

Subsequently, the Federal Government put to tender a project to help fill the gaps and identify future priorities. The aims of the project were to produce:

- Agreed **guidelines for captive breeding, raising and restocking programs in Australian frogs** (#15/2004)

The Guidelines are intended for use nationally by conservation agencies, zoos, scientific research staff and other key interest groups. The current document fulfils the **final stage** of this project.

The completion of earlier stages of this project resulted in:

- A husbandry and hygiene conference ("**Captivity, Reintroduction and Disease Control Technologies for Amphibian Conservation"**, hosted by the Amphibian Research Centre in 2004). The conference brought together experts across diverse fields as a way of developing and seeking in-principal approval of agreed guidelines for captive breeding, raising and restocking programs for amphibians. All abstracts and presenter details can be accessed via the website: http://frogs.org.au/arc/conference.html.
- Numerous documents that are referenced herein as Appendices where relevant

Of particular relevance is:

1. The Australasian Regional Association of Zoological Parks and Aquaria **(ARAZPA) Amphibian Action Plan** (compiled by Gillespie, Traher & Banks, unpublshed report)

This document is a strategic plan of action for ARAZPA institutions to respond to the current conservation crisis facing amphibians. Its intent is to provide direction for zoological institutions to increase their capacity in amphibian *ex situ* management in ways that maximise their ability to support amphibian conservation priorities. This Plan was prepared in consultation with the ARAZPA Reptile & Amphibian TAG, external regional and global expertise in *ex situ* amphibian management, and the broader amphibian conservation and research community. The Plan can be found in Appendix 1.

2. **Cryopreservation and Reconstitution Technologies: A Proposal to Establish A Genome Resource Bank For Threatened Australian Amphibians** (compiled by Mahony & Clulow, unpublished report)

This document fulfils Stage 3 of the original tender and can be found in Appendix 2.

4. Key disease issues for amphibians

Here we review the most significant diseases of amphibians, including some that have zoonotic potential and some that have not been detected in Australia. There are many described diseases of amphibians but only a few are known to be an important threat to wild amphibians or other taxa including humans. Some become an issue in captive amphibian populations where management is inadequate. As research on this topic is limited, there are also likely to be many unknown diseases of amphibians which may pose a risk. Disinfection methods have not been validated for all pathogens. Any risk management strategy to minimise the impact of diseases of amphibians should take into account this uncertainty. For detailed reviews see Hemingway et al. (2009) and Berger et al (2009) for diseases in wild populations and Wright and Whitaker (2001) that also includes diseases in captivity.

4.1. Fungi

4.1.1. *Batrachochytrium dendrobatidis*

Batrachochytrium dendrobatidis (Bd) is a fungal pathogen capable of driving amphibian species to perilously low numbers or extinction. In Australia, the oldest record of Bd is from a museum frog specimen collected in south-east Queensland near Brisbane in 1978 (Department of the Environment and Heritage 2006a), which coincides with sudden frog declines in a number of species and two species extinctions in the region (Berger et al. 1998; Hines et al. 1999). Subsequent amphibian declines in central coastal Queensland (1985-86) and the Wet Tropics (1990-95) suggest that *B. dendrobatidis* spread north to its current northern limit at Big Tableland near Cooktown (Laurance et al. 1996; Berger et al. 1999; Skerratt et al. 2010). In southern Australia, the spread of *B. dendrobatidis* is poorly documented but its distribution extends down the entire east coast to Tasmania (first detected in 2004) (Obendorf and Dalton 2006; Pauza and Driessen 2008). Two separate foci occur in other states, one in southwest Western Australia, where the earliest record dates to 1985, and another around Adelaide in South Australia (earliest record 1995) (Murray et al. 2010). The Northern Territory is currently considered amphibian chytrid free (Skerratt et al. 2008; Skerratt et al. 2010; Murray et al. 2011).

In the majority of infected animals for most of the time, clinical signs of chytridiomycosis are absent. The period of showing signs is typically short and mostly limited to those amphibians that die. Central nervous system signs predominate, including behavioural change, slow and uncoordinated movement, abnormal sitting posture, tetanic spasms, loss of righting reflex and paralysis. Skin changes associated with chytridiomycosis are typically microscopic and not detectable at the clinical level with any degree of confidence, although abnormal skin shedding occurs (skin shed more frequently and in smaller amounts) and erythema (tissue reddening) of ventral surfaces and digits may be seen. For what to do if you encounter a sick or dead amphibian in Australia, see section 6.7. below. For a detailed factsheet about chytridiomycosis, see the Australian Wildlife Health Network website (http://www.wildlifehealth.org.au/AWHN/FactSheets/Fact_All.aspx).

4.1.2. *Mucor amphiborium*

This fungus is an important cause of morbidity and mortality in platypus in Tasmania and amphibians are a potential reservoir host (Gust et al. 2009). Amphibian mucormycosis is a systemic disease caused by the fungus, *Mucor amphibiorum*. Severely infected amphibians have fungi disseminated through their internal organs and skin. The fungi incite formation of granulomas that consist of inflammatory cells and fibrous tissue. At postmortem, the liver contains small pale nodules up to about 5 mm in diameter and usually in massive numbers. These nodules can also be seen in other organs such as the kidney, lung, mesentery, urinary bladder, subcutaneous sinuses and skin. The microscopic fungi are found inside these nodules. *M. amphibiorum* is a primary pathogen and can infect normal amphibians, but in the wild it appears to cause only sporadic infections. Possibly the usual inoculating dose in the wild is not high enough to cause epidemic disease. In captivity it can cause fatal outbreaks in collections. For more information on mucormycosis, see http://www.jcu.edu.au/school/phtm/PHTM/frogs/mucor/mucoramphibiorum.htm.

4.1.3. Oomycetes

Water moulds (family Saprolegniaceae, phylum Oomycota) are ubiquitous in surface water. High levels of infection with *Saprolegnia ferax* caused mortality of Western toad (*Bufo boreas*) egg masses in northwestern United States and were sufficient to affect local populations (Kiesecker et al. 2001). Epidemics may be associated with fish stocking or environmental cofactors.

4.2. Viruses

There are a number of viruses that are known to cause disease and mortality in amphibians, including ranaviruses, frog erythrocytic virus, Lucké tumor herpesvirus, herpes-like virus of skin, calicivirus and leucocyte viruses (Hemingway et al. 2009). In Europe and America the most important of these for their ability to cause mass mortalities and potentially population declines are the ranaviruses (Hyatt et al. 2000). Ranaviruses have been identified in a range of ectothermic vertebrates, including fish, amphibians (frogs, toads, salamanders) and reptiles (lizards, turtles, snakes). Some species can infect a broad host range across all these taxa.

Ranaviral disease is an emerging infectious disease overseas as it is being detected over an increasing geographic range and in more species (Hemingway et al. 2009). While ranaviral disease in wild amphibians has not been frequently observed in Australia, antibodies to ranaviruses have been detected widely (NSW, Qld, NT) in cane toads (*Bufo marinus*) (Zupanovic et al. 1998). Bohle iridoviris (BIV) was first found causing death in wild caught metamorphs of *Limnodynastes ornatus* and has since been detected in wild, moribund adult *Litoria caerulea* from Townsville and captive juvenile *Pseudophryne coriacea* from Sydney (Speare et al. 2001; Cullen and Owens 2002). Laboratory studies in Australia have also shown that cane toads (*Bufo marinus*) and a range of native frogs are susceptible to BIV (Speare et al. 2001). Tadpoles appear the most susceptible, while juvenile frogs were more susceptible than adults.

Data on the geographical origin and time of emergence or introduction of ranaviruses in Australia is not known. Ranaviruses not currently found in Australia can cause disease in native Australian amphibians in experimental challenges; for example, Venezuelan Guatopo virus was able to kill *Litoria caerulea* in experimental trials (http://www.jcu.edu.au/school/phtm/PHTM/frogs/otherdiseases-viruses.htm). We need to prevent the introduction of pathogenic ranaviruses into Australia.

Clinical signs of acute ranaviral disease may be seen in tadpoles, metamorphs, juveniles and adults. In general, amphibians infected with ranavirus may show decreased activity, ascites (accumulation of fluid in the peritoneal cavity), anasarca (accumulation of serous fluid in various tissues and cavities of the body), skin ulceration, focal and systemic haemorrhages and death. For what to do if you encounter a sick or dead amphibian in Australia, see section 6.7. below. For a detailed factsheet about ranaviral disease, see the Australian Wildlife Health Network website (http://www.wildlifehealth.org.au/AWHN/FactSheets/Fact_All.aspx).

4.3. Bacteria

The range of bacteria reported as causing disease in amphibians is small. Bacterial septicaemia can cause significant disease in captivity. Infection with Aeromonas spp., non-haemolytic group B Streptococcus, Flavobacteria and chlamydia have caused outbreaks in captive amphibians and Mycobacteria can cause chronic problems. Another group of bacteria can be carried by amphibians with minimal effect and are potentially capable of causing infections in humans (zoonotic diseases). Salmonella and Leptospira are in this category and are a potential risk to humans, livestock and domestic pets, see below.

4.4. Myxozoa

Myxosporean parasites (*Myxidium* spp.) in the brain and liver of declining Australian frogs, the Green and Golden Bell frog (*Litoria aurea*) and the Southern Bell frog (*Litoria raniformis*), have recently been reported to be associated with disease and may have a significant impact on wild frogs (Hartigan et al. 2011).

4.5. Mesomycetozoa

Ichthyophonus sp. occurs the USA where it is often an incidental finding in tadpoles, frogs and salamanders but may cause morbidity and mortality. It infects muscles and adult frogs with massive infections become lethargic and emaciated. Massive acute lethal infections with numerous mortalities occur infrequently in ranid larvae (D. Green, unpubl., Mikaelian et al. 2000)

4.6. Alveolates

A *Perkinsus*-like organism is a major cause of mortality events in tadpoles in the US. Occurs predominantly in tadpoles of *Rana* spp. and may cause mortality rates of 80-99% in a pond over the course of 2-6 weeks (Davis et al. 2007). Weakly swimming, bloated and floating tadpoles are found.

4.7. Other parasites associated with captive breeding programs

Infections with rhabditiform nematodes, such as the amphibian lungworm *Rhabdias,* and the intestinal nematode *Strongyloides* can be significant in captive populations. While they can be quite common subclinical infections in wild amphibians and not normally of significant concern, when brought into a captive setting they may cause more serious problems. Rhabditiform nematodes, for example, have a direct life cycle which may be completed in as little as 48 hours and hyperinfections can occur. Whereas adult Rhabdias sp. inhabit the lungs, the recently hatched larvae burrow across skin and may invade various internal organs – large numbers can be fatal. Rhabditiform nematodes are controlled in captive situations by combinations of faecal parasite monitoring, good enclosure and facility hygiene and anthelminthic treatment (Pessier & Mendelson 2010).

4.8. Zoonotic Diseases

Guidelines for preventing human exposure to amphibian disease are available at the Centre for Disease Control website- http://www.cdc.gov/healthypets/animals/reptiles.htm

4.8.1. *Salmonella*

Amphibians may carry pathogenic *Salmonella* species, but rarely show signs of disease (Anver and Pond 1984). Prevalence of salmonellas isolated in clinically normal amphibians is generally greater than 10% and bacterial levels can be high (Sharma et al. 1974). In Australia, *Salmonella* were isolated from 12.7% (19/150) of *B. marinus* collected from the wild and 9 serotypes were identified. All nine had previously been isolated in Australia from humans and livestock (O'Shea et al. 1990). An outbreak of gastroenteritis in humans near Rockhampton possibly originated from green tree frogs (*Litoria caerulea*) contaminating drinking water in rainwater tanks (Taylor et al. 2000). Some strains of salmonellae are cosmopolitan while others are not found in Australia, but could be imported.

4.8.2. *Leptospira*

Leptospira are spirochaetal bacteria that usually invade the kidney of vertebrates and are excreted in the urine. Humans and domestic animals are susceptible to various strains of *Leptospira* usually from the species *Leptospira interrogans*. Serious acute and chronic disease occasionally with death can result. Little is known about the occurrence of *Leptospira* in amphibians, and on their significance as reservoir hosts for leptospirosis in humans. No studies appear to have been done on leptospires in amphibians in Australia. However in Barbados, toads (*Bufo marinus*) and frogs (*Eleutherodactylus johnstonei*) were found to be reservoirs for serovars of *Leptospira* pathogenic to humans (Gravekamp 1991).

4.8.3. *Spirometra erinacei*

The adult stage of the tape worm *Spirometra erinacei* inhabits the small intestine of carnivores such as the cat, dog, fox and dingo. The first larval stage occurs in copepods and the second larval stage (spargana) are long, flat white worms that can infect amphibians and other vertebrates in muscles and under the skin. Sparganosis occurs in around 5% of Australian frogs and heavy burdens are associated with severe disease (Berger et al. 2009).

Sparganosis is a public health problem in Asia, usually occurring as subcutaneous or intramuscular infections. Humans become infected by drinking water with infected copepods, eating undercooked frogs, and the worms can also migrate from frog flesh into skin wounds

5. National and border biosecurity

Unregulated trade in animals, as well as unintentional shipment, is suspected to have been a major contributor to the spread of emerging infectious diseases such as chytridiomycosis. There are numerous bodies and regulatory levels that attempt to provide guidance about how to minimise the risk of pathogen spread and transmission in amphibians.

5.1. World Organisation for Animal Health (OIE)

The World Organisation for Animal Health (OIE) lists key diseases as "notifiable" to promote the reporting and management of diseases among member countries. Preventing the spread of amphibian diseases across international borders is important, and both chytridiomycosis (Article 8.1.1) and ranavirus (Article 8.2.1:) are now listed as notifiable diseases in the OIE Aquatic Animal Health Code (http://web.oie.int/eng/normes/fcode/). To access these codes, follow these links:

- **Chytridiomycosis**: http://web.oie.int/eng/normes/fcode/en_chapitre_1.8.1.pdf
- **Ranavirus**: http://web.oie.int/eng/normes/fcode/en_chapitre_1.8.2.pdf

The codes outline recommendations for the "**Importation or transit of *aquatic animals and aquatic animal products* for any purpose from a country, zone or compartment**". The key recommendations are outlined below:

- **Provided commodities are treated in a manner that inactivates the disease agent (Bd or ranaviruses),** Competent Authorities should not require any disease conditions when authorising the above activities, regardless of the disease status of the exporting country
- However, in cases where it could otherwise reasonably be expected that commodities pose a risk of Bd or ranavirus transmission, a risk assessment should be carried out in accordance with the recommendations in the Aquatic Code.

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Where commodities do not meet this condition and/or a reasonable risk remains, there are additional requirements that depend on the disease status of the country, zone or compartment.

Freedom from disease:

Importation of live aquatic animals from a country, zone or compartment declared free from disease (Bd or ranavirus) requires an international aquatic animal health **certificate confirming disease free status** issued by the Competent Authority.

- A country may make a **self declaration of freedom from disease** (Bd or ranaviruses) if one of the following conditions is met:

1. It has no amphibians or other susceptible species AND basic biosecurity conditions have been continuously met for a period of 2 years
2. There has been no observed occurrence of the disease for at least the past 10 years despite conditions that are conducive to its clinical expression AND basic biosecurity conditions have been continuously met for a period of 10 years
3. Targeted surveillance has been in place for at least the past 2 years without detection of disease (Bd or ranaviruses) AND basic biosecurity conditions have been continuously met for a period of 2 years
4. For a country that previously made a self declaration of freedom from disease, it may regain that status after detection of the disease if the affected area was declared an infected zone and a protection zone was established AND infected populations have been destroyed or removed from the infected zone by means that minimise the risk of further spread of the disease AND the appropriate disinfection procedures have been completed AND if the conditions of 3.) above are met.

- A zone or compartment may also be declared free from disease by the Competent Authority if it meets similar conditions to the above. Where a zone or compartment extends over more than one country, declarations must be made by all the Competent Authorities involved.

- A disease free status can be maintained if basic biosecurity conditions are continuously maintained. Targeted surveillance may be discontinued provided conditions that are conducive to clinical expression of disease exist. However, in infected countries and in all other cases where conditions are not conducive to clinical expression of disease, zones or compartments can only maintain a disease free status if targeted surveillance is maintained.

Unknown or known infected country, zone or compartment:

For the importation of live aquatic animals and aquatic animal products for any purpose (e.g., aquaculture, processing for human consumption, use in animal feed, agricultural, laboratory, zoo, pet trade, industrial or pharmaceutical use), in general the Competent Authority of the importing country should:

- require an international aquatic animal health **certificate** stating the commodities have been **appropriately treated to inactivate disease agents (Bd and ranavirus)**
- undertake a risk assessment and apply appropriate risk mitigation measures

The risk assessment and risk mitigation measures will vary with purpose of the importation or transit of commodities. See the Aquatic Code at the links provided above for further details.

5.2. AUSVETPLAN and AQUAVETPLAN

In Australia, management of animal disease emergencies normally defaults to protocols outlined in the Australian Veterinary Emergency Plan (AUSVETPLAN - http://www.animalhealthaustralia.com.au/programs/eadp/ausvetplan/ausvetplan_home.cfm) or the Australian Aquatic Veterinary Emergency Plan (AQUAVETPLAN - http://www.daff.gov.au/animal-plant-health/aquatic/aquavetplan). However, few of the diseases for which specific plans have been developed concern diseases of free-ranging wildlife. No amphibian diseases are currently included in AUSVETPLAN or AQUAVETPLAN.

5.3. Key Threatening Process and Threat Abatement Plan (TAP)

Chytridiomycosis was listed as a Key Threatening Process in Australia in 2002. A Threat Abatement Plan (TAP) for infection of amphibians with chytrid fungus resulting in chytridiomycosis was subsequently prepared by the Commonwealth Government. These documents can be accessed here:

- **Key Threatening Process:** http://www.environment.gov.au/biodiversity/threatened/ktp/frog-fungus.html
- **TAP:** http://www.environment.gov.au/biodiversity/threatened/publications/tap/chytrid.html
- **TAP Background document:** http://www.environment.gov.au/biodiversity/threatened/publications/tap/pubs/chytrid-background.pdf

Recommendation 1.1.3 of the TAP proposes that a risk-based approach be used for chytridiomycosis using AUSVETPLAN as a model (Department of the Environment and Heritage 2006b). However, this has not progressed nationally (but see Phillips et al. 2010), and recommendations from the Aquatic Code (see above) should be adhered to.

5.4. Biosecurity Australia

Risk analysis performed by Biosecurity Australia in **"Quarantine requirements for the importation of amphibians or their eggs into zoological facilities"** and **"Quarantine requirements for the importation of amphibians or their eggs for laboratory purposes"** (Animal Biosecurity Policy Memorandum 2003/26) does not list chytridiomycosis as a risk since it is endemic in Australia. However, this disregards the risk of importation into chytrid free areas or the introduction of novel strains. Although chytridiomycosis is not specifically mentioned, the general hygiene strategies recommended should still prevent the release of imported strains of *B. dendrobatidis* during the initial two years. After two years the amphibians can be released without testing for *B. dendrobatidis*. However, if being released into a chytrid free area, the same requirements imposed on Australian bred amphibians under the Threat Abatement Plan and the Aquatic Code would apply.

Risk analysis performed by Biosecurity Australia in **"Quarantine requirements for the importation of amphibians or their eggs into zoological facilities"** and **"Quarantine requirements for the importation of amphibians or their eggs for laboratory purposes"** (Animal Biosecurity Policy Memorandum 2003/26) mentions ranaviruses:

- "The veterinary certificate must… certify that… for both live amphibians or amphibian eggs…, as far as can be determined, no case of ranavirus infection (including frog virus 3, Redwood Park virus, Regina ranavirus), or ranid herpesviruses has been diagnosed at the premises of origin during the 12 months prior to certification."

Importation of amphibians must meet the requirements of two Commonwealth departments, 1) Department of Agriculture, Fisheries and Forestry (DAFF) and 2) Department of the Environment and Heritage (DEH, now DSEWPaC). The relevant documents can be accessed here:

- **DAFF:**
  Zoological facilities - http://www.jcu.edu.au/school/phtm/PHTM/frogs/aqis/2003-26a.pdf
  Laboratory purposes - http://www.jcu.edu.au/school/phtm/PHTM/frogs/aqis/2003-26b.pdf
- **DEH:** http://www.deh.gov.au/biodiversity/trade-use . This site also has the requirements for export of amphibians from Australia.

6. General risk assessment and mitigation strategies for reintroduction and translocation programs

Maintaining amphibians in captive breeding programs prior to reintroduction to the wild or during translocation from one location to another has inherent risks for the introduction of infectious diseases to new locations or populations.

These risks extend beyond the facility itself and so must be managed with an appropriate level of responsibility. Every program will differ somewhat and absolute recommendations that apply to every circumstance are not a realistic goal.

All facilities that keep captive amphibians for any purpose (e.g., education, commerce, laboratory research, conservation) should therefore take responsibility for tailoring and implementing infectious disease surveillance and control measures that prevent the introduction of amphibian pathogens to new locations or populations.

In general:

- It is impossible to create programs that are completely free of disease risk
- Each program must therefore determine what level of risk is acceptable to its stakeholders
- The disease risk of a reintroduction or translocation may be so high in some circumstances that important decisions may need to be reconsidered (e.g., not to reintroduce animals because the disease risk is too high)
- The disease risk of reintroduction or translocation can be substantially reduced by careful planning and adherence to recommendations that help to mitigate risk

The IUCN Species Survival Commission has developed generic tools to help guide veterinarians and animal managers through a process of disease risk assessment (not amphibian specific):

- The **Animal Movements and Disease Risk workbook** published by the IUCN/SSC Conservation Breeding Specialist Group is available for download at www.cbsg.org/cbsg/content/files/Disease_Risk/disease.risk_manual.pdf

Suggestions for disease risk mitigation that are relevant for amphibian programs include:

- When possible, reintroduction or translocation programs should be conducted **within the native range of the species**
    - Regional facility capacity is thus important
    - Programs that keep amphibians outside of the native range of the species (e.g., amphibians from Australia kept in New Zealand) have higher disease risks than programs located within the native range of the species. Increased risks include introducing non native pathogens into the local environment and, conversely, having novel local pathogens introduced to captive held amphibians
- Amphibians used in translocation or reintroduction programs should be kept in **isolation** from other amphibians
    - Isolation is particularly important for mixed collections where participation in amphibian conservation programs is desired
- When translocating, **the time that amphibians spend in captivity should be minimized**
- Reintroduction and translocation programs should **collect background disease and health related information** on both the captive population and the wild population/community where introduction will take place. This aids risk assessments. Useful methods include:
    - Necropsy (including histopathology) of all animals that die to help detect new or unsuspected diseases
    - Surveillance via necropsy of sacrificial animals from populations to obtain optimal samples for laboratory investigation
    - Targeted surveillance of captive and wild populations for specific pathogens (e.g., Bd or ranaviruses)
    - Ensure disease monitoring occurs prior to and after animal release
    - All mortality events in the wild population should be investigated and documented

6.1. Risk mitigation: captive breeding and reintroduction

Programs that bring amphibians from wild populations into captivity for breeding and release of progeny back into the wild are important for amphibian conservation. Strategies for disease risk mitigation and disease screening of populations prior to release of animals back into the wild were stratified by Pessier and Mendelson (2010) into three risk categories:

- **Low Risk:** amphibians are held in captivity in their native country and the facility only keeps amphibians from within that country.
- **Medium Risk**: amphibians are held in facilities outside their native country, or in facilities that keep amphibians from other countries.

- **High Risk:** amphibians are held in facilities with species from other countries, or facilities with infectious amphibian pathogens, without proper biosecurity practices

Risk mitigation strategies that should be common to all captive facilities involved in reintroduction programs include:

High and medium risk situations are to be avoided.

- Facilities maintain best practices of biosecurity
- A quarantine program should be in place for all animals entering a facility
- Isolation of animals destined for release from other animals should be enforced, particularly in mixed collections
- Morbidity and mortality events should be thoroughly investigated by necropsy and histopathology
- Development of specific pathogen-free captive populations should be considered along with appropriate screening to detect breaches in freedom from disease status
- Animals should be preferentially released into their native range
- Decision thresholds should be in place to recognise when disease mitigation strategies are unlikely to be effective. In cases where risk is unacceptably high, reintroduction should not occur
- Pre-release disease testing and treatment protocols should be designed and implemented according to the level of risk of novel disease introduction to the wild

6.2. Pre-release disease testing and treatment protocols

For all levels of risk:

Do not release animals to the wild:

- that are sick
- from facilities experiencing a mortality event
- from facilities that have an incomplete population health history
- from facilities that have low biosecurity standards
- from facilities that house exotic amphibian species

If an infectious disease problem is identified during routine necropsy and histopathology of the captive population:

- Assess the risk of releasing the pathogen into the wild population. If the identical pathogen is endemic to the wild population, it may not impact the decision to release. If the pathogen is not identical (i.e., different strain, different species) an appropriate impact study/assessment is required
- Treatable pathogens should be eliminated prior to release where possible

Consider pre-release testing and treatment regimes for pathogens known to be significant to wild populations (e.g., Bd, ranaviruses). Routine surveillance with appropriate diagnostic methods yielding a complete population health history should occur for a sufficient period (1-2 years) prior to release. This should include identifying pathogens currently present in source and host wild populations, which can be used as a reference for screening pre-release animals. Reintroduction from a facility should not proceed if pathogens are identified that are either causing illness or have not been identified as already present in the host population.

In high risk situations:

If infectious diseases are identified that are not easily treatable or eliminated a new population (re-derivation) should be considered and created by:

- Removing eggs from the wild and hatching in permanent isolation
- Re-derivation of multiple generations should be considered
- Disinfection of eggs prior to rearing should be considered where tests have deemed it safe to do so

Experiments should be considered in the laboratory to expose animals destined for release to their sympatric species. Sympatric species can then be monitored for disease via surveillance necropsy and histopathology (including sacrificial animals). Appropriate diagnostic samples should be taken (and preserved if testing is not immediate) at this time (e.g., liver and kidney). Experiments should run for an appropriate period of time to allow manifestation of diseases (typically 60-90 days).

6.3. Translocation

Translocation involves the transport of animals from one geographic location to another, typically with little or no time held in captivity. Disease risks include:

- Spread of a significant pathogen
- Acquisition and release of a significant pathogen from captivity if animals are held for any period of time in transit

For guidelines on risk assessment and mitigation for animals that will be held in captivity for any period of time, see above. In general, all translocated animals should be isolated securely from other animals held in a captive situation.

If illness or deaths occur during translocation, they should be investigated thoroughly as for captive amphibians (necropsy and histopathology).

To avoid the spread of pathogens in field based activities, strict **hygiene protocols** should be adhered to (for example see report found here: http://www.environment.gov.au/biodiversity/invasive/projects/index.html#threat-10-11.

Where possible, basic disease and health surveillance should be undertaken from both source and target populations to inform risk analyses. This may include targeted surveillance for key diseases (e.g., Bd, ranaviruses).

Where infectious diseases are identified, the risks of translocation for spreading disease should be formally assessed:

- Assess the risk of releasing or spreading the pathogen to/between wild populations. If the identical pathogen is endemic to both wild populations, it may not impact the decision to release. If the pathogen is not identical (i.e., different strain, different species) an appropriate impact study/assessment is required
- Treatable pathogens should be eliminated prior to release where possible. In the case that treatment is deemed necessary and requires animals to be taken into captivity for any period of time, guidelines for handling captive animals should be followed (see above).

7. Husbandry and Facility Biosecurity

The role of diseases in global amphibian declines has emphasised the need for improved husbandry and biosecurity practices in facilities that keep captive amphibians. Amphibians are routinely moved globally for use as laboratory research subjects, pets, educational or display animals and as part of conservation and breeding programs. These movements can increase the risk that amphibian pathogens will be moved to new locations, both in the wild and to facilities.

7.1. Facility Biosecurity

Implementation of biosecurity practices that reduce the potential for the introduction of infectious diseases to new locations:

- **are the responsibility of all institutions that maintain or move captive amphibians**
- also help reduce the risk posed by infectious diseases to the success and sustainability of captive amphibian programs

The major concepts of facility biosecurity are:

- The simplest and least expensive biosecurity measures are to:
  - **maintain captive amphibians within the native range of the species**
  - **avoid geographically mixed collections**
- In all other cases, facilities must ensure that **appropriate biosecurity practices** that reduce the risk of disease transmission and spread are in place
- Facilities intending to reintroduce amphibians to the wild should maintain animals in permanent isolation (e.g., dedicated rooms or buildings)
- Good husbandry practices, such as the use of dedicated footwear, protective clothing, dedicated tools and equipment, and following specific work flow patterns, reduce the risk of spreading diseases
- Use of simple but strategic husbandry routines and practices reduces the risk of introducing and spreading infectious diseases within an amphibian facility

- Procedures for disposal of solid waste and wastewater are considered whenever captive animals are held outside the native range of the species or in mixed collections
- Sources of food and water in the facility are scrutinized for the potential to introduce amphibian pathogens
- Facilities should be:
  - pest proof
  - amphibian proof (for escape of captives or for entry of or contact with free ranging native amphibians)
  - designed for automation in feeding, watering and cleaning
  - easy to clean and maintain
- Ideally, facilities should provide for the unique environmental needs of species in regard to the temperature, humidity, lighting, and water quality they would experience in their native ranges

It is important to keep in mind that is not possible to achieve 100% facility biosecurity in any *ex situ* amphibian population. However:

- The opportunities for pathogen movement can be reduced by identifying vectors and husbandry practices that present potential risks and designing protocols to remove or reduce these risks
- Implementing and maintaining appropriate biosecurity is a continual process of risk assessment followed by risk reduction
- A realistic level of appropriate biosecurity can be achieved with protocols that are simple and inexpensive to put into practice. Significant investment in facilities and equipment is only required to achieve the highest level of biosecurity.

7.2. Husbandry types and basic standards

Husbandry is practiced for a number of reasons but viewed from conservation and biosecurity perspectives can be broken into three main categories:

1. Animals that may/will be returned to the wild
2. Animals kept for conservation research or other research that will never be released to the wild
3. Animals kept for all other purposes

Husbandry type 1 requires animals to be of a high quality and not represent a threat to the ecosystem into which they are released. Examples include ark, rescue, reintroduction/translocation and supplementation programs.

Husbandry type 2 requires an understanding of the quality of the animals to interpret the research. Examples include conservation research or other research. Animals should never be released to the wild.

Husbandry type 3, as a general rule, only requires the animals to breed and survive. Examples include education, farming and pet trade. Animals should never be released to the wild.

In general, the minimum standards of animal husbandry increase with husbandry type from 3 (lowest) to 1 (highest).

These standards are:

Type 3: Minimum standards

1. Escape-proof housing of size appropriate for species
2. Scheduled water changes
3. Water free of pathogens or chemistry damaging to the species
4. Exposure to appropriate light
5. Appropriate temperature
6. Appropriate cage furnishings
7. Appropriate food

Type 2: Medium standards – the above plus:

8. Maximize use of automation in water quality maintenance
9. Maintain consistent/directional flow of routine during all maintenance
10. Design of enclosure should minimize keeper/animal contact
11. Climate conditions should fluctuate in keeping with the species' natural requirements unless experimental requirements prohibit this
12. Adequate level of record-keeping, as required

Type 1: Highest standards - the above standards plus:

13. One species or local assemblage per room/unit
14. Food must come from secure sources and animals well fed just prior to release; three-month familiarization with natural food types prior to release if possible
15. Climate conditions should fluctuate in keeping with the species' natural requirements; three-month familiarization with "natural" conditions prior to release
16. During familiarization, monitor condition of specimens to determine fitness for release
17. Highest level of record-keeping, as required

7.2.1. Husbandry risks

Hygiene considerations can be tailored to three major risk categories:

1. Facilities from which there is a risk of parasites/pathogens exiting and causing harm to natural populations
2. Facilities for which there is a risk of parasites/pathogens entering and causing harm to valuable captive populations
3. Facilities that present neither of the above risks

Note: in certain situations 1 and 2 may exist simultaneously.

While it is commonplace for a facility to prioritise and regard a captive population above all else, there is never a circumstance where the risk of spreading parasites/pathogens to external populations should be given lower priority than the risk to captive populations.

As a general rule:

- **the level of risk to the facility and to biodiversity surrounding the facility increases with distance from the native range of the species held.** Consequently there is a need for increased biosecurity with increased separation of the project, or parts of the project, from range.

7.3. Quarantine

Pathogens that pose the greatest threat to conservation programs are unlikely to be identified by standard zoo quarantine periods. For example, Bd and some ranaviruses can remain unnoticed in resistant hosts or as subclinical infections indefinitely.

New pathogens that could threaten Australian amphibians could come from similar circumstances.

For this reason:

- **the only safe form of quarantine is permanent quarantine**

Three levels of quarantine are suggested to cater to the various husbandry and hygiene situations outlined above. The standards are cumulative; i.e., each level incorporates all the standards of lower levels.

Quarantine level 1: Highest level - Applies to animals held far from range

The imperative is to prioritise the local biodiversity above all else, regardless of how important or threatened a species in captivity may be. For this reason this level of quarantine is suggested in all situations where species are held far from range.

Standards:

- The quarantine facility is a completely separate building or isolatable area
- Only single species or species assemblages (a faunal group that naturally occurs in the range area) are permitted per isolated area
- Maintenance conducted in a way that minimises the risk to other areas of the collection or the external environment
- The EXIT of staff and materials must be with maximum precautions including:
    - Shower out
    - Dedicated clothing that remains inside
    - Dedicated personal and other equipment that remains inside
    - Isolation and sterilization procedures for all exiting materials including wastewater

    - Security, capture and control procedures against all entry and exit of potential vectors (insects, vermin etc.)
- Within the facility gloves and protective clothing should be worn at all times to avoid contact between keepers and pathogens

Quarantine level 2: Medium level – minimum requirements for Husbandry type 1 (e.g., ark, rescue, reintroduction and supplementation programs).

The imperatives are to 1) protect the natural populations of the target and other species by preventing exposure of specimens held to other species in mixed collections, and 2) to protect the animals held if they represent important or valuable assets. This level of quarantine is the minimum suggested in all situations where species held fall into Husbandry type 1.

Standards:

- Isolation. Captive colonies do not come into contact with any amphibians that would not naturally interact with it in a wild setting
- Entry of animals, water and other materials handled to minimise the risk of entry of pathogens
- Practices to control or reduce flow of pathogens in the event of their presence or entry should be adopted to a level appropriate to the value of the animals and continued at all times. These include:
    - Unidirectional maintenance
    - Use of gloves and other isolation procedures and equipment
    - Maximisation of automation
    - Minimisation of human interaction with enclosures and inhabitants

Quarantine level 3: Lowest level – No risk to the external environment and low value collection

This quarantine is recommended only where there is no disease risk to the natural environment and generally where the animals held are of limited value. The majority of native display and pet trade animals held in Australia would fall into this category.

Standards:

- Animals should be processed through normal health screening procedures
- Treatment for known disease (e.g., chytridiomycosis) should be applied as/when required
- Mixing with animals unable to be held to this standard should not occur
- Unknown disease outbreaks should be treated with extreme caution including:
    - Culling of animals that may have had exposure
    - Disinfection or safe disposal of all enclosure materials

Current practices in some institutions and many areas of the pet trade do not meet even these basic standards. There are also current disease risks to local biodiversity associated with the significant vector distribution network supported by the pet trade and the lack of regulation in some areas. Adequate enforcement of biosecurity standards is currently lacking but should be ensured.

7.3.1. Long term isolation

Amphibians kept in conservation programs that have a goal of reintroducing captive animals or their progeny to the wild should be permanently separated and protected from other amphibians in 'long term isolation' (also known as 'permanent quarantine' – see above).

The principles of long-term isolation include:

- Housing only a single species or native species assemblage in a freestanding building or inside an isolated room or rooms within a building
- The greater the physical isolation of a species or species assemblage from a mixed amphibian collection the simpler it is to establish and maintain long-term isolation and effective biosecurity practices. A separate building for long-term isolation is better than separate rooms within a building
- Animals in long-term isolation are never housed in the same room with amphibians from outside their native range
- Preventing indirect contact with amphibians from outside the native range by potential vectors including animal care staff, cages, substrate, water and food systems or tools. This involves implementation of specific biosecurity practices

8. Hygiene management

Hygiene management issues can be broadly classed into *in-situ* (field based) and *ex-situ* (facility based) categories. While general **isolation and disinfection** hygiene management principles apply to both, greater detail on *in-situ* hygiene guidelines can be found in a separate document **'Hygiene protocols for the control of diseases in Australian frogs'** (see http://www.environment.gov.au/biodiversity/invasive/projects/index.html#threat-10-11).

8.1. Staff training and implementation of biosecurity practices

Animal husbandry staff members are one of the most significant vectors by which infectious diseases may be transmitted to, around and from an amphibian facility.

Suggestions that can help animal husbandry staff in carrying out biosecurity practices include:

- Development of and adherence to customised , user-friendly, easily accessible, written Standard Operating Procedures (SOPs) that provide an overview of biosecurity practices
- Review biosecurity practices with new staff before they begin working with animals and regular (e.g., annual) review of biosecurity practices with all animal care staff
- Advanced training in biosecurity measures and husbandry practices can be promoted by specialist educational programs for amphibian keepers
- Provide husbandry staff with the resources, tools and equipment necessary to manage captive populations at appropriate biosecurity levels

- Encourage a work environment where staff members feel comfortable reporting biosecurity errors as soon as they occur
- Design procedures that reduce staff contact with amphibians (e.g., automation). These procedures should still allow staff to regularly observe subtle signs of disease or abnormal behaviour

8.2. Husbandry staff hygiene and protective clothing

Procedures for amphibian facility husbandry staff hygiene and protective clothing are important for the success of biosecurity protocols.

- Invitation of visitors to biosecure areas should, in general, be avoided. When necessary, visitors are to follow the same guidelines as regular staff members.

Footwear and disinfectant baths:

- Dedicated footwear should be used for each building that houses captive amphibians
- Dedicated footwear is required for each long-term isolation room. Dedicated shoes or boots can be stored within the isolation room
- An alternative to dedicated footwear is the use of disposable plastic foot covers (e.g., ‘Shubees’)
- Foot baths for disinfection are less preferable to dedicated footwear
- Footbaths are most effective if footwear is made of easily disinfected material (e.g., rubber boots) and is not heavily contaminated with soil or other organic material
    - Foot baths require regular maintenance and cleaning to preserve efficacy
    - Place footbaths at the entrance and exit of biosecure areas
    - Disinfectants for use in foot baths include sodium hypochlorite (bleach), Virkon, and F10. Virkon may have advantages for use in footbaths because it maintains greater activity in the presence of organic materials

Dedicated clothing:

- Dedicated clothing should be used for each building that houses captive amphibians
- Dedicated clothing is required for each long-term isolation room. In most instances, a laboratory coat over normal clothes is sufficient unless clothes are heavily soiled
- An alternative to dedicated clothing is the use of disposable protective clothing (e.g., ‘Tyvec’ jumpsuits or surgical ‘scrubs’).

Hand washing and protective gloves:

- Frequent washing of the hands and arms with a disinfectant soap is recommended for husbandry staff members:
    - Before entering each isolation room or facility
    - Between working on different enclosures

- Disposable gloves should be used when handling amphibians or cleaning enclosures:
    - A new pair of gloves should be worn for each enclosure

  - Non-powdered gloves should be used or gloves should be thoroughly rinsed before handling animals
  - Latex and nitrile gloves might be toxic to some tadpoles. Ensure adequate tests have been performed before handling animals (toxicity associated with glove use has not been observed in postmetamorphic animals)

8.3. Husbandry routines

Husbandry routines and work-flow patterns used by amphibian husbandry staff are important for minimizing the potential to move pathogens within a captive amphibian facility, including transmission of pathogens between:

- animals kept at different levels of biosecurity
- amphibian enclosures
- animals in quarantine, animals in an established amphibian collection or animals held in isolation

Some basic husbandry routine principles include:

- Animals that are kept in isolation should be cared for first in the day
- Automation of amphibian husbandry tasks can reduce possibilities for disease transmission
- A systematic organized routine for the daily care of a collection is recommended
- Enclosures that contain amphibians that are least likely to be infected with pathogens of concern should be arranged so that they are serviced first in a directional sequence
- Enclosures and equipment should be labelled to clearly identify each unit in the sequence of enclosures
- If sick or dead animals are found during the husbandry routine they should be immediately removed from the enclosure
- Dead animals should be submitted for necropsy examination
- Sick animals should be removed for veterinary attention
- At a minimum, staff members should wash their hands before returning to complete the directional servicing in that room or facility

8.4. Water sources

The source and composition of water for an amphibian facility is an important consideration for the success of any captive amphibian program.

Items that must be addressed include:

- pH
- water hardness
- trace elements
- presence of potential toxic metals (e.g., copper)
- presence of potentially toxic additives (e.g., fluoride, chlorine or chloramines in municipal water supplies or environmental contaminants such as pesticides)

It is important that water used in a captive amphibian facility be free of important amphibian pathogens.

- If modern municipal water supplies are used as an initial source for facility water, the risk of disease introduction is very low
- If natural water sources are used, consideration should be given to disinfection of incoming water prior to use in amphibian enclosures

8.5. Food sources

Food items for amphibians in captive facilities are a potential source for introduction of pathogens. Food items may act as a mechanical vector for disease transmission (e.g., wild-caught insects may transfer water with infective Bd zoospores). A clean, reliable and trusted source of food is required for all facilities that keep captive amphibians:

- Invertebrate food items should be cultured and reared on-site if possible
- Aquatic food items (e.g., *Daphnia* or brine shrimp [*Artemia*]) should be produced from eggs if possible
- If food items are purchased from a commercial supplier, the amphibian facility should make sure that practices that reduce disease risk are used. The supplier's facility should be clean and use high quality materials
- Potential benefits to feeding wild-caught food items to captive amphibians (e.g., superior nutrition, tailoring to a specific diet) must be balanced with potential disease risks when making decisions for a captive population. Animals kept in isolation should not be fed wild-caught food items from outside of their native range
- If wild-caught food items are fed to captive amphibians, these food items should only be collected from areas that are known to be free of pesticides or other chemicals
- Wild-caught food items should not be collected from locations known to be experiencing outbreaks of infectious diseases

8.6. Cleaning and Disinfection

The ability to thoroughly clean and disinfect a diverse range of equipment, animal enclosures, cage decorations and furniture and water is essential for good biosecurity and control of infectious diseases in captive amphibian populations. The guidelines presented below have an emphasis on Bd and ranaviruses; however, the concepts are applicable to a wide variety of amphibian pathogens.

Key concepts of cleaning and disinfection include:

- Most disinfectants are inactivated by the presence of dirt or organic materials. Thorough cleaning of objects prior to disinfection is essential
- A single method or type of disinfection will not work for all amphibian pathogens or applications. Careful selection of the disinfectant method and type is necessary for different situations
- Correct disinfectant concentrations and exposure times are important for effective disinfection
- Environmental impacts should be considered when selecting a disinfectant

- Glass or metal surfaces and materials are easy to properly disinfect with chemical disinfectants
- Plastics of varying types (including silicon) are easy to disinfect but may absorb chemicals from the disinfectant (e.g., solvents) thus rendering the enclosure toxic
- Natural materials such as rocks, wood or dehydrated plant materials are difficult to disinfect. Application of heat may be most effective on these materials
- Living plants are difficult to disinfect. Careful plant selection, removal of dirt and gentle chemical or physical disinfection may reduce risks
- Methods for water disinfection include heat, filtration, chemical disinfection or ozonation

Of the disinfectants available:

- Sodium hypochlorite (bleach), quaternary ammonium compounds and potassium peroxymonosulfate (Virkon®) are experimentally effective against Bd
- Bd is susceptible to relatively low levels of heat and to complete drying
- Potassium peroxymonosulfate (Virkon®), sodium hypochlorite (bleach) and chlorhexidine are experimentally effective against ranaviruses. These viruses may nevertheless be somewhat resistant to disinfection on dry surfaces

8.6.1. Principles of cleaning and disinfection

Designing an effective disinfection protocol requires understanding of the properties of disinfectants and target pathogens, and practical consideration of the equipment, facilities or processes requiring disinfection. As well as understanding the efficacy of various disinfecting processes, it is important to consider the safety of any disinfection protocol to the environment and the animals on which they will be used. Key distinctions include:

- **Cleaning**: The physical removal of all visible organic and inorganic debris from items
- **Disinfection:** A physical (e.g., UV light) or chemical (e.g., bleach) process to reduce the numbers and/or viability of microorganisms (e.g., bacteria, fungi or viruses) on an object, surface or material
- **Sterilization:** A physical or chemical process that removes all microorganisms from an object, surface or material

Thorough cleaning and disinfection reduces most of the risk of transferring amphibian pathogens. Sterilization of objects is labour intensive and less practical for most routine applications.

Guidelines available for cleaning and disinfection of commercial aquaculture facilities may be applicable to amphibian facilities, especially for amphibian farming and mass production facilities. For detailed information, see:

- **The World Organization for Animal Health (OIE) Manual of Diagnostic Tests for Aquatic Animals**, which has an overview of aquaculture disinfection methods: www.oie.int/eng/normes/fmanual/1.1.3_DISINFECTION.pdf

Cleaning:

Cleaning alone does not render an object free of pathogens. However, it is important to thoroughly clean objects prior to disinfection or sterilization.

- Thorough cleaning physically removes many or most pathogens that are trapped in organic debris
- Thorough cleaning makes successful disinfection more likely
- Cleaning allows disinfectants to directly contact the surfaces of an object
- Warm or hot water improves the ability to remove organic materials from objects
- Regular cleaning of all items used in animal husbandry should be performed
- Use of detergents aid cleaning by loosening organic material from the surface of objects and help to break apart biofilms of microorganisms that can resist disinfection
- Thorough rinsing of detergents from objects is essential after cleaning

Disinfection:

Disinfection of an item by application of an appropriate chemical agent after cleaning reduces pathogen numbers and viability and minimises potential for disease transmission. Things to consider include:

- **Efficacy of the disinfectant and the type of pathogens that must be eliminated**. For example, some microorganisms such as *Mycobacterium* spp. or *Cryptosporidium* spp. are very resistant to most common disinfectants
- **The potential for toxicity to amphibians that are exposed to the disinfectant.** Amphibians are very sensitive to some disinfectant residues and thorough rinsing of all disinfectants is required after use
- **Concerns about human exposure to disinfectants and about discharge of disinfectants into the environment**
- **Safety for use on different materials.** Some disinfectants may be corrosive to materials or tools used in amphibian facilities
- **Ease of use and disposal**
- **Cost**

A list of suitable disinfectants, their required concentrations and exposure times for various purposes is summarised by Phillott et al. (2010) and is reproduced in Table 1 below.

Table 1. Disinfection strategies suitable for killing *Batrachochytrium dendrobatidis, Mucor amphibiorum* and ranaviruses in field studies. From Phillott et al. (2010) and Webb et al. (submitted).

Application	Disinfectant	Strength	Time	Target pathogen
Surgical equipment and other instruments (e.g. scales, callipers)	Benzalkonium chloride	1 mg ml–1	1 min	*B. dendrobatidis*
	Ethanol	70%	1 min	*B. dendrobatidis*
				Ranaviruses
Collection equipment and containers	Sodium hypochlorite (bleach contains 4% sodium hypochlorite)	1%	1 min	*B. dendrobatidis*
		3%	1 min	Ranaviruses
	Path X or quaternary ammonium compound 128	1 in 500 dilution	0.5 min	*B. dendrobatidis*
		1 in 100 dilution	10 min	*M. amphibiorum*
	Trigene	1 in 5000 dilution	1 min	*B. dendrobatidis*
	F10	1 in 1500 dilution	1 min	*B. dendrobatidis*
	Virkon	2 mg ml–1	1 min	*B. dendrobatidis*
		1%	1 min	Ranaviruses
	Nolvasan	0.75%	1 min	Ranaviruses
	Potassium permanganate	1%	10 min	*B. dendrobatidis*
	Complete drying		>3 h	*B. dendrobatidis*
	Heat 60°C		30 min	*B. dendrobatidis*
				Ranaviruses
	Heat 37°C		8 h	*B. dendrobatidis*
	Sterilising UV light		1 min	Ranaviruses only
Footwear	Sodium hypochlorite (bleach contains 4% sodium hypochlorite)	1%	1 min	*B. dendrobatidis*
		3%	1 min	Ranaviruses
	Path X or quaternary ammonium compound 128	1 in 500 dilution	0.5 min	*B. dendrobatidis*
		1 in 100 dilution	10 min	*M. amphibiorum*
	Trigene	1 in 5000 dilution	1 min	*B. dendrobatidis*
	F10	1 in 1500 dilution	1 min	*B. dendrobatidis*
	Phytoclean (30% benzalkonium chloride)	0.075%	1 min	*B. dendrobatidis*
		5%	1 min	*M. amphibiorum*
	Complete drying		>3 h	*B. dendrobatidis*
Cloth (e.g. carry bags, clothes)	Hot wash 60°C or greater		30 min	*B. dendrobatidis*
				Ranaviruses

8.6.2. Enclosures

General cleaning schedules for amphibian enclosures will vary depending on the species, the number of animals kept in the enclosure, the enclosure type, the types of substrates used, and the use of filtration systems.

- Design of enclosures to facilitate flow through of wastes will reduce the build up of waste and pathogens and reduce the quarantine and animal stress risks associated with disturbance and manual cleaning.
- Use layered materials of course grade to allow wash through of animal waste and uneaten food
- Avoid use of materials that will become excessively waterlogged under the irrigation regime in use
- Position rain/water inlet systems to maximise suspension of waste materials and flow to waste outlet
- Frequent flushing of enclosure substrates with water (where applicable) should be performed.
- Flushing regimes should be determined by animal density, rate of waste production and build up of organic material and will vary with species, season and feed regimes
- Flushing will almost certainly be more frequent than the occupant’s natural exposure to precipitation as it is usually required to clean an enclosure housing animals at greater density than in nature
- Substrate flushing by passage of water through the substrate without “raining” on the occupants can mitigate concerns about excess precipitation.
- Organic substrates like sphagnum moss, peat, coconut husk, and soil-based substrates should be disposed of after use and not re-used in another enclosure
- The rate of replacement should be determined by enclosure density, waste production and flushing regime efficacy
- pH, ammonia, and nitrate are important parameters to monitor for many aquatic species and can help to determine the need for partial or complete water changes
- In cases where the above systems cannot be achieved build up of organic wastes and of certain pathogens (e.g., rhabditiform nematodes) can be reduced by use of:
- Disposable substrates like paper towels or paper pulp that are frequently changed
- Reusable substrates that are easily disinfected, such as untreated foam rubber or ‘Astroturf’. Duplicate sets of these items for each enclosure will simplify the task as they can be swapped in an out

Disinfection of animal enclosures is performed:

- At the time of periodic substrate changes
- As part of an approach to controlling an outbreak of an infectious disease in a captive amphibian population
- Before previously used enclosures are re-used with different animals

Disinfectants must be thoroughly rinsed from enclosure surfaces to avoid exposing amphibians to the disinfectant chemicals.

8.6.3. Equipment and tools

Tools and equipment should be cleaned and disinfected between use in different enclosures.

- Having multiple sets of equipment or dedicated equipment for each enclosure is helpful for workflow efficiency
- Isolation enclosures should have dedicated sets of equipment

8.6.4. Substrates and cage furniture

There is a huge variety of substrates and cage furniture used in captive amphibian enclosures and each require different approaches to disinfection. Thorough cleaning and removal of organic material is necessary for effective disinfection.

In general:

- Plastics, glass and metals are easy to clean and disinfect
- Natural or organic materials (e.g., wood, plant material) are generally more difficult to clean and disinfect
    - Heat treatment may be the most effective
    - Consider the source of these materials following a risk assessment framework
    - Autoclaving and microwave sterilisation may be a viable option
    - Use of plants that come from within the range of the species is preferable
    - Plants should be removed from pots, their roots and surfaces rinsed of soil and other matter and transferred to new pots with clean soil. Hydroponic production of plants within facilities is the safest option
    - In isolation, plastic or silk plants are preferable for their ability to be washed and thoroughly disinfected

8.6.5. Water

Disinfection or sterilization of water may be necessary to ensure that water coming into a facility is free of pathogens and contaminants or to ensure that wastewater exiting a facility does not contain pathogens or contaminants that may pose a risk to native amphibians.

If necessary, wastewater treatment from an amphibian facility can be accomplished by application of heat, ozone or bleach.

- Guidelines for aquaculture facilities are provided by the World Organization for Animal Health (OIE) (www.oie.int/eng/normes/fmanual/1.1.3_DISINFECTION.pdf)
- For disinfection of water by heat (either wastewater leaving the facility or incoming water) relatively low temperatures of 71°C for 15–20 minutes are effective for Bd and ranaviruses in a laboratory setting. These temperatures may not be effective for bacterial spores or all viruses. If sterilization of water is desired, boiling is necessary. Heating under pressure (autoclave or pressure cooker) increases the effectiveness
- Filtering water through one-micron (1µm) cartridge filters, available at hardware stores, is one method that may be successful at removing zoospores of the amphibian chytrid fungus. Water filtration is not effective at removing viruses

- Ultraviolet light may be effective at removing ranaviruses from water, but efficacy against Bd is questionable
- Correct application of reverse osmosis (RO) filtration will remove all pathogens but is only useful as a sterilisation mechanism for inlet water as pathogens are concentrated and expelled in the RO waste stream. RO can assist in waste treatment by concentrating pathogens and reducing the use of other more costly or environmentally problematic treatments

9. Treatment and Control of Diseases

Among the most important infectious disease issues identified in amphibian captive breeding programs are chytridiomycosis, *Ranavirus* infection and infection with the rhabditiform nematodes *Rhabdias* and *Strongyloides*. The goals of treatment and other disease control measures are to:

- Mitigate the effects of infectious diseases on the success and sustainability of captive populations
- Reduce the risk that captive populations could serve as sources of infectious diseases (e.g., chytridiomycosis and *Ranavirus*) for wild amphibian populations
- Identify methods that can be used to create specific pathogen free amphibian populations

For captive breeding programs a decision to treat animals for a specific infectious disease or parasite will depend on:

- The significance of the pathogen to the health of the captive population and any intended release populations
- The necessity to maintain or develop host species adaptability or immunity to a specific pathogen that is naturally found in the wild population
- In some cases, developing immunity may be a suitable goal for combating infectious diseases that are likely to spread into endangered populations (e.g., Bd)
- Decisions to treat an individual or population for a pathogen will also depend upon the occurrence of the disease in conspecific and contact animals, other captive populations and in wild populations

9.1. Treatment and control methods for chrytridiomycosis

Treatment methods for chytridiomycosis are necessary for:

- Reducing morbidity and mortality in captive populations due to infection with Bd
- Salvage of wild amphibian populations experiencing mortality due to chytridiomycosis
- Reducing the risk of disease spread posed to both captive and wild amphibian populations
- Creation of disease-free breeding populations for use as survival assurance populations or for commercial purposes (e.g., food, laboratory animals and pets)

Bd can infect a huge range of amphibian hosts but the course of infection is highly variable - not all species or individuals develop fatal chytridiomycosis. For this reason, infections can be maintained and rapidly spread from more resistant individuals and species. It is important that both clinical and aclinical infections are treated in captive populations.

Some common problems to all forms of treatment:

- Further trials using rigorous experiments are needed to validate treatments
- Treatments are not consistently successful in eliminating Bd infection across all species or individuals
- There is an unknown risk of treatment regimes for most amphibians (most treatments are described for one or a few species only)
- Some treatment regimes are known to be toxic or are not tolerated by some amphibian species or specific amphibian life-stages (e.g., tadpoles)
- Treatment associated deaths are poorly documented
- Some treatments have the potential to impact human health

Some general comments about all treatment methods for Bd are:

- There are species and life-stage (e.g., tadpole, juvenile, and adult) differences in the ability to tolerate medication
- If there is no experience with a specific treatment in a species or life-stage it is advisable to first evaluate treatment safety with appropriate experiments
- Because the infection is located in a superficial location on the skin, treatments are usually applied topically as a bath
- Periodically agitate the treatment solution to ensure contact of the medication with all skin surfaces (dorsal and ventral)
- Bd may persist on enclosure materials and in water so it is important that animals are placed into a Bd free enclosure after every treatment
- Use of post-treatment PCR testing to evaluate animals for treatment success or failure is strongly recommended. Multiple tests may be required to be confident that animals are free of infection after treatment
- Multiple cycles of treatment and testing may be needed to ensure clearance of infection
- If animals are sick or dying of chytridiomycosis it is helpful to provide supportive care with supplemental electrolytes and antibiotics (e.g., Baytril)
- Keep good records of treatment regimes and characteristics of individuals treated

There are several options for treating infection with Bd (reviewed by Berger et al. 2010). The most common include:

- Itraconazole
    - Treatment protocol well described (see http://www.cbsg.org/cbsg/workshopreports/26/amphibian_disease_manual.pdf#search=%22amphibian%22)
    - Successful use in numerous programs treating adult frogs
    - Short daily exposure (5-10 mins)
    - Few human safety concerns

- Standard 0.01% protocol toxic to tadpoles and some metamorphic frogs; however, low dose treatment in tadpoles appears promising (Garner et al. 2009)
- Expensive

- Elevated temperatures
  - Exploits inability of Bd to grow above 30°C
  - Combines well with other medication treatments
  - 32°C for 5 days cured Bd in a temperate species (*Pseudacris triserata*) (Retallick and Miera 2007)
  - High temperatures may be detrimental to some amphibians

Other methods that have been used less frequently include:

- Chloramphenicol (and Florfenicol)
  - Treatment protocol well described: www.nzfrogs.org/site/nzfrog/files/Treatment Protocol.pdf
  - Tolerated by tadpoles and post-metamorphs in treatment trials
  - Continuous immersion treatments possible (good for aquatic amphibians and tadpoles)
  - Continuous immersion potentially unsuitable for terrestrial amphibians
  - Caused death in juvenile spotted treefrogs (Marantelli unpubl. data)
  - Inexpensive

- Voriconazole
  - Effective and safe in tadpoles and post-metamorphic animals in recent trial (Martel et al. 2010)
  - Concentration remains stable in water for at least a week
  - Further trials warranted

- Malachite green and formalin
  - Not generally recommended due to toxicity of chemicals to amphibians or humans

- Benzalkonium chloride
  - Not effective for treatment

- Azole antifungal medications other than itraconazole
  - Not in wide use and/or have limited efficacy

- Trimethoprim sulfadiazine
  - Limited trials exist demonstrating efficacy against Bd

- Terbinafine (Lamisil®)
  - Not generally recommended due to lack of trials demonstrating efficacy against Bd

9.2. Control of ranavirus infections

The extent and significance of ranavirus infections is unknown in captive collections. However, mass mortality events in captive and wild populations are possible. Treatment options for viral pathogens are limited so focus is given to disease control rather than treatment. The goals of the control methods are:

- Avoid transmission of infection to other amphibians in the population or facility
- Avoid transmission of infection to native amphibian populations
- Creation of breeding populations of amphibians that are known to be free of ranaviral infection

Subclinical infections with ranaviruses have been documented with periods of persistence ranging up to six months, and possibly more.

- Subclinically infected animals have the potential to spread infection to other animals
- Reliable diagnostic tests to detect animals subclinically infected with ranaviruses are not yet available, complicating implementation of disease control measures

If an outbreak of ranaviral disease is suspected or identified in a captive population, control methods that are useful include:

1. Isolation of sick animals from healthy animals
2. Strict adherence to biosecurity practices that minimize or eliminate transmission of pathogens between animal enclosures. Practices to control infection and transmission are:
    a. Avoid the transfer of soiled substrates (e.g., soil, gravel, moss, plants), cage furniture or water between different enclosures
    b. Disinfect tools and equipment between use in different enclosures (Disinfectants known to inactivate ranaviruses are listed in Table 1)
3. There are limited options available for treating individual animals with *Ranavirus* infection. Treatment with antibiotics (e.g., enrofloxacin) could help control secondary bacterial infections
4. A risk assessment should be performed for animals that are known to be infected with a ranavirus or that have survived a ranavirus outbreak

10. Surveillance and Diagnostic Testing

Good captive husbandry programs depend on disease surveillance protocols, appropriate laboratory methods and sound interpretation of laboratory results.

Important aspects of disease testing and surveillance include:

- Principles of sampling populations for infectious diseases (e.g., determining the number of animals that must be sampled to be confident that a population is free from disease)

- Knowledge of qualified laboratories that can perform diagnostic testing
- Selection of an appropriate diagnostic test
- Collection of the best sample type for the test that will be performed
- Correct interpretation of test results
- Use of postmortem examination for identifying and managing disease problems in amphibian colonies (both infectious and non-infectious diseases) and for performing disease risk assessments in amphibian reintroduction programs

10.1. Disease surveillance

Disease surveillance may allow monitoring of a population for pathogens, determining the disease status of a population, determining disease burden (prevalence) or rate of disease spread (incidence) within the population, or estimating other parameters such as disease related morbidity or mortality. Disease surveillance of both *ex situ* (captive) and *in situ* (wild populations) populations is necessary to detect disease problems that could be threatening to the health of captive breeding populations and the success of reintroduction programs.

Disease surveillance is necessary to:

- Detect and mitigate disease problems before they result in significant mortality events
- Rapidly determine the cause of mortality to limit the impacts of disease outbreaks
- Limit the potential for spread of infectious diseases
- Gather information needed for disease risk assessments when planning amphibian quarantine or reintroduction
- Detection of potential zoonotic diseases that could affect staff

Disease surveillance must be tailored to each amphibian species or population and depends on factors such as susceptibility to a pathogen of interest, presence of the pathogen, long-term conservation goals and practical consideration of the resources available. For a review of the critical elements of surveillance programs for chytridiomycosis (applies to other diseases) in Australia see (Skerratt et al. 2008; Skerratt et al. 2010; Murray et al. 2011).

The basic components of a disease surveillance program are:

- Collect a history of the (wild or captive) population or assemblage of interest. Information that may be of interest includes population size, age structure, original source (e.g., captive /wild caught), dietary history, history of body condition, etc.
- Determine how the sampling regime or pathogen detectability may influence surveillance parameters (i.e., high risk species, life-history stages, sick and dead animals, seasons, weather etc. may all influence a picture of pathogen prevalence within a population)
- Determine an efficient approach to sampling (a census is rarely required)
- Determine required sample sizes depending on requirements for confidence in parameter estimates

Differences in sampling methodology can:

- Bias the results obtained and affect the perceived prevalence or presence of disease in the population
- Affect how well the diagnostic test performs
- Impact the ability to answer a research question

Appropriate resources (e.g., veterinary epidemiology texts, statisticians etc.) should be consulted to aid the design of surveillance programs. This will ensure that the correct sampling strategy is selected (e.g., stratified or cluster sampling etc.) for the goals of a particular project. For example, sample size calculations can be specific to a study design, expected disease prevalence, estimated population sizes, questions of interest, diagnostic test characteristics and so on.

10.2. Diagnostic testing

Diagnostic testing for generic amphibian diseases requires extensive expertise that falls beyond the scope of these guidelines. However, it is useful to provide some basic information about the diagnostic test procedures for some of the most significant or common problems encountered in captive breeding facilities. These include chytridiomycosis, ranaviruses and some parasites. An excellent manual for wildlife health investigations has been produced that contains instructions on general approaches as well as details on collecting mortality data, euthanasia, necropsies, sample collection and sample submission (Rose 2007).

Chytridiomycosis:

When selecting a diagnostic method it is helpful to distinguish between disease or death due to Bd infection and subclinical infection.

- **Morphologic methods** (e.g., wet mounts, cytology and histopathology) are useful for diagnosis in sick animals because the organism is present in large numbers in the skin. Morphologic methods are not reliable for detecting subclinical infections and should not be used for quarantine screening of animals
- Conventional or Taqman polymerase chain reaction (**PCR**) tests are more sensitive than morphologic methods and are useful for detecting smaller numbers of organisms present (e.g., in subclinical infections)
- **PCR** is the preferred diagnostic test for screening wild or captive populations for the presence of Bd infection and for quarantine screening of animals
- Bd requires specialized techniques for diagnosis by fungal culture

Samples:

- Morphologic methods require skin samples or histologic sections (e.g., of toe tips)
- PCR methods can be used to process skin swabs (the preferred method), water baths and tissue samples (toe tip or tadpole mouthparts)

Swabbing for Bd:

Most PCR for diagnosis of infection with Bd conducted in Australia uses the Taqman real-time quantitative PCR (Hyatt et al. 2007). The most reliable swab to be used in conjunction with this method is the:

- MW 100 (a fine tipped, individually housed rayon swab with a plastic handle manufactured by the Medical Wire and Equipment Co.)
- In general, swabs with wooden handles should be avoided if possible, unless conventional PCR is used

The basic swabbing procedure for post-metamorphic animals is:

1. Label the swab housing with useful information (e.g., date, species, individual or capture number, location etc.)
2. Put on a fresh pair of disposable plastic gloves
3. Open the swab
4. Gently rub the swab across the skin of the animal. Areas to be targeted include the feet and hands, thighs and abdomen. A standardised number of swabs should be used (e.g., 5 strokes per body region).
5. Replace the swab into its housing
6. Store swabs at or below room temperature. Freeze swabs at or below -20°C if they will not be analysed within six months of collection
7. Send swabs in batches convenient to the diagnostic facility by courier (conventional post may be less reliable)

In tadpoles, Bd is restricted to the keratinised mouthparts so sampling techniques must target this area. Sampling can be by dissection of mouthparts (lethal sampling, euthanasia required) or by swabbing. Suitable swabs include the same swabs as used for post-metamorphic animals (see above) if tadpoles are large enough, or by smaller swabs (such as wooden toothpicks) for smaller animals. Note that the rayon swab is more reliable and should be used where possible. The basic procedure for obtaining swab samples from tadpoles follows the above but:

- Insert the swab into the mouth of the tadpole and gently swirl
- If using toothpicks, gently scrape tooth rows and keratinised beak and preserve in 70% ethanol
- Disposable gloves are potentially toxic to tadpoles. See Cashins et al. (2008) for guidelines

Cross contamination:

PCR techniques are extremely sensitive to the presence of Bd DNA in a sample. While there are many benefits of this high sensitivity, it also increases the possibility of false-positive results from contamination. It is critical that researchers do everything possible to avoid potential sources of contamination.

Methods to help minimise the chances of contamination include:

- Use a fresh pair of disposable gloves between each animal
- Swab the animal first in a processing routine (e.g., before measuring body size, weighing, marking etc.)
- Open, use and store the swab efficiently without it ever coming into contact with anything other than the skin of the animal being tested
- Ensure swab storage is appropriately secure

Inhibition:

Foreign material (e.g., soil, plant matter) on the swab can inhibit PCR reactions. This may increase the chance of obtaining false-negative errors.

Methods to help minimise the chance of inhibition include:

- Remove dirt, soil and other matter on the surface of the animal being tested
- In some cases, gently rinsing the animal with fresh water is acceptable (do not wash vigorously and do not use potentially contaminated water)
- Ensure that the diagnostic laboratory uses appropriate controls (internal positive controls) to detect PCR inhibitors

Pooling samples:

In situations where test results from individuals is not important (e.g., testing a population for the simple presence of Bd), pooling or batch testing of swabs from multiple animals can reduce costs. Pooling of up to 5 swabs may be acceptable without the loss of overall test sensitivity.

Ranaviruses:

Ranaviruses form a large group of related viruses that vary in their biological behaviour (e.g., host range, virulence to different amphibian species etc.). The ranaviruses of concern to amphibian conservation programs include the Frog Virus 3 (FV3) like viruses, *Ambystoma tigrinum* (ATV) like viruses and the Bohle iridovirus (BIV).

Diagnosis of *Ranavirus* infection is most straightforward in animals that are sick and have systemic ranaviral disease. Unlike PCR testing for Bd, the available diagnostic tests for ranaviruses have not been validated for detecting subclinical infections. As such, there are no reliable tests for detecting infections in living animals for purposes such as:

- Surveys of wild or captive populations for occurrence or prevalence of *Ranavirus* infection
- Screening new animals in quarantine prior to entry into a captive collection
- Screening prior to use in a reintroduction or translocation program.

Iridovirus infections cannot be diagnosed from clinical signs alone, and amphibians showing no signs of disease may be infected. However, high mortality rates associated with haemorrhages, oedema, ascites or ulcers are suggestive of ranaviral disease. Histopathology showing severe necrosis of haematopoetic or other tissues indicates the use of specific tests. These include immunostaining, viral culture on cell monolayers, serology and molecular techniques to identify the aetiologic agent. Transmission electron microscopy (TEM) can classify the agent as a member of the *Iridoviridae*. ELISA and other serological tests can identify the genus *Ranavirus.* PCR analyses are required to distinguish among virus species and strains (Chinchar and Mao 2000).

Viral culture or PCR are needed to detect low grade carrier infections. Marsh et al. (2002) developed tests based on variation of the major capsid protein (MCP) gene sequence to distinguish among important ranaviruses from the regions of Australia, Europe and America.

Taqman real-time PCR is useful as a research and screening tool, with the potential given further development to become a sensitive and specific method for detection and differentiation of ranaviruses (Pallister et al. 2007).

The CSIRO Australian Animal Health Laboratory maintains an Iridovirus reference collection and can do a range of diagnostic tests. The main research groups in Australia are led by Dr Alex Hyatt (Australian Animal Health Laboratories) and Professor Richard Whittington (University of Sydney).

Internal parasites:

Faecal examination can be used to screen for the presence of internal parasites in wild and captive amphibians. Simple faecal examination techniques require minimal equipment.

- Faecal examination may detect protozoal parasites and various helminth worms (cestodes, trematodes or nematodes) and/or their eggs
- Parasites that are likely to cause disease problems in captive amphibians as well as commensal organisms not associated with disease may be detected
- Wild amphibians frequently carry a variety of internal parasites. Most are not significant population limiting factors in wild populations. However, in captive populations some of these may become problematic because of increased animal density, poor hygiene, inadequate husbandry or stress
- Parasite surveillance is necessary for maintaining healthy captive populations. The rhabditiform nematodes, *Rhabdias* (amphibian lungworm) and *Strongyloides* (an intestinal worm) can be significant problems in amphibian captive breeding facilities

The techniques used for faecal examination include faecal wet mounts, faecal flotations, faecal sedimentation and the Baermann technique. All of these are described in detail in standard parasitology texts and should be familiar to veterinarians.

10.3. Diagnostic facilities

There are several institutions in Australia that specialise in diagnostic testing for amphibian diseases. In circumstances where a captive breeding facility cannot resolve a diagnostic problem, consideration should be given to contacting and/or submitting samples to an experienced laboratory. Such laboratories will have rigorous standards for dealing with issues that may affect the interpretation of diagnostic results (e.g., sensitivity and specificity of tests, quality controls such as standardised test quantities and appropriate controls). They will also be aware of the need to inform appropriate State/Commonwealth agencies of diagnostic results if necessary.

A procedure for the preparation and transport of a sick or dead frog is given below. Adherence to this procedure will ensure the animal is maintained in a suitable condition for pathological examination and assist determining the extent of the disease and the number of species affected. For more information about sick and dead amphibians, see http://www.jcu.edu.au/school/phtm/PHTM/frogs/pmfrog.htm).

Collection:

- Do not use bare hands to handle sick or dead frogs
- Disposable gloves should be worn when handling sick or dead frogs
- New gloves and a clean plastic bag should be used for each frog specimen to prevent cross-contamination
- If the frog is dead, keep the specimen cool and preserve as soon as possible to avoid decomposition

Preserving specimens:

- Specimens can be **preserved/fixed in 70% ethanol or 10% buffered formalin**
- Cut open the belly and place the frog in about 10 times its own volume of preservative
- Where no preservative is available, **specimens can also be frozen**. If numerous frogs are collected, some should be preserved and some should be frozen. Portions of a dead frog can also be sent for analysis (e.g., a preserved foot, leg or a portion of abdominal skin)

Transportation:

- If the frog is alive and likely to survive transportation, place the frog into either a moistened cloth bag with some damp leaf litter or into a plastic bag with damp leaf litter and partially inflated before sealing
- Remember to **keep all frogs separated** during transportation
- **If the frog is alive but unlikely to survive transportation** (death appears imminent), euthanize the frog and place the specimen in a freezer or preservative. Once frozen/preserved the specimen is ready for shipment
- **All containers should be labelled** showing at least the species (if known), date and collection location
- Preserved samples can be sent in jars or wrapped in wet cloth, sealed in bags and placed inside a padded box

- Send frozen samples in an esky with dry ice
- Place live or frozen specimens into a small Styrofoam esky. Seal esky with packaging tape before sending
- Send the package by courier and declare any hazardous or flammable contents (e.g., 70% ethanol)

To arrange receipt and analyse sick and dead frogs, make contact with experts at either of the organisations below prior to dispatching package:

Australian Registry if Wildlife Health
Taronga Conservation Society,
Australia
PO Box 20
MOSMAN NSW 2088
Phone: 02 9978 4749
Fax: 02 9978 4516

School of Public Health, Tropical Medicine and Rehabilitation Sciences
James Cook University
Douglas Campus
TOWNSVILLE QLD 4811
Phone: 07 4796 1735
Fax: 07 4796 1767

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Appendix 1: The Australasian Regional Association of Zoological Parks and Aquaria (ARAZPA) *Amphibian Action Plan* (compiled by Gillespie, Traher & Banks, unpublshed report) is included as a separate document.

Appendix 2: *Cryopreservation and Reconstitution Technologies: A Proposal to Establish A Genome Resource Bank For Threatened Australian Amphibians* (compiled by Mahony & Clulow, unpublished report) is included as a separate document.