WHAT TO DO WITH ILL OR DEAD FROGS

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1. INTRODUCTION

At our present stage of knowledge about diseases of frogs, we need all the additional specimens that we can get. Only by thorough studies of more dead and ill frogs will we be able to piece together the overall disease picture. For that reason every ill or dead frog is valuable, and efforts to get the maximum amount of information from each specimen are well worth while.

Deciding what to do with an ill or dead frog is a complex process. At the current time, if you find a frog that is ill, do not allow it to die in the wild; collect it and allow it to be used. This document will assist you in deciding what to do with a particular ill or dead frog given the particular circumstances associated with it.

If possible, identify a wildlife pathologist available to examine frogs to discuss preferred preservation methods.

2. ASSUMPTIONS

More information will be obtained from a post mortem examination done by an experienced pathologist than from one done by a person with little experience in collecting specimens from frogs.

Less information will be obtained from a dead frog than from a frog that is processed in the terminal state. Therefore, if a collector has dissecting skills and equipment, examining a terminal frog will provide more information than shipping the frog and having it die in transit.

Processing of specimens will depend on:

- whether the frogs are ill or dead
- if ill, whether they are terminal or non-terminal
- time from collection to processing
- level of expertise of finder/dispatcher
- facilities available

Any stage (eggs, tadpoles, metamorphs, juveniles and adults) should be sent if dead, ill or abnormal.

Dead or ill cane toads should be treated in the same way as frogs.

3. BASIC INFORMATION REQUIRED ON ALL SPECIMENS

To make the most of every specimen, please record and pass on the following information:

- Species of frog
- Date and time
- Where found - be very detailed. Use map if helpful
- Brief description of area where specimen was found
- Why you think it is abnormal. In this include details on appearance and behaviour
- Whether other frogs were ill or dead
- What proportion of population was affected
- Is finding ill or dead frogs a common occurrence? If "yes", please provide details on locations, dates, signs, and species.

The submission form at the end of this document can be used to record this information.

4. PRESERVATION TECHNIQUES

Once a frog dies, its tissues start to degenerate (post mortem degeneration). The rate of degeneration is dependent on ambient temperature. If a frog is dead, action has to be swift to get the maximum amount of information possible from the specimen. Post mortem change decreases the amount of histological information as well as the chances of isolating an infectious agent. Post mortem degeneration can be stopped by:

- chemicals called fixatives (alcohol, formalin, or glutaraldehyde), or
- freezing (though this is not ideal)

Preserving agents, while protecting the tissues from post mortem degeneration, also kill infectious agents. So if viruses or bacteria are present, once the tissue is preserved, the infectious agent cannot be grown. This is the major disadvantage of preserving specimens. Infectious agents in tissues are best protected by freezing the whole specimen, or pieces of certain tissues.

Fixatives stop post mortem degeneration and protect the histology

Fixatives kill infectious agents

Freezing protects infectious agents and keeps them alive, also toxicology can be done.

Freezing stops post mortem degeneration, but ice crystals damage the tissues for histology

4.1 IDEAL PROCESSING TECHNIQUE

The ideal solution to get the maximum amount of data is to:

- necropsy the frog using an aseptic technique
- cut organs into pieces:
    - preserve parts of organs in 10% buffered neutral formalin for histology (please remember that fresh well buffered formalin is best).
    - preserve small parts of organs in 2.5% glutaraldehyde for transmission electron microscopy; this enhances the possibility of identifying intracellular pathogens, and is very important when looking for a new infectious agent.
- freeze the remainder of organs at -70^{o}C for isolation of infectious agents. Freezing at -20^{o}C is better than leaving in the fridge at 4^{o}C.

Obviously this option requires skill, knowledge and access to the correct chemicals.

4.2 SPECIMENS TO COLLECT

Specimens for disease investigation are mainly collected for 4 reasons:

1. Histopathology - to observe the pattern of changes in cells in tissues and visualisation of some infectious organisms. Organs affected by disease can be identified. For histopathology, tissues have to be preserved in formalin or alcohol based liquids. Please try to use quality fixatives, e.g. fresh well buffered formalin, as this enables follow-up electron microscopy to be used.
2. Identification of agent - if disease is caused by an infectious agent, fresh or frozen specimens may allow the micro-organism to be isolated, grown in-vitro, identified using antigen detection techniques, or identified using molecular biological techniques.
3. Transmission electron microscopy - if an infectious agent is present, it may be identified using TEM even if the micro-organism cannot be isolated. To get the

best result, tissues are preserved in glutaraldehyde (if glutaraldehyde is not available, use formalin).

4. Toxicology - frozen tissues are necessary. Liver, kidney, fat and stomach contents are considered likely to have concentrated levels of toxins.

For histology, collect the following in 10% formalin:

- Any abnormal changes or lesions
- Skin
- Skeletal muscle
- Liver
- Kidney
- Spleen
- Lung
- Heart
- Stomach
- Intestine
- Urinary bladder
- Eye
- Brain
- Bone of femur
- Gonads

For isolation of infectious agents or toxicology, collect the following organs and freeze:

- Any lesions
- Liver
- Kidney
- Lung
- Skeletal muscle
- Brain

- Stomach contents
- Fat bodies
- Remainder of carcase.

To detect chytridiomycosis by PCR, swab ventral skin – see separate instructions for molecular diagnosis.

5. DEAD FROGS

Dead frogs can be handled in three ways:

- Frozen whole
- Preserved whole in formalin or alcohol after opening the belly
- Necropsy for gross examination and removal of individual organs

5.1 PROBLEMS WITH DEAD FROGS

The main problem is that post mortem degeneration in dead frogs is very rapid; the higher the temperature, the greater the rate of degeneration. The aim is to get the maximum amount of information from the frog before it decays any further.

5.2 PROCESSING OF DEAD FROGS

If a post mortem can be done immediately by someone with experience and the right chemicals, then do a post mortem, divide organs if possible into two, put specimens for histology in 10% buffered neutral formalin, freeze the other half of the organs, freeze the carcase.

6. ILL FROGS

The key question here is mainly for people that have found a terminal (almost dead) frog, and are trying to decide whether:

- to forward the frog to the laboratory, and run the risk of it dying in transit, and have the lab get a dead frog; or
- to kill the frog on site, and process it for subsequent examination by the laboratory.

6.1 SEVERITY OF ILLNESS

An "ill" frog for our purposes has at least one of the following:

- behaves in an abnormal fashion;

- is very thin;
- has abnormalities of eyes or skin;
- is deformed

Ill frogs should be classified into two groups: terminal and non-terminal.

Terminal: a frog which will probably die within 24 hours.
Signs: not able to move; moves very slowly; very thin; can be turned onto back and does not attempt to right itself.

Non-terminal: a frog that is ill, but will probably not die in 24 hours.
Signs: attempts to escape; moves actively.

6.2 PROBLEMS WITH ILL FROGS

An ill frog may become a dead frog during transit. Terminally ill frogs and toads usually die in transit. Once an amphibian dies, degeneration occurs rapidly. If a frog is terminal, it is better to process it prior to dispatch, or to dispatch it in a cooled container.

6.3 BENEFITS OF COLLECTOR DOING PROCESSING

Since more comprehensive information can be obtained from live amphibians, it is better for us to receive live, ill frogs than dead frogs. So the main aim should be to get a live, ill frog to the Amphibian Disease Group. However, after considering the various factors below, the best option for the collector may be to process the frog, and then to send the specimens to us.

6.4 PROCESSING OF ILL FROGS

6.4.1 TERMINAL FROGS

Collector Has Minimal Dissection Experience

If you have minimal scientific facilities and skill: Put frog in plastic bag in freezer to kill it.

If there are many ill frogs, some can be fixed and the rest frozen. 10% formalin is preferred, although 70% alcohol can also be used. After euthanasia, cut through the skin and muscle on the belly before placing in fixative.

Collector has Dissection Experience

If you have appropriate facilities and skill:

- Weigh frog

- Measure snout-vent length
- Anaesthetise with chloroform, or barbiturate
- Open ventrally along abdomen
- Collect blood from heart using needle, capillary tube or incision
- Make smears (dry at room temperature)
- Collect into plain tube (store in fridge at $4^{o}C$)
- Collect 0.3 ml into EDTA or sequestrene tube for haematology (store in fridge at $4^{o}C$)
- Examine internal organs for abnormalities
- Divide organs into 2, one part for histology (placed in 10% formalin), and other into sterile tube (for culture of micro-organisms - freeze in fridge at - $20^{o}C$ or - $80^{o}C$)
- If possible, a small section of the organs and lesions (~1mm thick) should be placed in 2.5% glutaraldehyde at $4^{o}C$ for electron microscopy. 25% glutaraldehyde is usually obtained which needs to be diluted in 0.1M phosphate buffered saline shortly before use. After 24 hours the samples should be transferred to phosphate buffered saline.
- Fresh tissue specimens should be frozen at $-20^{o}C$.

6.4.2 NON-TERMINAL FROGS

If the frog is ill, but it seems likely that it will not die within 24 hours, do the following:

- Source a interested pathologist if possible
- Place frog in a bag made of cloth
- Wet bag
- Dispatch so air supply is not restricted (i.e., don't put into airtight bottle or plastic container).

6.5 SENDING SPECIMENS

6.5.1 TERMINAL FROGS

Full post mortem

To be useful, blood specimens should arrive within 2 days of collection. If delivery is impossible within 2 days, the blood tubes should be frozen as for fresh tissue samples.

Dispatch frozen samples with dry ice or if not possible, with an ice brick.

Dispatch fixed samples in 10% formalin.

Frozen Specimens

Forward with dry ice or, if not possible, with an ice brick.

6.5.2 NON-TERMINAL FROGS

Forward as livestock.

7. TRANSPORTATION OF SPECIMENS

If sending specimens by air, the fresh tissues are regarded as diagnostic specimens and are not classified as dangerous goods. They have to be packed and dispatched according to IATA Regulation 650. Labelled "Diagnostic specimen" on consignment note.

If dry ice is included, the package is treated as a Dangerous Good. Label "Diagnostic specimen and dry ice" on consignment note, but no shipping bill required. If 10% formalin is included, since formalin is a dangerous good, the package has to be packed in accordance with Dangerous Goods Regulations. Shipping bill required.

8. AMPHIBIAN SUBMISSION FORM

SENDER'S NAME AND ADDRESS:
TELEPHONE.....
EMAIL.....
SENDERS ID NUMBER.....
SPECIES..... STAGE..... AGE..... SEX.....
LENGTH Snout-vent..... WEIGHT.....
DATE & TIME COLLECTED.....DATE SUBMITTED.....
LOCATION FOUND(lat/long).....
TYPE OF ENVIRONMENT.....
ARE PESTICIDES etc USED? .....
ABNORMAL BEHAVIOUR.....
EFFECT OF HANDLING.....
ABNORMAL APPEARANCE: Skin .....
Eyes.....Orifices.....
Other.....
.....
ARE OTHER FROGS ILL? .....
PROPORTION OF FROGS AFFECTED.....
IS FINDING ILL/DEAD FROGS A COMMON OCCURRENCE? .....
EUTHANASED? ..... HOW? .....
FIXATIVE USED (if any)? .....
FROZEN? .....WHAT TEMPERATURE? .....
TIME FROM DEATH TO AUTOPSY/FREEZING.....
IF CAPTIVE, HAS ANY MEDICATION/DISINFECTANT BEEN USED? .....
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COMMENTS.....
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Thank you for your cooperation.

9. HOW TO MAKE GOOD QUALITY FORMALIN

10% NEUTRAL BUFFERED FORMALIN

The quality of the histology depends on using good quality fixatives. The pH of the fixative is critical. The best histology is obtained using 10% formalin with a pH about neutral. The following is a formula that will produce 10% formalin of neutral pH.

CHEMICALS NEEDED

Di-sodium orthophosphate (Na2HPO4) -- 6.5 gm

Potassium dihydrogen orthophosphate (KH2P04) -- 4.0 gm

40% formaldehyde solution -- 100 ml

Distilled water -- 900 ml

PROCEDURE

Dissolve salts in small part of the water with heating.

Add remaining water, then formaldehyde.

Add 1-2 ml of methylene blue solution as a colour indicator.