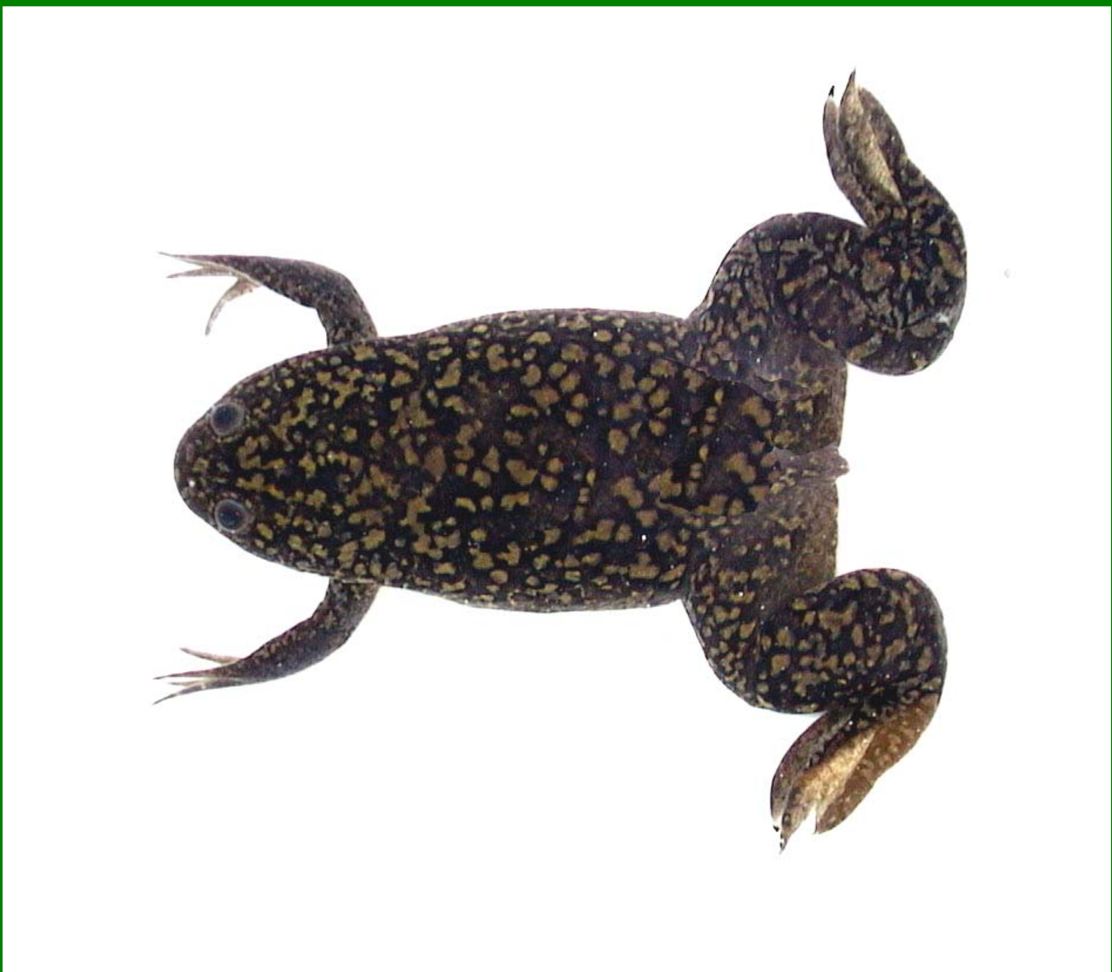
Guidance on the housing and care of the

African clawed frog

Xenopus laevis



Barney T Reed

Research Animals Department - RSPCA

May 2005

Acknowledgements

The author would like to sincerely thank the following people for their helpful and constructive comments during the preparation of this report:

Mr. D. Anderson - Home Office, Animals Scientific Procedures Division
Mr. M. Brown - MRC Laboratory of Molecular Biology, Cambridge
Dr. G. Griffin - Canadian Council for Animal Care (CCAC)
Dr. M. Guille - School of Biological Sciences, University of Portsmouth
Dr. P. Hawkins - Research Animals Department, RSPCA
Dr. R. Hubrecht - Universities Federation for Animal Welfare (UFAW)
Dr. M. Jennings - Research Animals Department, RSPCA
Dr. K. Mathers - MRC National Institute for Medical Research
Dr. G. Sanders - School of Medicine, University of Washington
Prof. R. Tinsley - School of Biological Sciences, University of Bristol

Special thanks also to those organisations whose establishments I visited and whose scientific and animal care staff provided valuable input and advice.

<u>Note</u>:

The views expressed in this document are those of the author, and may not necessarily represent those of the persons named above or their affiliated organisations.

About the author

Barney Reed studied psychology and biology at the University of Exeter before obtaining a MSc in applied animal behaviour and animal welfare from the University of Edinburgh. He worked in the Animals Scientific Procedures Division of the Home Office before joining the research animals department of the RSPCA as a scientific officer.

[Front cover photo: Xenbase]

RSPCA policy

The prime objectives of the Royal Society for the Prevention of Cruelty to Animals (RSPCA) are to promote kindness and prevent or suppress cruelty to animals. With regard to laboratory animals, the RSPCA is opposed to all experiments or procedures that cause pain, suffering or distress and believes the necessity and justification for the use of animals must be critically challenged on a case by case basis. As long as animals continue to be used, the Society believes every possible effort should be made to prevent suffering at every stage of the animals' lives, not just during experiments but also throughout their acquisition, transport, housing, husbandry and care.

The Society adopts a constructive, practical approach to this issue, supporting and promoting the development and adoption of techniques that will result in the replacement, reduction or refinement of animal experiments. This report is part of the Society's work on refinement.

For further information regarding this publication please contact:

Research Animals Department
RSPCA
Wilberforce Way
Southwater, Horsham
West Sussex
RH13 9RS

www.rspca.org.uk

Or email: research_animals@rspca.org.uk

CONTENTS

1 Introduction

The refinement of all aspects of the husbandry, care and use of laboratory animals is important for many reasons - legal, ethical, scientific and not least animal welfare. The latter two are inextricably linked, since it is increasingly being demonstrated that good animal welfare is essential to produce good scientific results (e.g. Poole, 1997).

Refinement encompasses both the reduction of suffering (i.e. negative effects on physical or psychological wellbeing) and the improvement of animal welfare. This can be achieved through the development and subsequent application of comprehensive guidelines for every stage of animal care and use, always striving to define not just appropriate standards and protocols, but optimal ones. Such guidelines should be based on the findings of behavioural, health and welfare research, and are best developed through inclusive discussions with all relevant interested parties.

Amphibians have been a relatively neglected group in this respect, and are frequently given only the briefest, if any, mention in handbooks for the care and management of laboratory animals. In addition, there is a widely held belief that *Xenopus laevis* frogs are hardy (Wolfensohn & Lloyd, 2003) and robust (The Wellcome Trust, 2003), capable of tolerating a range of environmental conditions and experimental procedures. There has historically been little investigation into the capacity of these animals to suffer and failure to appreciate that they are at times housed in sub-optimal environments.

The information that is available on *Xenopus laevis* in the scientific and commercial literature tends to deal with the intricate issues at the molecular or cellular level that are associated with studies utilising *Xenopus* eggs or oocytes, in the fields of biochemistry, developmental biology, genetic technologies or physiology. There is relatively little information with regard to how the animals from whom the raw materials for such studies are obtained, should *optimally* be cared for, both so that the oocytes and eggs produced are of consistent high quality and so that each individual animal's welfare is safeguarded.

Where information relating to the care and use of *Xenopus laevis* can be found, it is often inconsistent or incomplete. Thus, with respect to *Xenopus laevis* frogs specifically there is:

- an absence of thorough ecological studies of population biology, interaction within groups, generation times and growth rates derived from long-term research of *Xenopus laevis* in the field (Tinsley & Kobel,1996);

- little systematic research to ascertain conditions for: optimal growth rate and group homogeneity (Hilken at al, 1995); housing requirements (The Berlin Workshop, 1994; O'Rourke, 2002); or the environmental conditions, water quality and nutritional regime necessary to optimise health, egg / oocyte production (Green, 2002) and welfare.

It is therefore difficult for establishments to decide upon the standards and conditions necessary to achieve the highest standards of animal wellbeing, and most appear to act on the principle of, "if what they are doing seems to be working, then there is no need to change". This can have serious implications for scientific studies as well as animal welfare since egg quality may be affected by the environmental conditions experienced by the female frog. Egg quantity can also be affected by poor environmental conditions leading to an unnecessary increase in animal use.

There is a lot of interest in *Xenopus* welfare within the animal care and use community, and a clear demand for a better understanding of the behaviours and requirements of these animals, together with much more comprehensive and consistent guidance in this respect. This report aims to assist in achieving this goal. It is based on information obtained from: a) comprehensive searches of the relevant UK, European and other legislation; b) the scientific literature; c) material produced by commercial organisations; d) information from ethics committees around the world; e) observations from visits to research establishments; and f) from discussions with animal research regulators, animal users and animal care staff including veterinarians.

1.1 Aims of the report

The purpose of this report is:

- to draw together previous research undertaken and recommendations made, in order to illustrate areas of both consensus and inconsistency;
- to highlight potential welfare and ethical concerns relating to the supply, housing and care of adult *Xenopus laevis* frogs;
- to facilitate understanding of *Xenopus laevis* behaviour and a subsequent improved appreciation of their requirements;
- to arrive, where possible, at consensus based on available data and sound scientific argument for *optimal* environmental and care conditions for keeping *Xenopus laevis* frogs;
- to provide some basic recommendations for improving health, welfare and egg quality, for reducing the potential for stress and suffering and possibly, the total number of animals required;
- in areas where current knowledge is sparse or inconclusive, to stimulate discussion that will enable movement towards identifying what should be regarded as ‘best practice’ [see text in boxes].

The care of eggs and tadpoles will not be addressed here, nor will the intricacies of all the various experimental techniques associated with research involving *Xenopus laevis* eggs and oocytes. However, a number of the more common procedures involving adult frogs of this species will be discussed[1].

[1] *Xenopus tropicalis* is used as an alternative amphibian species for some areas of study (Sive et al 2000). *Xenopus tropicalis* has a shorter generation time (around 5 months) and is diploid in genetic disposition as opposed to the tetraploid *Xenopus laevis*. It is unlikely however, that use of this species will widely replace that of *Xenopus laevis* because its eggs are smaller and are produced in clumps rather than singly, so are perceived as being trickier to do experiments with (The Wellcome Trust 2003). Differences in physiological characteristics and natural habitat mean the optimal conditions for keeping *Xenopus tropicalis* are likely to be different to that for *Xenopus laevis* and so similar future stringent consideration must also be given to how best to care for *Xenopus tropicalis*.

2 Background information on *Xenopus laevis*

Understanding the behavioural biology of those species kept and used in laboratories is crucial to improving both animal welfare and the quality of scientific research (Olsson et al, 2003). The following section therefore includes details of the anatomy, physiology and behaviour of wild *Xenopus laevis* frogs. These are some of the characteristics that need to be taken into consideration by animal care staff and others when designing and implementing laboratory housing and husbandry regimes for these animals.

2.1 Natural geographic range and habitat

Xenopus laevis, also known as the African clawed frog (or toad) or the common platanna, is comprised of a series of sub-species that may be found ranging from the Cape of South Africa, to Sudan to the north-east and Nigeria to the north-west. The sub-species studied in most laboratories is *Xenopus laevis laevis* (though for the purposes of this report the generic term *Xenopus laevis* will be used). Although members of this sub-species occur throughout South Africa and parts of Zimbabwe, virtually all the individuals used in research actually originate from the Cape of South Africa (Tinsley, pers.comm), or are probably the descendants of such individuals. Aided by the past actions of humans who released them after use in laboratories or as unwanted pets, they can now be found in established populations, as an invasive species, in areas of freshwater elsewhere in the world, such as California, USA (Gampper, 2002) and in Britain (Measey, 1998; 2001; Mattison, 1998).

Xenopus laevis can typically be found in the stagnant water (Deuchar, 1975) of pools, ponds, lakes or ditches, usually based on a substrate of deep mud. Under normal circumstances these frogs are totally aquatic (Mattison, 1998, Tyler 1999) and typically never leave the water, although they have been known to migrate between connected water bodies or overland (Wilson et al, 2002), or to aestivate[2] in a burrowed hole with an opening for air (Garvey, 2000; Deuchar, 1975) if their home pond dries up. Migration is limited by distance, and time of year, because once out of water and exposed, they are susceptible to rapid dehydration (van Zutphen et al, 2001) and possible death (Kelley, n.d.).

[2] To aestivate, means to pass the summer or periods of drought, in a state of dormancy or torpor

2.2 Species characteristics

- *Xenopus laevis* is one of 17 named species in the *Xenopus* genus (Tinsley & Kobel, 1996) included in the Pipidae family.
- *Xenopus* are naturally nocturnal (Elepfandt, 1996a).
- They have a head smaller than their flat body, with small, round, upward-gazing eyes with convex cornea (Schultz & Dawson, 2003) that lack eyelids (USGS, 2003). The anatomy and positioning of the eyes on top of the head (Elepfandt, 1996a) are suited for atmospheric rather than underwater vision (Schultz & Dawson, 2003). These features allow the free-living animals a better chance of detecting prey or reacting to attacks from above by potential predators such as birds, when they are near to, or at, the water's surface.
- Vision is not the main sense used for gathering information from the dark and murky aquatic environment that these animals typically inhabit. Blind individuals have been held without observed disadvantage in the same tank as sighted individuals with no apparent impairment of the animal's ability to locate movement of, and within, water (Elepfandt, 1996a). It is suggested that vision has a more important role where these frogs attempt to catch terrestrial prey (Measey, 1998).
- The ears are not visible externally, but these animals have a highly developed auditory system facilitating complex underwater acoustic communication between individuals (Elepfandt, 1996b). The full repertoire of this communication in the form of 'clicks' may be difficult for humans to perceive due to the reflection of sound at the water's surface.
- It is also thought probable that pheromones are involved in *Xenopus* communication, though this has yet to be proven (Elepfandt, 2004).
- Nostrils are positioned on the top of the head on the snout, and the presence of two nasal cavities (the vomeronasal and the rostral) affords the ability to smell in both air and water (Elepfandt, 1996a). Animals are aroused by the odour of food nearby and swim around in search of it until they touch it, often only making a decision about its palatability after the object reaches the mouth (Elepfandt, 1996a). A highly developed olfactory sense affords this species the rare ability amongst frogs of locating and consuming non-living food items (Beck, 1994).
- The front limbs, used to direct food towards the mouth (Phillips, 1979; O'Rourke, 2002), are short and the four fingers are not webbed. The hind legs are larger and primarily used to powerfully propel the frog through the water. The five toes on the hind legs are webbed and the inner three have small claws at their end. These claws are useful for digging out insects and other food items from the mud, and for shredding larger prey into more manageable-sized portions (Garvey, 2000).
- These frogs lack a moveable 'fleshy' tongue.
- When *Xenopus* do have to occasionally traverse land surfaces, they are capable of moving reasonably fast by a series of lolloping leaps, landing flat on their belly each time (Deuchar, 1975).
- African clawed frogs elevate their urea levels in periods of drought as an adaptive strategy that may help them to survive (Wray & Wilkie, 1995).
- Like fish, these frogs have a lateral line of specialised sensory organs (around 180 individual organs in an adult – Elepfandt, 1996a) that run in a circle around the back of the body, the head and around the eyes. This highly sensitive system, retained to adulthood only by some amphibian species (usually those who are predominantly aquatic) is utilised to locate prey and disturbances in the environment via the perception of water movement and velocity. The lateral line is visible with the naked eye and is seen as a symmetrical pattern of elongated greyish dots or white lines, each 2-3 mm in length (van Zutphen et al, 2001).

- Cutaneous respiration is not well developed in this genus (Deuchar, 1975) so these animals mainly rely on well-developed lungs for gaseous exchange while the animal is at the water's surface (Garvey, 2000). A clawed frog will drown if it is prevented from breathing air as the intake of oxygen via the skin alone will be insufficient (van Zutphen et al, 2001).
- *Xenopus laevis* have dorsal skin colouring in various shades of olive grey with large darkened blotches (although albino strains have been purposely maintained for use in some laboratories). The undersides of *Xenopus laevis* frogs are yellowish-white (Deuchar, 1975). The skin contains chromatophores (Phillips, 1979), which are cells containing pigment, used for camouflage. This mechanism is under hormonal control.
- The skin is damp and slippery. Mucous glands secrete a slimy protective layer, which helps prevent mechanical damage to the skin and provides a barrier against pathogens. Serous glands, which are typically present on the head and shoulders, synthesise and secrete a variety of compounds that help protect against predators and chemicals, or that have antibacterial or anti-fungal properties (Schultz & Dawson, 2003).
- Females are larger than males, growing up to 15cms from snout to vent compared to 10cms in the male (University of Arizona, 2001).
- Cloacal flaps are only visible in the females, while the males develop darkened nuptial pads on inner forearms and fingers to hold on to the females during mating (Sive et al, 2000). Whilst performing amplexus (a mating embrace where the male grasps the female from behind with his forearms) both frogs will perform a series of somersaults in the water, with hundreds of eggs – usually between 500 and 1000 (Deuchar, 1975) though possibly as many as 5,000 (Dawson et al, 1992) – being released and fertilised. The eggs are scattered over the area and as there is no further parental investment, they are left unprotected. Environmental conditions experienced by the adult female prior to egg-laying, and subsequently by the fertilised eggs, will largely dictate the ratio developing successfully into tadpoles.
- There is much field information on wild *Xenopus laevis* frogs from areas within its range in South Africa, to suggest that breeding is seasonal - notably September (early spring) to March (late summer) (Tinsley, pers.comm).
- Gravid females are recorded as containing from 1,000 to 27,000 eggs with larger females producing larger clutches. In the wild, they will produce multiple clutches in a season under favourable conditions (Measey, 2004).
- The tadpoles are herbivorous, eating very small plant particles. They adopt a carnivorous diet after metamorphosis into a froglet, which depending on conditions, is completed by between two and three months (University of Arizona, 2001). Male frogs typically reach sexual maturity in around 10 to 12 months, while females can, again depending on environmental conditions, take up to two years (Kelly n.d.). Optimal laboratory rearing conditions can shorten this time to one year for females (Gurdon, 1996) or even 8 months (Tinsley & Kobel, 1996).
- Individuals regularly live for 10-15 years in captivity. It has been suggested that they can survive for up to 25 years in the laboratory (Wolfensohn and Lloyd, 2003) or even have a life span as long as 30 years (Kelly, n.d.). No comprehensive data has been collected on the longevity of wild individuals in South Africa though there has been a recording of at least 18 years for an individual living in the wild in the UK (Tinsley, unpublished).
- Normal behaviour for these frogs is to spend most of their time lying motionless below the surface of the water (University of Arizona, 2001) with outstretched arms, waiting for food to pass by (Mattison, 1998).

- The unique structure of the sliding pelvis allows these frogs to attempt to avoid predators by diving backwards from the water surface (Videler & Jorna, 1985) and is also a useful adaptation which helps this animal to burrow in mud (Tinsley, pers.comm).
- It was believed that *Xenopus laevis* did not hold physical territory in the same manner some of the more terrestrial species might. However, studies are now revealing that interactions within populations include the maintenance of territories in underwater habitats (Elepfandt and Yager, in Tinsley and Kobel, 1996).

2.3 Use of *Xenopus laevis* in research and testing

2.3.1 Past and present use

There is a long tradition of using this species in scientific study (Halliday, 1998) and it is certainly the most widely used and easily recognised amphibian research animal (Schultz & Dawson, 2003). During the 1940s, before the development of more efficient endocrinological techniques, female *Xenopus laevis* were commonly housed and used in hospital laboratories as a means of detecting human pregnancy. Upon injection of urine from a human female, the frogs start to produce eggs if the hormones associated with human pregnancy (primarily, human chorionic gonadotrophin) are present (Mattison, 1998).

Later on*, Xenopus laevis* was the first vertebrate animal to be cloned (Gurdon et al, 1975), and recent years have seen their continued use with the advent of further developments in genetic technologies. **They are now one of the most widely used vertebrate species in developmental, cell and molecular biology research** (Gurdon, 1996).

Developmental biologists, embryologists or geneticists use *Xenopus laevis* because the eggs and early embryos of this animal are large and experimental manipulations are thus relatively easy (Woodland, 2003). Their growth outside of the uterus means that embryos can be observed throughout development (Kelley, n.d.) which is extremely rapid[3]. Manipulations of the cells within fertilised developing eggs, or of the environment in which the eggs are maintained are often undertaken to discover how subsequent growth and development is affected. A researcher may manipulate embryos directly following fertilisation, allow the embryos to develop in a simple saline solution for a couple of days, and then examine the tadpoles to determine whether and how the experimental intervention influences development. The role of genes in development can for example, be assayed by injecting a small amount of mRNA encoding the gene of interest into an early embryo, then allowing the embryo to grow into a tadpole (Xenbase, 2003).

Xenopus display a circannual cycle in relation to reproduction and this initially limited their use. Seasonal effects impact on breeding frequency and success and in addition, for over five months of the year, no breeding usually takes place in wild individuals (Tinsley, pers.comm). Once this was overcome with

[3] Cell division proceeds soon after fertilisation and within a few hours the blastula stage is reached. Hatching occurs after two to three days whereupon the transparent, filter-feeding tadpoles measure around 4mm in length (Phillips, 1979).

the inducement of egg laying behaviour through hormonal injection (see Section 5.1), demand for these frogs for research purposes increased.

This species, like most frogs, has a high degree of physiological sensitivity to their surrounding environment. For this reason, in some countries, **they are used in the safety assessment testing regimes of many potential environmental pollutants and other toxicological substances** (Dumpert, 1983, in Hilken et al, 1995). Since normal *Xenopus laevis* development was described (initially by Nieuwkoop and Faber in 1956), the use of this frog has become common for many *in vitro* development toxicology investigations. Indeed, the '*Frog Embryo Teratogenesis Assay: Xenopus*' (FETAX) system has become standard protocol (University of Arizona, 2001) and has been used to evaluate hundreds of substances since its inception (Wright & Whitaker, 2001). The test is based on assessing the toxicity of a substance on its capacity to induce embryonic mortality, malformation or growth inhibition (Altafaj et al, 2004).

These frogs are also kept and **used for educational purposes** in a small number of schools and universities (e.g. dissection classes). However, this use of amphibians, and animals in general, appears to be declining markedly (Halliday, 1999) due, in particular, to ethical concerns voiced by students, parents, teachers, animal welfare organisations and others, and due to financial constraints.

The wide-ranging use of this species is further illustrated by the fact that they have been used in research studies aboard various space-shuttle missions (The Wellcome Trust, 2003).

2.3.2 Numbers of *Xenopus laevis* used

It is extremely difficult to gain an accurate assessment of the current use of *Xenopus laevis* for scientific research and testing purposes. In the UK for example, the Home Office publishes details of the numbers of animals used under the *Animals (Scientific Procedures) Act 1986* each year. These figures show 9,288 amphibians were used in 14,985 procedures in 2003 (Home Office, 2004). However, although it is acknowledged that the main amphibian species used is *Xenopus laevis* (Mrozek et al, 1995), the figures for each amphibian species are not provided separately, so it is not possible to determine the exact number.

A further obstacle to discovering the full extent of the numbers maintained in UK laboratories is that there are some practices routinely carried out on *Xenopus laevis* that do not require reporting in the annual Home Office statistics. These include:

- the euthanasia by an approved (*Schedule 1)* method of frogs for tissues, organs, or oocytes (if oocytes are collected only once);
- the euthanasia by an approved (*Schedule 1)* method of those frogs bred but not then required for experimental purposes.

Current UK figures are therefore likely to be a significant underestimate of the actual numbers kept in research and testing establishments, which are likely to run into many thousands.

The most recent European wide figures, produced for 2002 (EC, 2005) from data provided by the 15 member states, are also likely to be an underestimate of the total numbers *kept* in laboratories, for similar reasons. These list 59,689 amphibians having being used in ‘scientific procedures with the potential to cause pain, suffering, distress or lasting harm’. This was predominantly for fundamental biological research (28,891), but also for education and training purposes (14,057), and a smaller number for toxicological and other safety evaluation studies (5,535), and for research and development of products and devices for human or veterinary medicine (5,411).

Elsewhere it is even harder to estimate the use of this species. During 2001, in Canada, 49,350 ‘amphibians’ were used in research (CCAC, 2004). In New Zealand for 2002, this figure was 409 (NAEAC, 2003). It is unknown how many of these animals were *Xenopus laevis*. The United States of America does not even categorise the total number of ‘amphibians’, let alone the numbers used of this particular frog species (e.g. USDA, 2002).

Amphibian species are becoming better understood and scientists are claiming an increasing number of advantages and applications for using these animals in research (AWIC, 2001). Many view *Xenopus laevis* in particular as an ‘ideal model organism’ (The Wellcome Trust, 2003). In addition, researchers in many countries are looking to shift where possible to those species *perceived* to have less capacity for experiencing pain and distress and for which there tends to be less emotive appeal and public concern. Furthermore, in Germany, the European frogs (*Rana spp*.) now fall under endangered species regulations, and so their use is often replaced where possible and appropriate by *Xenopus laevis* (Hilken et al, 1995). As a result of all these factors, many establishments now house *Xenopus* frogs as routinely as rodent species (O’Rourke, 2002) and it is likely that *Xenopus* use will increase further in the future (Green, 2002). The need to define factors that affect their welfare and seek ways of refining all aspects of their husbandry, care and use is therefore of paramount importance.

3 Supply and transport

For many years, large numbers of *Xenopus laevis* frogs have been taken directly from the wild in southern Africa for the purposes of breeding and/or supply to research and testing establishments around the world. More recently, an increasing number of captive frogs have been purpose-bred for scientific use. The source of animals is an important consideration for both animal welfare and scientific reasons, as are other factors relating to supply such as transport and quarantine.

3.1 Wild-caught versus purpose-bred

An estimated third of *Xenopus laevis* frogs used are taken from the wild (*anon., pers.comm.*). The impact of large-scale capture is not currently a direct threat to their existence, as this species is not endangered. However, for animal health and welfare, scientific reliability, and consequently ethical reasons, it is better that animals obtained are from captive colonies bred specifically for the purpose.

Xenopus laevis can be bred and reared easily under laboratory conditions (Council of Europe, 2003) and many establishments currently already routinely breed their own frogs. *Xenopus laevis* frogs of either sex and at any stage of development can also usually be readily obtained from commercial suppliers (Kelly, n.d).

Commercial organisations may supply: wild-caught animals; wild-caught animals that have been held in captivity for a period of time; or captive-bred animals. Some companies selling purpose-bred animals reared specifically for research state that the animals they are selling are a minimum of 5 generations removed from wild-caught stock (e.g. NASCO, 2003).

A comparison of the perceived benefits of using either captive-bred or wild-caught *Xenopus laevis* frogs is given in Figure 1.

Figure 1: Comparison of perceived benefits of using either captive-bred or wild-caught *Xenopus laevis* frogs

Perceived benefits of using *captive-bred* frogs	Perceived benefits of using *wild-caught* frogs
Laboratory or captive-bred individuals have a known life history, age and diet, and the potential for introducing unwanted disease or parasites to an existing colony can be reduced.	Wild caught animals are usually slightly lower in price to buy than their captive-bred conspecifics.
Captive-bred animals have experience of artificial rearing, housing and husbandry conditions similar to those they are likely to experience for the rest of their lives. Events such as handling by humans, tank and water cleaning practices are likely to cause less stress, health and welfare problems than in naïve wild-caught animals. It is noted that lab bred *Xenopus* may remain completely 'unmoved' by sudden sharp loud noise and can habituate to people moving frequently in the room (Council of Europe, 2003).	Wild-caught *Xenopus* are considered by some to be more fecund than lab-bred counterparts, resulting in scientists using fewer animals to obtain the amount of biological material that is required (Council of Europe, 2003). Wild-caught animals may be generally larger and therefore may produce more eggs (Xenopus Express, 2003) though this size and productivity differential may be age related.
Lab-reared frogs inbred over a number of generations will be more genetically similar which could be beneficial to the reliability of results in many studies as it reduces the effects of potential variables.	It allows genetic diversity to be maintained in a colony if this is required.
Amphibians are an important part of the ecological balance of many habitats. By not taking these frogs from the wild, the balance of the local ecosystem remains less disturbed	

Comprehensive data on the comparative health of wild-caught versus purpose-bred *Xenopus laevis* frogs throughout the duration of their use in a laboratory setting should be recorded and critically evaluated. Currently, many of the comparisons made regarding egg production and quality, and health, are anecdotal.

Purpose-bred frogs should be used in preference to animals taken from the wild (Council of Europe, 2004) **- a researcher must be able to provide specific justification for the need to use wild-caught individuals.**

3.2 Transport considerations

Animals bred off-site, or wild-caught and transported to the place of use, may have to endure a long journey, often to another country or continent. For example, many *Xenopus laevis* frogs are imported into Europe from the United States of America, South America or South Africa. Long journeys could pose potential health and welfare problems to animals, so suppliers often go to great lengths to ensure the health of the frogs in transit. When all measures are taken to safeguard welfare, long-distance flights should not pose serious dangers to *Xenopus laevis* and in most instances these animals seem to travel well and appear healthy on arrival. However there are major problems that need to be avoided.

The factors to consider when transporting *Xenopus laevis* are the same as those relating to the movement of other species. Relevant codes of practice such as the European Convention on the Protection of Animals during International Transport (1968: CETS 65) should be adhered to. Companies should also follow the recommendations for adequate animal transportation of the International Air Transport Association and the Animal Air Transport Association (Council of Europe, 2003).

More information on general principles for the transport of laboratory animals can be found in the report of the LASA Transport Working Group (2005).

The biggest problem for *Xenopus laevis* frogs in transit, whether it is by air, road or other means is **exposure to excessive heat.** Even a few hours at temperatures above 25ºC can damage egg quality in the females (Sive et al, 2000) though the frogs themselves may *appear* unharmed. Where egg quality is affected by such heat exposure (potentially a problem in the warm summer months), more females may need to be supplied and overall numbers required would rise. It has also been illustrated that exposure to temperatures above 25ºC may lead to skin cell disintegration in *Xenopus* (Callaghan, 1953, in Deuchar, 1975).

It is preferable for frogs to be bred at the establishment where they will be used - this will avoid any potential risks to health or welfare associated with their transport.

3.2.1 Packing and insulation

When transporting animals, the potential for causing them injury or stress must be minimised. Some suppliers use a primary transport box made of water-resistant cardboard or polystyrene, and pack the frogs with damp sphagnum moss (e.g. NASCO) while others prefer to use damp foam cubes (e.g. Xenopus Express), into which the frogs can burrow. Primary transport boxes placed together in a larger container, are usually then surrounded by protective polystyrene or styrofoam insulation packing chips.

Traditionally, shipments were often prevented during the winter or summer months due to the frogs' sensitivity to temperature. More recently, it has become regular practice for commercial suppliers to add ice packets or chemical heat packets to the containers (making sure that they do not come into direct contact with the animals) as necessary, depending on the time of year and climate. Weather conditions thus now have less impact on delivery reliability, whatever the time of year.

- **Transport containers must have an adequate air supply.**
- **During transport frogs must be kept within acceptable temperature limits** (see Section 4.3.2) **and measures should be taken to prevent desiccation.**
- **Transportation of frogs should be postponed if extreme weather conditions (e.g. extreme cold or heat) could threaten the health or welfare of the animals.**

3.2.2 Travel delays

The second biggest consideration regarding the transport of *Xenopus laevis* frogs and a potential welfare concern, are **delays in transit**.

- **Journey times should be the minimum practicable.**
- **Adverse weather, mechanical problems or industrial action can cause flight delays or cancellations and it is important to check and plan for such eventualities.**

3.2.3 Grouping animals together during travel

It is unlikely that *Xenopus* frogs travelling together will cause each other injury. Nevertheless, given their carnivorous nature and observed instances of cannibalism of younger and smaller individuals (McCoid and Fritts, 1980 in Tinsley and Kobel, 1996), frogs travelling in the same box should be of similar size and weight.

- **Frogs being transported together in the same box should be at a similar stage of development, size and weight, and should not be overcrowded.**

3.2.4 Arrival

Sufficient preparation and communication between supplier and buyer regarding transport itinerary and conditions, breeding history of frogs, dietary background and health status should take place before shipment. This should also occur *within* the establishment receiving the animals, to ensure that should the primary people involved in the transfer be absent, other, suitably trained and informed animal care staff are on hand to receive the shipment, checking the health and welfare of each individual animal upon arrival.

Care for the first two weeks following arrival after transport is particularly critical for amphibian survival (University of California, 2000). Animals which arrive in ill health and do not have a chance to recover, should be euthanased immediately and the sender of the frogs should be informed (Council of Europe, 2003).

- **An appropriately trained and competent person must check the health and welfare of frogs on arrival. Animals should then be closely monitored and, to avoid introducing disease, should be quarantined away from any existing resident colonies.**
- **To help prevent the potential spread of disease or bacteria, when animals are transferred from shipping containers to the new holding tanks, the sphagnum moss and foam cubes with which they were transported should not be transferred with them.**

3.3 Quarantine

The principle of quarantining newly arrived frogs away from those already present in an establishment (in order to reduce the opportunity for infections or disease to be passed between the two groups) is widely agreed, however, the period of quarantine is debated. The *Xenopus laevis* supply company Xenopus Express (2003) recommend that all new amphibians should be quarantined for 7 to 10 days post-arrival, the University of California (2002) guidelines suggest two weeks, while DeNardo (1995) suggests a minimum period of 30 days. Wright & Whitaker (2001) suggest wild-caught amphibians, whether obtained directly or indirectly, should be held for an extended quarantine of 90 days or more. O'Rourke (2002) states that captive-bred animals coming from sources of known health status can generally spend less time in quarantine than wild caught animals but does not provide subsequent guidance on how long these relative periods should be, simply stating that recommendations that have been made vary, from weeks to months.

The lifecycles of many of the major parasites of *Xenopus laevis* are around four to six weeks (Sanders, 2004a). It would therefore seem wise for the quarantine period to be *at least* of this length. During this time frogs should be examined daily for signs of ill health, with regard to activity level, skin discoloration, ulceration, petechial (pinpoint) haemorrhages on the legs, coelomic swelling or any other unusual changes (Xenopus Express, 2003). Frogs can also be given prophylactic treatments for pathogens during this time (see Section 4.10)

- **A minimum quarantine period of 30 days is advised for all new animals entering an establishment with strong consideration given to extending this period to 90 days for those animals obtained directly from the wild.**
- **Routine husbandry procedures for frogs in quarantine should be undertaken after the resident colony has been attended to and must be carried out with separate dedicated equipment.**
- **Frogs should be observed daily for signs of ill health.**

4 Housing and care

It is important that frogs are kept under *optimal* rather than *tolerable* conditions both to maintain a healthy colony and in order to stimulate good quality egg production throughout the year. This section highlights and assesses current practice, guidance and research in relation to the range of environmental parameters that need to be considered, with the aim of helping develop consensus on best practice.

4.1 Lighting provision

4.1.1 Photoperiod

African clawed frogs can grow and reproduce over a fairly broad range of lighting regimes (Major & Wassersug, 1998). However, continuous light, light deprivation, and inappropriate photoperiods can cause varying symptoms ranging from lethargy to sterility and even death (Hayes et al, 1998).

A number of laboratories house *Xenopus* species under natural lighting, but the majority now employ artificial sources and block out all outdoor lighting cues. A 14-hour light and 10-hour dark period has been recommended (e.g. Council of Europe, 2003) as roughly corresponding to nature, though this is presumably seasonally-affected. Many establishments choose to use a constant regime of 12 hours light, 12 hours dark (Major & Wassersug,1998). Either of these protocols is suitable, as frogs appear to respond well to lighting regimes of between 12-14 hours of light and 12-10 hours of dark (Sive et al, 2000).

Gradual dimming/brightening of the lights is more akin to natural conditions and allows animals to adapt to the change. This measure also removes a potential stressor from the environment if lights had previously been abruptly turned on or off. Although vision is not the primary sense utilised by these frogs, it is worth giving these animals the benefit of the doubt with regard to potential sensitivity to such rapid changes in environmental lighting levels.

- **Continuous 24-hour lighting should not be used.**
- **A lighting regime of between 12 and 14 hours light, and 12 and 10 hours dark is recommended.**
- **Where artificial lighting is used, a gradual brightening/dimming period of around 20-30 minutes in the morning/evening should be incorporated.**

4.1.2 Spectrum

When frogs are exposed to sunlight-equivalent light levels, proper vitamin D levels and correct calcium/phosphorous balance can be maintained (University of Arizona, 2001) and many researchers believe egg quality improves (Sive et al, 2000). It is therefore important to consider providing full spectrum lighting, including ultraviolet (UV) range[4] (University of California, 2000; NASCO, 2003; Wolfensohn and Lloyd, 2003; Tyler, 1999), particularly for animals kept over a long period such as six months or more.

Any artificial lighting used should simulate outdoor sunlight levels, with consideration given to including the ultraviolet (UV) part of the spectrum.

4.1.2 Intensity

Frogs should not be subjected to bright light, though illumination in the animal house must be of an adequate level to allow observation of the animals and for routine housing and husbandry procedures to be carried out. Many laboratories employ standard lighting that may be particularly aversive to frogs in tanks in the highest position of tank racking i.e. those closest to the light source. Frogs should have access to places where they can avoid light (Xenopus Express, 2003) as the stress caused by inappropriate lighting will be exacerbated where no hiding refuges are provided.

There appears to be a lack of conclusive evidence as to whether albino *Xenopus* frogs suffer from light-induced retinal damage in the same way albino mammals can. However, a precautionary principle should be followed and such frogs should not be exposed to bright lighting.

Tanks should not be openly exposed to direct sunlight or very bright artificial light and frogs should have access to darkened refuges (see Section 4.9).

4.1.3 Nocturnal nature

Xenopus laevis are naturally nocturnal animals (Elepfandt, 1996a). Few establishments appear to have developed husbandry protocols that take account of this behaviour. Therefore, there may be potential, particularly for the welfare of *wild-caught* animals, to be compromised through disturbance caused by human activity or an inappropriate lighting regime. The Council of Europe (2003) suggests frogs *bred and reared under laboratory conditions* are commonly active by day and night, but literature and information is extremely hard to find regarding their behaviour in laboratories during the dark phase.

---

[4] Animal care staff and laboratory personnel who enter the animal house and who are exposed to UV light should wear protective eye goggles and long-sleeved garments.

Establishments may wish to review current lighting, feeding and tank cleaning protocols to determine the practicality of alternative regimes that may better cater for the nocturnal nature of these animals.

4.2 Humidity

Under normal circumstances *Xenopus laevis* frogs are totally aquatic (i.e. equivalent of 100% humidity). Controlling the humidity levels in the room holding these animals in tanks is therefore less important than for those species that naturally spend large periods of time on land. In any case, it is difficult to control environmental humidity in rooms holding open water tanks as the humidity at the water's surface is likely to be different from that elsewhere in the room.

4.3 Water provision

4.3.1 Quantity

There is inconsistency in the literature and between establishment practices regarding the amount of water to be provided per animal. In some cases this variation may be related to differences in other husbandry routines such as group size, feeding or water changing regime. However, there does not seem to be a clear and consistent basis for the decisions made.

Potential constraints imposed on frogs (and their behaviour) by enclosure size and the amount of water provided may be overlooked because of a general belief that amphibians are insensitive to changes in the size of the captive enclosure (Hayes et al, 1998). However, little research has been undertaken on the capacity of this species to perceive changes in the spatial environment. It is generally accepted that supplying suitable depth and volume is very important, but that water quality is the most important factor (DeNardo, 1995; British *Xenopus* Group, 2001; Wolfensohn and Lloyd, 2003).

The dimensions of the tank and the volume of water must be great enough to allow frogs:

- to move or swim around;
- to lie fully submerged well away from the surface of the water;
- to avoid contact with other animals if desired;
- to turn fully in any direction without impediment either from tank walls or other animals.

There should also be sufficient space within the tank to allow the addition of suitable environmental enrichment (see Section 3.4).

i) Depth

It has been shown that *Xenopus* can 'live' in standing or slowly moving water of any depth (Elepfandt, 1996b) but there is no evidence with respect to the *optimal* depth. Although Hilken et al (1995) observed no effect on growth rate of frogs after placing them in tanks with differing water depths (of 5, 10 or 20 cms), water which is too shallow can increase stress, startle and escape responses in these wholly aquatic frogs. Insufficient water levels can also cause ovary regression in females (Alexander & Bellerby, 1938). Furthermore, a field study of natural behaviour (Elepfandt, 1996b) showed that in any given water body, calling males favoured deeper areas where their calls propagate better.

A range of recommendations for water depth can be found in the literature (see Figure 2). The rationale behind the recommendations is rarely provided:

Figure 2: Summary of recommendations for water depth for housing *Xenopus laevis* frogs

Source	Recommendation	Rationale (where provided)
University of California ARC guidelines (2000)	5-10cms (**approx. 2-4 inches**)	
Council of Europe (2004)	Minimum depth depends on the size (snout to vent length) of the animal: ▪ **< 6cms body length** = water depth of 6cms (**2.4 inches**) ▪ **6-9 cms body length** = water depth of 8 cms (**3.1 inches**) ▪ **10-12 cms body length** = water depth of 10 cms (**3.9 inches**) ▪ **13-15 cms body length** = water depth of 12.5 cms (**4.9 inches**)	
University of Arizona IACUC guidelines (2001)	**4-5 inches**	Allows 'serenade behaviour' where frogs stand on their hind legs and protrude heads from water (and may even 'chirp', particularly in the evenings).
Beck (1994)	**At least 6 but no more than 12 inches**	
Williams (1991)	20cms (approx. **8 inches**)	
LASA Good Practice Guidelines (2001)	At least 25 cms (approx. **10 inches**)	
Van Zutphen et al (2001)	30 cms (approx. **12 inches**)	
Wolfensohn and Lloyd (2003)	Up to 40 cms (approx. **16 inches**)	
The Berlin Workshop (1994)	**Should not exceed** 50cms (approx. **20 inches**)	To facilitate lung breathing at low energy costs.

A water depth of *at least* 8 inches is recommended for housing adult *Xenopus laevis* frogs. This is based on current evidence and knowledge, and should allow them to perform most normal behaviours.

ii) Volume

When maintained under natural conditions, *Xenopus* do not live in direct contact with each other (Council of Europe, 2003). Without doubt, population density is one of the most important factors affecting the growth of *Xenopus laevis.* Frogs with more area available per animal grow significantly faster than animals kept in a tank with a higher population density (Hilken et al, 1995).

Suggestions in the literature for volume of water to be provided per frog at breeding age vary (see Figure 3), usually from around 2 litres (0.53 gallons) of water per frog to 8 litres (2.11 gallons) of water per frog (Schultz and Dawson, 2003). However, figures can be up to around 19 litres (5 gallons) per frog.

Figure 3: Summary of recommendations for water volume for housing *Xenopus laevis* frogs

Source	Recommendation	Rationale (where provided)
University of California ARC guidelines (2000)	One adult frog per 2 litres of water.	
Marine Biotech, Inc	Tanks such as the XR_3 and XR_4 are designed with a rationale of around one adult *Xenopus laevis* frog per 2.64 litres of water.	
NASCO (2003)	At least one gallon (US) (3.79 litres) per frog.	
University of Arizona IACUC guidelines (2001)	Maximum of four frogs in 5 to 10 litres of water (an approximate minimum of 1.25 - 2.5 litres of water per frog).	
LASA Good Practice Guidelines (2001)	15 animals may be kept in 70 litres of still water (approx. 4.7 litres per frog).	
Sive et al (2000)	Four females or six males in approximately 16 litres of water (2.7 to 4 litres per frog).	
Wolfensohn & Lloyd (2003)	Approximately 6-10 litres of water per animal.	
Xenopus Express (2003)	2-5 gallons (US) (approx. 7½ to 19 litres) per adult.	Frogs like lots of room to swim.

[5] (for *Council of Europe* recommended volumes see footnote at bottom of this page).

There is wide variation in the volume of water recommended per frog. More discussion and research is needed to determine best practice, with due regard to the behavioural needs of the animals (see Section 4.3.1).

4.3.2 Temperature

Xenopus laevis frogs have been found to exist in the wild across a wide geographical range and across a spread of environmental temperatures (Tinsley and Kobel, 1996). As with other amphibians, they are cold-blooded animals whose body temperature is dependent upon that of the surrounding environment, and as a consequence, they are very susceptible to health problems if the surrounding environmental conditions are unsuitable.

In their natural environment, cold-blooded animals will literally ‘select’ their body temperature by finding the appropriate thermal environment through basking, burrowing, hiding under rocks or logs, or entering water (Kreger, 2002). For *Xenopus laevis,* who, it is stated, typically prefer water around 21ºC to 24ºC (Green, 2002), this behavioural modifier may be expressed by the depth at which they rest in the water. Other changes in behaviour as a result of exposure to unfavourable environmental temperature have also been observed in this species in the wild*.* At water temperatures of 30ºC (a level which can in fact be lethal to *Xenopus* (Green, 2002)), these frogs have been observed to excavate pits 30-40cm deep in the soft bottom mud where the temperature remained a more comfortable 20ºC (Tinsley and McCoid, 1996). Their ability to adapt their bodily systems (the locomotor system for example) to surrounding temperatures has also been noted (e.g. Wilson et al, 2000).

i) Adverse effects associated with temperature

If water temperature is sub-optimal, frogs will not eat and their metabolism and immune systems will be depressed (University of Arizona, 2001). The *Xenopus* immune system operates best above 21ºC (Tinsley and Kobel, 1996) and is depressed at low temperatures. Keeping these frogs in laboratories at unsuitable temperatures, such as 15ºC or lower, may be the reason for pathogenic infection occuring (Tinsley and Kobel, 1996). It is certainly believed

[5] Council of Europe (2003) recommended minimum space requirements are provided in a different format to that given by others and are as follows:

Body length (snout to vent) (cms)	Min. water surface area (cm^2)	Min. additional water surface area per additional animal (cm^2)	Min. water depth (cm)
<6	160	40	6
6-9	300	75	8
10-12	600	150	10
13-15	920	230	12.5

that *Xenopus* are stressed by prolonged exposure to temperatures below 14ºC and above 26ºC (Green, 2002).

At marked departures from the preferred range, *Xenopus* growth and development is delayed (The Berlin Workshop, 1994). Nevertheless, in a rare study undertaken to investigate temperature preference in *Xenopus*, Hilken et al (1995) observed no differences in growth development between frogs kept at 19, 22 or 24 º C over a five month period starting when the animals were 3.5 months old. However, this may have been because the temperature levels used fell within a relatively narrow range (5º C), all of which may have been suitable. It should also be borne in mind that growth rate is just one potential indicator of health.

ii) Optimal temperatures

In order to offer the best conditions for overall frog health and egg quality, the *optimal* conditions should be provided not simply those that the frogs can tolerate. However, it is not clear what the optimal temperature range should be. The recommendations in existing guidelines are shown in Figure 4.

Providing areas of slightly varying temperature which allow animals to seek their preferred microenvironment is beneficial (Council of Europe 2003). However, this is not usually possible in the type of housing usually provided in the laboratory (Hayes et al 1998). Research is needed to determine both the optimal conditions and how best to provide them.

It is also important to minimise significant fluctuations of either room or water temperature. Although frogs may be tolerant to gradual increases or decreases in temperature, they may be susceptible to thermal shock and suffer mortality if they are abruptly exposed to variation in temperature of 2ºC to 5ºC (Green, 2003). In addition, where frogs are to be moved between tanks with water of different temperature, gradual and staged acclimatisation in intermediary holding tanks is advised.

Figure 4: Summary of recommendations for water temperature for housing *Xenopus laevis* frogs[6]

Source	Recommendation	Rationale (where provided)
Wolfensohn and Lloyd (2003)	15 - 18ºC	
NASCO (2003)	Optimal temperature is 16 - 18ºC	
Sive et al (2000)	16 - 20ºC	
Van Zutphen et al (2001)	18 - 20ºC	Note: Variations in the ambient temperature in excess of 5ºC per 24h may harm the animals.
Council of Europe (2004)	Optimal temperature is 18-20ºC	

[6] A survey of establishments using *Xenopus* mainly in the United States (Major & Wassersug, 1998) revealed temperatures are maintained at levels within the wide range of 15 to 25ºC (59-77ºF).

University of California ARC guidelines (2000)	15 – 27ºC (60 to 80 º F) though believe the ideal is 19 - 22ºC (66 to 72 º F)	
Xenopus Express (2003)	20 - 23ºC	Sub-optimal temperature will affect eating, metabolism and the immune system will be depressed. *Xenopus* are sensitive to temperature changes within the room as well as the water.
Schultz and Dawson (2003)	18-24ºC	Ranges of 18-24ºC are considered acceptable for adequate growth. 'Laboratories have discovered' that water temperature maintained at 20-22ºC optimises oocyte production.
University of Arizona IACUC guidelines (2001)	Avoid temperatures over 24ºC	Warmer temperatures will affect breeding.
LASA Good Practice Guidelines (2001)	Ambient room temperature of 20ºC	

- **Water temperature should not be maintained below 16ºC or above 24ºC, with strong consideration given to providing a relatively controlled temperature at a point between 18 and 22ºC.**
- **Allowing an individual animal some control over the immediate microhabitat is desirable. Current animal housing practices should be reviewed to determine if measures can be taken (such as the inclusion of temperature gradients) within the constraints of the laboratory environment to allow more scope for this.**
- **Abrupt significant fluctuations of either room or water temperature should be avoided.**

4.3.3 Water quality

i) pH level

The pH tolerance range for *Xenopus* described in the literature is variable. In the Cape, South Africa, *Xenopus laevis* are noted to breed in both acidic and alkaline water (Tinsley & Kobel, 1996). Some authors state that *Xenopus* frogs can tolerate a wide range of pH and that a suitable level might be between 5 and 9 (e.g. NASCO, 2003). However, a far narrower tolerance zone (e.g. between 6.5 and 8.5) is given by others (University of Arizona, 2001) with a warning that deviation from this (where water is too acidic or too alkaline) could even cause the sudden death of an entire colony (Wolfensohn & Lloyd, 2003). Sive et al (2000) consider a pH of 6.5 to be optimal for these frogs, while the commercial frog supplier Xenopus Express (2003) recommend maintenance between pH 6.5 and 8.5.

A potential danger to frog health and welfare is that a higher pH increases the toxicity of chemicals, such as ammonia, in the water. Even without ammonia present, it is believed a change of 1pH unit (e.g. from 6.5 to 7.5) can lead to the loss of the frogs' protective mucous, a higher susceptibility to pathogen attack, and other stress-related conditions (Sive et al, 2000).

Clearly it is important to monitor the pH of the water in the tanks regularly, using pH test tape or preferably, a more precise electronic meter.

ii) General hardness

Analysis of samples taken from ponds containing wild *Xenopus laevis* in South Africa often show the general hardness of the water to be moderate to very hard (Godfrey & Sanders, 2004). It has also been shown that housing adult females in hard water (containing a high concentration of calcium and magnesium ions) can greatly improve the firmness of their oocytes and early embryos and their survival and normal development (Godfrey & Sanders, 2004). However, these frogs have been held in 'soft' water in some establishments without apparent problem.

iii) Other water quality parameters

It is important to have a full knowledge of the origin and properties of the water used for maintaining frogs. Properties will vary widely depending on whether water is obtained from municipal sources (e.g. tap water, which in effect is treated sewage), natural springs, lakes or rivers, or whether it is distilled/desalinised.

Water should be completely de-chlorinated before use (Sive et al, 2000) as chlorine attacks the protective mucous layer over the skin and can predispose frogs to infections (Wolfensohn & Lloyd, 1998). This can be achieved by exposure to air for several days in standing tubs. If local water authorities add chlorine agents to the water then this can be removed by running the water through a carbon filter (Sive et al, 2000). Allowing the water to stand before use and using filters should also help deal with any chlorine surges in the water supply. The pipes used for transporting water into and around the aquatic system should not be galvanised or copper, since heavy metals can leach from such pipes and may be toxic (Wolfensohn & Lloyd, 2003).

The water quality standards in Figure 5 have been proposed and successfully implemented for *Xenopus laevis*[7] (Sanders, 2004b).

[7] Note: this is just one recommendation for good practice, other sources suggest different parameters - for example, the NASCO (2003) water supply has 350mg/L alkalinity, 340mg/L hardness and 490µS conductivity

Figure 5: Water quality standards for *Xenopus laevis*

Alkalinity	≥	50 mg/litre $CaCO_3$
Hardness	=	75-150 mg/litre
PH	=	6.5-8.5
Salinity	=	0.4 mg/litre(ppt)
Conductivity	=	50 -2000 µS
Un-ionised ammonia (NH_3)	<	0.02 mg/litre
Nitrate (NO_2)	<	1 mg/litre
Nitrate (NO_3)	<	50 mg/litre
Chlorine	=	0 mg/litre
Dissolved Oxygen content	>	80% saturation
CO_2	<	5 mg/litre

- **Maintenance of a stable water pH between 6.5 and 8.5 is advised.**
- **Water quality and pH level should be routinely monitored. Contingency plans should be made in case of system breakdown or other emergency.**
- **When devising water quality assurance schemes for frog housing, a water expert knowledgeable of the local water treatment procedures should be consulted** (Dawson et al, 1992).

4.3.4 Cleaning

Frogs are naturally found in murky water, which provides a visual barrier to potential predators (Schultz & Dawson, 2003). However, there is a difference between murky, and dirty. Frogs routinely shed skin particles, and release ammonia and faeces into the surroundings. This along with floating decaying food particles will foul the water and may have implications for frog health where space and animal movement is limited, as in the laboratory tank. Consideration must therefore be given to how best to maintain the quality of the water whilst at the same time minimising disturbance to the animals.

Xenopus laevis are routinely housed either in tanks of standing water (periodically 'dumped and refilled' every day or few days), or in tanks where a drip-through system continuously and slowly changes the water. In drip-through systems the water coming in may be new, or to reduce overall water use, may be treated and cleaned re-circulating water. Static systems require frequent cleaning of tanks but have the benefit of enabling disease outbreaks to be more easily controlled. This can be harder in re-circulating systems (Sanders, 2004b). Recommendations for cleaning practices will be influenced not only by the tank or system design in place but also by the feeding regime and quality of water entering the system. There is no current universally applicable regime, but there are many suggestions for suitable general practice.

i) Standing water tanks

Some suggest changing the water in standing tanks at least three times a week (Sive et al, 2000). A number of establishments change the water even more frequently. However, it has been discovered that frogs disturbed daily for water changes have a slower growth rate and this might negate any improvement gained by better water quality (Hilken et al, 1995). Indeed, the authors observed that frogs grew better in a tank where water was replaced only once a week.

If complete water changes are undertaken, then this should be done at around the same time each day (Schultz & Dawson, 2003), preferably post-feeding (see Section 3.3.6) since this is when the water quality is at its worst (DeNardo, 1995).

Establishments using individual standing water tanks should review their water changing protocols. Although most establishments change the water in tanks at least three times a week, and maybe even everyday, the benefit of such frequent water changes in view of the increased disturbance and stress caused to the animals has yet to be comprehensively evaluated.

ii) Drip-through water systems

It has been proposed that drip-through systems are the optimal way to house *Xenopus laevis* since levels of toxic waste are kept low and solid waste (in suspension) can be drained continuously (Sive et al, 2000). The downside of these systems is that they use a lot of water (if not re-circulating) and the quality of the input water must be monitored constantly which often means a significant capital investment. In addition, these animals would naturally favour still, stagnant areas of water or, at most, slight and slow moving waters, so a strong water inflow may cause them stress.

As frogs sense water movement through a highly developed lateral line system, the position of in- and out- flowing taps in the tanks and the rate of water flow should be set so water turbulence or motion is minimised. When using continuous drip-through systems (which are more cost-effective when housing large numbers of frogs) a rate of 10-50ml per minute has been recommended by Sive et al (2000). Consideration should also be given to submerging the incoming water jet as this may lead to less water turbulence (and noise) than where the water filters down from a height onto the surface of the existing body of water.

If exposing frogs in tanks to constantly running water, the flow rate should be the minimum required in order to reduce the potential for causing the animals distress.

iii) Minimising temperature variation in the water supply

Though frogs may be able to tolerate a wide range of variation in temperature, e.g. a 5ºF increase or 10ºF decrease (NASCO, 2003), abrupt changes greater than just a degree or two could sometimes induce shock or even death (University of Arizona, 2001). Therefore, whatever the system, incoming replacement water should be consistent in temperature with that it is replacing (see Section 4.3.2).

iv) Removing dissolved gases

Water used directly out of the tap is saturated with dissolved gases and will cause bubbles under the skin and in the toe webs of frogs known as 'gas bubble disease' (University of Arizona, 2001; Xenopus Express, 2003) leading to possible emboli, emphysema, and even death (Orwicz, 1985, in DeNardo, 1995). Therefore, incoming water should be left for 24 hours prior to use to equilibrate with the surrounding environment. This allows the dissolved gases in the water to dissipate before the water is used.

Incoming water can also be passed through a de-gassing device (vertical packed column) to speed up the process of equilibration of the water (Sanders, 2004b).

v) Careful use of cleaning agents

The use of bleaches and detergents in aquaria must be treated with considerable caution (LASA, 2001). Most amphibians are susceptible to intoxication by phenol and cresol-type disinfectants such as Lysol® and will exhibit symptoms including convulsions, flaccid paralysis and death (University of Arizona, 2001).

LASA (2001) suggest that tanks can be sterilised using food grade hypochlorite, (e.g. 'Klorite') at 200ml/70L (0.28% solution) but emphasise the importance of rinsing them thoroughly several times before frogs are returned. Other recommendations for use in tanks housing amphibians, are 10% bleach (University of Arizona, 2001), 3% bleach (DeNardo, 1995) or betadine scrub, followed by thorough rinsing.

Cleaning strategies should be designed to minimise disturbance and distress to the frogs. Disinfectants should be used with extreme caution.

4.4 Tank housing

4.4.1 Labelling

Tank housing should always be clearly labelled with the number, sex and species housed inside. If the animals contained are currently being used in an experiment, the reference of that research should be clearly identifiable. This is so that all personnel involved in the research are fully aware of the experimental procedures involved, the objectives of the work, the potential adverse effects the animals may experience and the agreed humane endpoints (if applicable).

4.4.2 Tank material

Tanks should be constructed from material that is smooth, impervious, easy to clean and without sharp edges. Plastics used must not add toxicants to the water; acceptable plastics include those used for the storage of human food (Schultz & Dawson, 2003). Tanks for *Xenopus laevis* have traditionally been constructed from fibreglass, stainless steel (Schultz & Dawson, 2003), glass or polycarbonate (Wolfensohn and Lloyd, 2003). Whether the materials that are currently being used have any effect on growth or reproduction in *Xenopus* species has not yet been established (Major & Wassersug, 1998).

4.4.3 Colour and transparency

Tanks with darkened or opaque sides better approximate pond conditions (Sive et al, 2000) and are also likely to be less stressful for the frogs. Indeed, the literature suggests that both young and adult frogs show preference for dark background colouring (Goldin, 1992) and that a black background colouring may be better for the frogs' wellbeing than a grey or white one (Hilken et al 1995). In addition, a dark floor may enhance these animals' sense of security (Council of Europe, 2004) thereby improving their wellbeing. This is not surprising given that light emanating from underneath the frogs where the tank has a transparent floor, is the exact opposite of conditions encountered in the natural environment.

In white opaque tanks it is easier to see the build-up of grime, and this may facilitate cleaning. However, white or completely transparent tanks are not considered best practice for housing these animals, particularly those held over long periods.

An argument may be made for leaving some part (e.g. just the front panel) of the tank transparent (Council of Europe, 2003) so that the wellbeing of the frogs can be checked with minimal disturbance to the animals[8].

[8] Note: where frogs are recovering from surgical procedures or treatment for disease for example, there may be some justification in the short-term for using a holding tank with a clear bottom, to allow the non-invasive assessment of wound healing.

4.4.4 Lids

A well-fitting wire or nylon mesh cover can be used to prevent animals from jumping out of the tank. It will also prevent objects accidentally falling into it. Tanks should never be completely covered with a solid cover as this will affect air quality and may possibly lead to hypoxia in the frogs.

4.4.5 A specialised design

Currently, many frogs are kept in containers not originally designed for the purpose. Containers of various volumes and dimensions are used, many of which were originally designed for other species (e.g. fish aquariums, rodent 'shoebox' cages filled with water) or those that were not originally designed for housing animals at all (e.g. bath tubs). Strong consideration must be given to the suitability of available containers to house these frogs. The development of specifically designed tanks by laboratory equipment manufacturers is increasing, and this is welcome.

4.4.6 Dimensions

Tank dimensions must allow adequate space for a suitable volume of water (see Section 4.3.1) and for environmental enrichment (see Section 4.9).

A tank size of 65 x 45 x 45cms has been recommended by LASA (2001) for holding four to five frogs in 5 to 10 litres of water. A tank of this depth should (depending upon the water depth) prevent *Xenopus* from jumping out. An overhanging lip will further act to prevent escapes. *Xenopus* escaping during the night from laboratory aquaria are often dehydrated, debilitated or dead by the next morning (Tinsley and Kobel, 1996), so such incidents should be prevented.

The shape of the tank is also important. Long, narrow designs should be avoided as they restrict locomotor activity and social behaviour such as feeding frenzies (Council of Europe, 2004).

- **Opaque containers or those made of darkened plastic are preferable to completely transparent ones – in particular, the bottom of the container should not be permeable to light.**
- **Containers must prevent escape and should allow space for sufficient volume and depth of water and for enrichment such as refuges to be added.**

4.5 Identification and marking techniques

Marking can have an effect on an animal and its wellbeing through the act of marking itself, through the wearing of the mark and/or through the procedures required for observing the mark (Mellor et al 2004). The actual need for individual or group identification must therefore first be established, as only some research protocols may require this. If identification of animals is necessary then it is of paramount importance that only the most humane methods are used. These will be the ones likely to cause the least pain or distress, and which minimise the risk of secondary health problems whilst also being effective over the required period of use. When considering the acceptability of potential methods it is important to bear in mind that **pain perception in amphibians is likely to be analogous to that in mammals** (CCAC, 2004; Green, 2003).

4.5.1 Criteria for an acceptable method

The method of identification employed must:

- cause minimal suffering or impact on the animal both during the marking process or anytime subsequently;
- be long-lasting (although this is dependent upon duration of study/use);
- be reasonably quick and simple to apply to the animal;
- be easy and quick for the human to read/identify;
- be economically viable (particularly pertinent for laboratories housing large numbers of animals).

4.5.2 Assessment of methods

There are numerous methods of identification that have been tried with *Xenopus laevis* frogs over the years, though many of the more traditional methods are now actively discouraged and should no longer be practised. This is usually because of the adverse effects on the animals, but also often because of their relative ineffectiveness. Some of the merits and shortcomings of the various methods described in the literature are considered in Figure 6. There is currently no 'ideal' method favoured by all.

Figure 6: Methods of identification and some points to consider regarding their suitability

Key:	✓	This method can be considered
	X	This method should *not* be used
	?	The merits of this method are debatable – it might be considered suitable in certain circumstances

Taking into account both animal welfare and scientific requirements, the 'pros' 🟩 'cons' 🟥 and 'other factors needing consideration' 🟨 are highlighted for each method.

Method	**Points to consider** (with reference)	
Record cards firmly secured on the front of tanks	▪ No impact on the animal. ▪ Inexpensive. ▪ Useful when just needing to distinguish between groups of animals. ▪ Does not allow comprehensive lifetime history to be maintained for each individual.	✓
Photographs for recognising individuals by body markings	▪ Non-invasive. ▪ Inexpensive. ▪ Already being used successfully (e.g. Mathers, 2004). ▪ Particularly useful for small numbers of animals. ▪ The pattern of markings on the frog will remain consistent but relative skin colour can alter as a result of stress (Wolfensohn & Lloyd, 2003), or fluctuations in the environmental lighting, humidity or air temperature and this may make the pattern harder to distinguish. ▪ Could be time consuming where large numbers of animals are kept in each tank. ▪ Development of electronic scanners (linked to a computer) for detecting marking patterns could increase potential of this method. ▪ Markings fade once the animal dies. ▪ Patterns in very young or albino individuals are not as easy to see.	✓
Microchip transponders are increasingly being suggested for use in amphibians and reptiles (Schaeffer 1999). Transponders around 12mm long by 1.2-2mm diameter are implanted in the lymphatic cavity (WSAVA 1999) or injected sub-cutaneously in the area behind the neck.	▪ Some suggest this to be one of the best methods (e.g. Wolfensohn & Lloyd, 2003). ▪ Very large numbers of animals can be individually identified (Halliday, 1999). ▪ Transponders have been found to be effective (Fineberg, 2003) practical and reliable (Mrozek et al, 1995) and able to serve as the method of identification for the duration of the frog's life, normally without adverse effect (Mrozek et al, 1995; Hoogstraten-Miller & Dunham, 1997). ▪ Chips can be retrieved, re-sterilised and re-used when a frog is euthanased. ▪ Immediate skin tissue damage should only be minimal and if it does occur then it can be treated with tissue adhesive such as VetBond® (anon, pers.comm). ▪ Frogs normally have to be removed from the water to allow scanning – but individual identification is usually only necessary when the frog is to be used in an experimental procedure or is being examined for illness and as such would probably need to be removed from the water anyway. ▪ Anecdotal evidence suggests that on rare occasions, a microchip when placed subcutaneously in the neck, may move around the scapular region of the frog and even be expelled from the body by the frog. More data is required to confirm the prevalence of this and the potential for the chip to cause a blockage (i.e. in a lymph heart) – such occurrences are apparently highly infrequent. ▪ If implantation of the transponder requires surgery (i.e. when it is placed into the dorsal sac) it would be classified in the UK as a regulated procedure under the ASPA 1986. ▪ Due to their smaller size, microchip transponders used for *Xenopus laevis* are not suitable for use in *Xenopus tropicalis.* ▪ Some people are concerned ulcers or inflammatory responses may occur around the injection site. ▪ Most expensive of assessed methods (around £2-3 per transponder).	**?**

Freeze branding - shaped copper wire is placed into liquid nitrogen and then placed on the belly of the frog (Measey 2001)	• Some may deem this method as being more acceptable for marking frogs in field studies where alternative methods are fewer in number. • Frogs regularly shed skin meaning branding may be relatively ineffective. In some cases, the mark may become illegible in a few months to a year (University of Arizona, 2001). • Some believe this method to be difficult to justify due to the significant adverse effects (anon, pers.comm).	x
Sewing coloured plastic beads onto the muscle mass of the leg or back or attaching studs or tags to the web of the feet	• Beads have been sewn onto the legs or back of anaesthetised frogs with no reported perceived health or welfare complications (e.g. Hoogstraten-Miller & Dunham, 1997). • The suturing of coloured plastic or glass beads of appropriate size into the toe webs of frogs with plastic suture or monofilament line is considered an acceptable method of marking by some e.g. University of Arizona (2001), Schaeffer (1999), Wolfensohn & Lloyd (2003). • The skin and feet web in particular is very delicate in frogs, containing many blood vessels (Fineberg, 2003) which may be damaged during this process. • It could be hazardous to the animal if the thread or beads/tags get caught by the hind claws of that or other animals as they move around (Verhoeff-De-Fremery & Vervoordeldonk, 1982). • It could disrupt the mucous membrane of the skin and increase the chance of opportunistic pathogenic infection.	? x
Tattooing of numbers or letters onto the skin	• If this method is undertaken, appropriate anaesthesia should be used and the size of the numbering or lettering should be no bigger than is necessary to enable clear interpretation, i.e. there is no need to cover the whole of the belly. • Tattooing is often carried out with no anaesthetic used (i.e. Fineberg, 2003). Because the frog does not struggle greatly whilst laid on its back, people may deem the procedure not to be aversive to the frogs. However, prey species often do not show overt distress behaviours that might draw the attention of predators to their plight (Green, 2003) and thus decrease the chances of survival. Furthermore, while on their backs, these animals may simply be exhibiting tonic immobility and as such humans might not recognise suffering. • There is concern the use of vibrating needles and the resultant disruption of the protective slime layer and dermis may predispose the frog to diseases such as red leg syndrome (Hoogstraten-Miller & Dunham, 1997). • Ink tattooing is not an ideal method due to its non-permanence and the need often to re-tattoo (Hoogstraten-Miller & Dunham, 1997). As repeated re-tattooing over scar tissue should be avoided, this method, if used, should only be considered where identification of the animal will be required for 18 months or less. • The presence of bruising, redness and swelling of the delicate skin, observed for a period of days after tattooing (even when done by a highly experienced professional), would suggest that this method does have a negative impact on the animal.	x
Applying acetic acid to the skin to mark the animal	• This method is painful for the frogs - it should not be undertaken.	x
Leg ringing	• If the bands are tight enough to stay on the slippery mucous covered skin, then they are probably too tight. Blood circulation around the foot could be restricted, causing the feet to swell and become necrotic within hours (University of Arizona, 2001).	x

Removal of toes or fingers (usually by scissors or nail clippers)	▪ Traditionally a commonly used method. ▪ Causes animals pain and may cause distress. ▪ Should not be done (Council of Europe, 2004; Wolfensohn & Lloyd, 2003). ▪ Causes tissue damage in frogs (Golay & Durrer, 1994). ▪ Could be detrimental to health – it appears to decrease frog survival in the wild (Clarke, 1972; McCarthy & Parris, 2004). ▪ Frogs can re-grow removed digits (University of Arizona, 2001) meaning the process may have to be undertaken more than once.	**x**
Skin grafts - lighter coloured abdominal wall skin is removed under anaesthesia and implanted onto the darker coloured back, or vice versa	▪ Proponents of this method (e.g. Van Zutphen et al, 2001) provide no information relating to the associated health or welfare problems or the longevity of the method – this makes it hard to assess. ▪ The procedure is time-consuming. ▪ Undertaking the procedure requires surgical expertise. ▪ Use is limited to small number of individuals.	**x**

Careful consideration should be given to whether identification of individual animals is necessary, and if so, then to the method of identification used, particularly where the methods are invasive and likely to cause a degree of trauma.

Non-invasive methods of identification such as using group cards or photographs of the dorsal markings of the frogs are the methods of choice.

4.6 Group housing

It is generally considered that *Xenopus laevis* frogs should be kept in a group, and in most establishments, this is how they are housed.

Grouping is advisable as:

- efficient group feeding in this species involves feeding frenzies that are not as prevalent when only very small numbers of animals are kept together (Council of Europe 2004);
- grouping animals can help reduce fear responses.

Maintaining stability in these groups is advisable because:

- *Xenopus laevis* naturally form hierachies within a territory (Elepfandt, in Tinsley and Kobel, 1996). In many other species, if group structure is regularly altered (or if groups are too big) con-specific relationships are more difficult to maintain and antagonistic encounters may be more frequent leading to an increased potential for stress or injury.

When keeping animals together in the same cage or tank, there are many factors to consider including group size and stability and stocking density. However, there is little evidence to suggest what the ideal group size should be. Many laboratories keep groups of between 5 and 20 frogs although these groups are small compared to one commercial breeder whose smallest aquarium contains 40 individuals and the largest, around 400.

Individuals must not be kept in overcrowded conditions as frenzied responses to feeding can lead to traumatic injury of tank-mates (DeNardo, 1995). In this respect, it is probable that once above a number of animals conducive to encouraging feeding behaviours, *area per frog* is more important than *actual number of frogs* in the tank. Hilken et al (1995) found that frogs with more area available per animal grew significantly faster than animals kept in a tank with a higher population density. Furthermore, it has long been known that too high a population density (along with insufficient water levels) affects oocyte quality in females by causing ovary regression (Alexander & Bellerby, 1938).

In the absence of comprehensive evidence it is difficult to gauge an optimum group size for African clawed frogs. The group should be small enough to allow the formation of a stable hierarchy yet large enough to promote feeding frenzies. Animal carers commonly consider a minimum of 5 or 6 animals per tank to be advisable.

Group housing of these frogs in relatively stable groups is advisable.

Overcrowding should be avoided.

It is important that animals kept together in the same tank are of similar size.

4.7 <u>Handling</u>

There are many factors requiring consideration in relation to handling these frogs:

- As potential prey species, amphibians do not like to be handled.
- The skin is highly glandular (secreting a mucous layer to help protect against abrasions) which can be easily damaged.
- The lateral line sensory system is highly sensitive (Elepfandt, 1996a).

For these reasons repeated disturbance for capture and handling should be minimised (Council of Europe, 2004), only being undertaken where absolutely necessary (Wolfensohn and Lloyd 2003).

4.7.1 Capture

Xenopus laevis should be caught by catching the head between the first two fingers as it crosses the palm of the hand, followed by the thumb gently restraining the neck. In this way the forward straining movement of the escaping animal will only serve to push it further into the finger grip (Williams, in Benyon & Cooper, 1991) and should prevent an animal jumping or falling and potentially injuring itself. Alternatively, a frog may be picked up by placing one hand across its back with a forefinger between the animal's hindlegs, wrapping the rest of the hand around the animal's middle. The other hand may be used to cover the frog's eyes. It is believed that this will 'calm' the animal whilst also allowing physical restraint (Sive et al, 2000). Picking frogs up by the hind legs is not acceptable.

Before handling a live animal for the first time, it is helpful for animal carers to practice holding a model frog (Sive et al, 2000) or if appropriate, one that has been recently euthanased (although frogs should not be killed specifically for this purpose).

4.7.2 Use of hands

The skin of amphibians is more delicate and sensitive than that of any other vertebrate (Halliday, 1999). Traces of hand lotion, colognes or medicated ointments may harm and even have the potential to kill these frogs. Additionally, some amphibians have been observed to die if handled by someone who smokes (Halliday, 1999). It is also believed that the thin epidermis of the frog's skin could be abraded by the ridges and callosities of the restrainer's hand (Wright & Whitaker, 2001). If personnel are going to handle frogs then their hands should be thoroughly cleaned and moistened with water.

4.7.3 Use of gloves

Many believe moistened gloves should be worn by personnel changing water or handling frogs to avoid the problems potentially arising when the bare hands of personnel come into contact with frogs skin (Schaeffer, 1999; University of Arizona, 2001; Wolfensohn and Lloyd 2003). If used, Sive et al (2000) state that gloves must not be powdered or textured since they may abrade the animal's skin, or cause skin irritation. However, there is also concern that gloves reduce the sensitivity of the human hand and so a frog might be harmed from over zealous handling (Xenopus Express, 2003).

Wearing plastic or latex gloves does afford additional protection to the handler. It is important for personnel to remember not to touch their own mucous membranes (e.g. rub their eyes) after handling frogs as secretions from the parotid glands rubbed into the conjunctival sac of the human eye can cause irritation (University of Arizona, 2001)[9].

4.7.4 Use of nets

Frogs in a tank will often struggle or try to evade being picked up. Therefore, some believe using a net may be the best method for capture and handling (University of Arizona, 2001).

Using a net to initially scoop the frog out of the water avoids damaging the mucous layer of the frog and is less traumatic than physical contact (handling)

[9] These frogs should pose no additional health risks to humans.

with the animal (University of Arizona, 2001). Swift capture can be made in fine nylon nets but care must also be exercised here to avoid trauma (Williams, in Benyon & Cooper, 1991). However, others believe nets might still cause micro-lesions of the skin and therefore should not be used (e.g. The Berlin Workshop, 1994; van Zutphen et al, 2001). Beck (1994) has even suggested that the risk of the animals having fingers inadvertently trapped and even amputated in netting should preclude use of this method. However, if soft and fine mesh is used for the net then these potential problems should be avoided. LASA (2001) state that such nets should be deep and strong.

4.7.5 Use of plastic containers

A small plastic bowl or container may be used to lift frogs from the tank along with the water immediately around it (Elepfandt, 2004). This method would appear both time-efficient and likely to cause less stress or harm to the animal. A cover should temporarily be placed over the container to prevent the frog from escaping.

A drawback of this technique is that it may be less practical in tanks with a large group size of animals, a deep water level and where trying to capture particular individuals.

4.7.6 Restraint

Restraint of these frogs might be difficult due to the secretion of a slippery mucous covering. Animals can be gripped gently but firmly and a soft damp clean cloth can be used to increase grip (LASA, 2001; Wolfensohn and Lloyd, 2003; IAT, 2003). It has been suggested that permanently aquatic species like *Xenopus* may be held with a *rough* cloth (Halliday, 1999). However, in view of the sensitive nature of the skin, this has the potential to cause mild skin abrasion and is therefore discouraged.

4.7.7 Other considerations

Whatever the method of handling, when approaching the tank (or when simply moving around in the vicinity of the tank) personnel should be aware of the potential effects of their own behaviour on that of the animals. The classic ‘escape’ response can be observed where frogs are startled and those previously hanging at the waters’ surface, dive to the bottom to hide when objects move rapidly *over the tank.* This response is not usually stimulated by objects passing *by the side of the tank* (Elepfandt 1996a). Unnecessary movement of hands or objects by personnel over the tank should therefore be avoided.

- **Handling should be kept to a minimum and every precaution to avoid stressing or injuring the animals should be taken.**
- **Personnel should consider taking a precautionary approach and wear gloves (non-powdered) whilst removing frogs from the water with soft-mesh non-abrasive nets.**

Unnecessary movements of objects or hands over the tank should be avoided.

4.8 Food type and feeding regime

4.8.1 Natural behaviour in the wild

Xenopus laevis are carnivorous by nature (Phillips, 1979) and although they will scavenge the carcasses of dead animals, they prefer live food (Green, 2002). They have a voracious appetite and will attack almost anything that passes in front of them (Garvey, 2000). They take whatever organisms can be ingested, with insects the most common prey in adult animals (Tinsley & Kobel, 1996). Field studies reinforce this finding - examination of the stomach contents of *Xenopus laevis* have found the remains of fish, birds, and other amphibians, but it is small insects, slugs, worms and other aquatic invertebrate which predominate (Green, 2002).

4.8.2 Feeding requirements of African clawed frogs

It has been recorded that *Xenopus* can usually survive long periods (months) of starvation (Tinsley and Kobel, 1996) but such events carry a physiological cost for the frog which breaks down body tissue in the attempt to cope. Food shortage strongly affects egg production and it has been cited as a major factor causing the ovaries of *Xenopus laevis* to regress (Bellerby & Hogben, 1938). Therefore, to help ensure optimal frog health and that egg quality is maintained, *Xenopus laevis* must be provided with sufficient quantity and quality of food.

The dietary requirements and feeding behaviours of *Xenopus laevis* are easier to provide for in the laboratory environment than many other amphibian species, due to their willing acceptance of non-living food items.

4.8.3 What food should be given?

A number of commercially prepared diets for captive frogs are available. For example, NASCO produce Frog Brittle®, which contains fish meal, meat meal, soybean meal, corn meal, wheat flour, dried yeast, distillers solubles, whey, wheat germ meal, salt, vitamin supplements-diacalcium phosphate[10].

---

[10] Vitamin content of Frog Brittle (NASCO 2003): Vitamin A - 14000 I.U. per kg, Vitamin D - 5000 I.U. per kg, Vitamin E - 88 I.U. per kg, Vitamin B12 - 0.04 mg per kg, Folic Acid - 1.5 mg per kg, Thiamine - 4.0 mg per kg, Riboflavin - 9.0 mg per kg, Pantothenote Acid - 11.0 mg per kg, Niacin - 60.0 mg per kg, Choline - 1550 mg per kg, Calcium - 2.0%, Phosphorus - 1.6%, Sodium chloride - 1.2%, Magnesium - 2%, Linoleic Acid - 2.8%, Iron - 300 mg per kg, Copper - 10 mg per kg, Cobalt - 2.2 mg per kg, Manganese - 9.0 mg per kg, Zinc - 100 mg per kg, Iodine - 48 mg per kg, Pyridoxine - 8.8 mg per kg

Beef liver is another item commonly provided but when given on its own has been implicated as a cause of vitamin A toxicity, metabolic bone disease and other amphibian diseases (University of Arizona, 2001; Wright & Whitaker, 2001). Meat (such as liver or heart) should therefore be mixed with vitamins (Mrozek et al, 1995).

Maggots and crickets are also provided by some establishments and are stated to appear to provide welcome stimulation and enrichment for the frogs.

4.8.4 How often should food be provided?

Establishments vary in the feeding regime they provide for their frogs. The regime is influenced by the quantity and frequency personnel deem to be appropriate and also by the type of food provided (see Figure 7).

Figure 7: Summary of recommendations for food type and feeding regime for *Xenopus laevis*

Source	What	How much	How often
Xenopus Express (2003)	A nutritional high quality balanced diet such as Xenopus Express' 'sinking frog food'.	As much as can be consumed in 15 minutes.	Daily
University of California ARC guidelines (2000)		As much as will be eaten in one hour	Daily or every other day.
LASA Good Practice Guidelines (2001)	*Xenopus laevis* can be fed proprietary pellets, minced ox heart or a meat and pellet mixture although meat should only be given as a supplement rather than a staple diet.	30-40g of dry diet for females with an average weight of 145g (mature males with an average weight of 60g should be fed about half as much as females). Adjust if necessary so that amount provided is consumed in about an hour.	3 times a week.
Wolfensohn & Lloyd (2003)	Pelleted food, or strips of beef heart (with tough tissue removed) with vitamin supplements.	Two or three strips of beef heart per animal. Pelleted food will normally be consumed within 30 minutes.	Two to three times per week.
NASCO (2003)	Frog Brittle	1 gram/frog	Twice a week.
University of Arizona IACUC guidelines (2001)	Frog Brittle, tubiflex worms, earthworms, commercial salmon pelleted diet, beef liver (though not on its own).	'Good' meals.	2 to 3 times a week.
Schultz and Dawson (2003)			Most facilities feed their frogs between 2 and 5 times a week.

Van Zutphen et al (2001)	Slices of meat into which a multi-vitamin and mineral mixture should have been intensively rubbed.	Ad libitum (leave food in tank for at least an hour and a half).	3 times a week.
Council of Europe (2004)	Captive animals should be maintained on their natural foods or on foodstuffs approximating those of their natural diets. Captive aquatic amphibians can successfully be maintained on pieces of fish fillet or scrapings from frozen liver and heart.	To satiation.	Daily feeding is not advisable for adult animals. Once to three times weekly is recommended.

4.8.5 Regurgitation of food

Uneaten particles of food may foul the water and so many establishments remove such debris after feeding has taken place. Care should be taken to leave a sufficient time interval between feeding and cleaning as frogs may regurgitate food if startled, disturbed, or handled shortly afterwards (University of California, 2000).

Some personnel (e.g. Schultz & Dawson, 2003) do not experience such a problem and recommend waiting only two hours before changing the water (for static water systems). However, the scientific literature shows that food regurgitation by frogs after disturbance can be a common occurrence and so waiting a little longer (i.e. between 3 and 5 hours as suggested in University of Arizona (2001)) before cleaning or introducing new water is probably best.

Some establishments (e.g. NASCO) feed their frogs at the end of the day so the frogs consume and digest their food undisturbed during the evening/overnight. This seems to more closely reflect the natural behaviour of this species and is recommended.

- **Frogs and their surrounding water should remain undisturbed for a 3-5 hour period after feeding.**
- **Feeding of frogs should preferably take place towards the end of the day – in this way, frogs can be observed to be eating adequately and then be left undisturbed to digest their meal.**

4.9 Environmental enrichment

4.9.1 What is it?

Environmental enrichment is a means of enhancing the quality of captive animal care by identifying and providing the environmental stimuli necessary for optimal psychological and physiological wellbeing (Shepherdson, 1998).

4.9.2 Why is it important?

The provision of space in terms of suitable cage or tank size alone, is not enough to ensure animal wellbeing, and must not be considered in isolation. What is provided *within* the housing by means of environment complexity is also critically important. Allowing animals the opportunity to exhibit a range of species typical behaviours can reduce the negative effects captivity may have on health and welfare. This is also important for the science - ‘happy animals make good science’ (Poole, 1997). Animals whose wellbeing is compromised (e.g. by being placed in unsuitable social groupings or an inadequate environment) are often physiologically and immunologically abnormal and thus experiments using them may reach unreliable conclusions.

Despite the wide acceptance and promotion of environmental enrichment, it remains an approach largely applied to mammals, with relatively little having been developed for other groups such as reptiles and amphibians (Hayes et al, 1998). In terms of complexity of both environment provided and behaviours shown, the *Xenopus laevis* frog can *seemingly* be catered for fairly simply. This belief is based on the information currently available regarding the natural behaviour of *Xenopus laevis* and their perceived needs. There may be a bias though, as humans are able to better empathise with the more familiar mammals than with amphibians, and may not be aware of, or may underestimate, the actual complexity of amphibian requirements.

Importantly, it has been shown that these frogs prefer an enriched environment to a barren tank, and that previously evaluated enrichment can be provided without any detrimental effect on egg production or quality (Brown & Nixon, 2004).

4.9.3 Hides and refuges

Frogs are a prey species, therefore it is highly important to provide them with a form of shelter to retreat and hide in. Despite this, a study of US establishments by Major & Wassersug (1998) found 54% were providing *no* form of refuge for adult *Xenopus laevis*. There are also many frogs in UK establishments who are not currently provided with a hiding place. One reason is that there may be concerns about the possible release of toxicants into the water or that frogs may injure themselves when these items are in the tanks.

However, the plastic pipes used commercially to safely deliver water to point of use for human drinking can be easily obtained and adapted, and where provided, appear to be frequently utilised by the frogs (Brown & Nixon, 2004).

The piping is extremely tough, durable and long lasting and has a very smooth surface, so the frogs will not harm or scrape themselves on it (one of the prerequisites of a good enrichment device). Ceramic tiles or terra cotta pots are also stated to provide suitable refuges and possible to decrease stress in these animals (Schultz & Dawson, 2003). These items should only be considered once their potential for leaching agents toxic to amphibians into the water has been properly evaluated.

Containers used for storing human food can also be customised and used as improvised ‘caves’ for the animals, as these too will not affect the water quality (Schultz and Dawson, 2003).

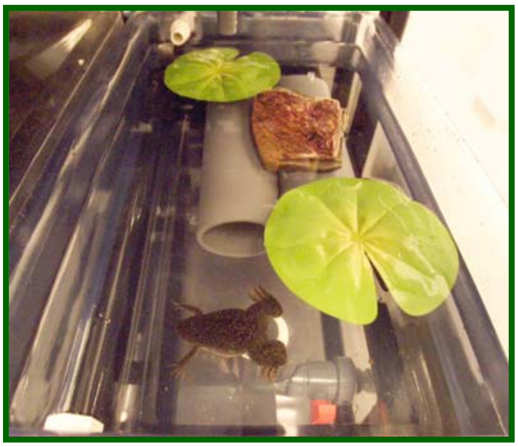


Photo credit:
M.Brown
(MRC)

Providing cover from above and additional hiding for frogs can also be achieved by placing floating objects in the water (Brown & Nixon, 2004). These vary from commercially available plastic 'lily pads' to simply cutting up black bin liners into various floating shapes. Objects placed on the surface of the water however, must still allow frogs to easily access the surface of the water to breathe.

Frogs must be provided with refuges to hide in or under (Kreger, 2002; CCAC, 1984; Schaeffer 1999).

4.9.4 Additional environmental complexity

The addition to the tank of a few medium-to-large stones or rocks will break up the physical monotony of a plain tank (Beck, 1994). If personnel have concerns that sterilisation of such items will not fully prevent leaching of minerals or kill any bacteria present, then perhaps plastic moulds of stones or rocks can be created from those materials previously assessed and classified as safe. If placed an inch or so away from the walls of the tank, the frogs can squeeze into the spaces between the objects and the tank floor (Kaplan, 1993). Enrichment structures should have smooth surfaces and rounded edges to reduce the risk of injury to the animals (Council of Europe, 2004).

A tank floor substrate of small stones should not be used as these can be accidentally ingested (Beck, 1994) and may also be impractical for regular cleaning. Furthermore, it is clear from ‘choice tests’ that frogs do *not* prefer gravel flooring (Brown & Nixon, 2004).

No rest area at the water’s surface is required in the tank as *Xenopus* frogs are totally aquatic (University of California, 2000).

Further research is required into the relative preferences of *Xenopus laevis* frogs for different objects in the surrounding environment

4.10 Assessment of health and disease prevention

An animal’s welfare can be compromised by poor health. This section will therefore deal with the identification of discomfort or clinical signs of disease and the subsequent treatment of common diseases in *Xenopus laevis* frogs.

Before frogs are acquired a veterinarian with a knowledge of *Xenopus* health and welfare should be consulted to agree issues such as health status of the incoming animals and those already at the establishment, how animals will be monitored, and the potential use of preventive medicine and treatment strategies. A vet should again be consulted before any treatments are given to the animals and animal carers should be made aware of any restrictions on the use of medicines.

Amphibians are prone to many diseases that are often difficult to treat and control (LASA, 2001). It is therefore far easier to prevent, than treat disease in these frogs (DeNardo, 1995). *Xenopus* may carry a range of pathogens without the development of disease, until there is physiological upset caused by additional environmental stressors (Tinsley, 1996). This can occur as a result of stress through overcrowding, improper handling, over-use, or poor water quality (Xenopus Express, 2003). Providing the most suitable housing conditions and care is vital for maintaining a healthy frog population (Sive et al, 2000).

4.10.1 Diagnosis of ill health

Diagnosis of disease and assessment of wellbeing can be difficult as *Xenopus* have little ability to express their state of welfare (The Berlin Workshop, 1994). *Xenopus laevis* is a potential ‘prey’ species and therefore may well have evolved adaptively to hide overt signs of distress, injury or susceptibility. This means that disease may become established, and pathogenesis may develop to an extreme degree before the frog exhibits obvious ill-effects (Tinsley 1996).

It is also harder for human carers to be able to recognise and assess signs of suffering the further away from humans (and mammals in general) the animals are on the phylogenetic scale. This is particularly so in those species which are not very animated or vocal during distress or sickness. Lack of a comprehensive understanding of amphibian behaviour, physiology and medicine, or inability to recognise health problems, may mean users obtain new animals rather than attempting to investigate or treat medical problems in existing animals (Crawshaw, 1991). Significant reductions in the numbers of animals used can be achieved when animals are kept healthy and when early signs of disease are recognised and appropriate veterinary care is provided.

Frogs should be observed daily for indicators of poor health.

4.10.2 What does a healthy frog look like?

It is very important to know how a healthy individual animal looks and behaves in order to be able to make a comparative judgement. Such animals should be:

- placid, with moderately slimy skin and a nice pear shape (Sive et al, 2000)

4.10.3 Some key signs of ill health in *Xenopus laevis*

Key signs to look out for include**:**

- dull skin with subcutaneous haemorrhaging (particularly petechial or ‘pinpoint’ haemorrhages on the ventral surfaces of the legs)
- failure to feed properly or weight loss
- erosion or tremors at its extremities (e.g. legs and feet)
- open cuts, lesions or abrasions of the skin or ulcerations
- excessive coelomic cavity distension / swelling ('bloating')
- changes in activity level e.g. lethargy
- postural changes
- skin discoloration or flaking
- diminished avoidance response and righting reflexes

Jumpy frogs, frogs with dry or excessively slimy skin, bloated frogs, and frogs that look grey and thin or reddish are not healthy and should not be used in experimental procedures as this would lead to further deterioration of the animals’ condition. Additionally, eggs collected at this time, for example, would be generally unsuitable for experimental purposes (Sive et al, 2000).

4.10.4 Common diseases

Sive et al (2000) and Wright & Whitaker (2001) observe that the two most common diseases in *Xenopus* are bacterial septicemia and nematode infection. Symptoms and some suggestions in the literature for treatment of these conditions are detailed in the following table:

Red leg syndrome (bacterial septicemia)

Background

- This is one of the most common diseases for frogs (and amphibians in general) in captivity
- It is not a single disease entity but a septicaemia caused by a number of gram-negative or gram-positive bacteria - the most commonly associated bacteria being *Aeromonas hydrophila* (Mahony, 1994).
- The bacteria forms a normal part of the fauna encountered by *Xenopus* in their environment and only under immunosuppressive conditions does it become pathogenic (Kelly, n.d.).
- Unsanitary conditions, prolonged exposure to cold conditions, overcrowding and other factors that lead to stress can increase the incidence of red leg syndrome (Xenopus Zoo, 2003).
- Red leg syndrome can spread through a whole colony and is only treatable if caught early (Xenopus Express, 2003).
- Sive et al (2000) recommends that infected individuals must be isolated immediately.

Clinical signs

- Include cutaneous haemorrage (especially in the legs and feet), subcutaneous oedema, and trembling (initially in the limbs). Frogs may also appear listless, bloated and may lose their appetite (Xenopus Zoo, 2003).
- In some instances death can occur without any visible evidence of illness (Mahony, 1994).

Proposals for prevention

- University of Arizona guidelines (2001) suggest placing frogs in a 0.6% calcium hypochlorite solution (or a 0.06% sodium chloride solution) when first acquired will help reduce the growth of the gram-negative bacteria *Aeromonas hydrophilia* and thus the occurrence of red leg syndrome.
- Care should be taken not to damage the skin of these animals as this might lead to a more severe systematic infection.

Recommendations for treatment (under appropriate veterinary instruction)

- Xenopus laevis frog colony care (2003) suggest that the salt solution of the tank water can be increased to 100mM, and 100microgrammes per ml of oxytetracycline should be added to the water for a week. They further state that antibiotics may also be used e.g. tetracycline oral: 1mg/5g body weight for 5 days.
  Note: Antibiotics should only be used after evaluation of bacterial culture and sensitivity. Appropriate drug selection, dosage and treatment length will minimise the development of antibiotic resistance by bacteria.
- Alternatively, for milder cases, LASA (2001) recommend frogs are placed in a bath of 1g/L potassium permanganate. The infected animal should be dipped for 30sec on alternate days, making sure that the water in the treatment tank is the same temperature as the stock tank.
- LASA (2001) also observe that Malachite Green can be effectively used as a 15 second dip at 0.2g/L on 3 successive days [11].
- A salt bath is also a good treatment for many of the problems that are found in amphibians. Treatment may either be a 10- minute dip in a solution of 25g salt/L or a bath for up to 3-5 days in a solution of 5-10g salt/L (LASA, 2001).

[11] Although Malachite Green has been recommended as an effective treatment for bacterial infection in *Xenopus laevis* frogs, its use for treating similar conditions in commercially produced fish has been banned due to the substance's carcinogenic properties. Laboratory

Nematode infection (e.g. *Pseudocapillaroides xenopodis*)

Background

- In many cases, the nematode (roundworm) parasite involved is non-pathogenic though its prevalence can be life threatening. The infection is highly contagious.
- An opportunistic infection may have arisen as a result of damage to the protective mucous of the skin and the infection can be maintained and increased by the ingestion of sloughed epidermis containing the parasite's eggs (Cunningham et al, 1996).
- This pathogen has a direct life cycle, therefore larval nematodes can penetrate into intact skin (Wright & Whitaker, 2001).
- In frogs with low levels of infection, the first clinical signs may not appear until 12 to 18 months after the initial infection (Wade, 1981, in Cunningham et al, 1996).

Clinical signs

- Include grey, rough, flaky skin usually starting on the thighs, excessive shedding of skin and rapid weight loss (Xenopus Express, 2003).
- Thread-like, generally whitish bodies around injection sites (Sive et al, 2000).

Proposals for prevention

- Continuous drip-through water systems (whether re-circulating or not) have the advantage (over standing water tanks) of removing much of the sloughed skin and infective larvae before it can be ingested by the frogs. As sloughed skin may contain pathogens, reduced ingestion of skin could lead to reduced potential for infection.

Recommendations for treatment (under appropriate veterinary instruction)

- Treatment is most effective if administered immediately symptoms are recognised.
- Xenopus laevis frog colony care (2003) recommend the use of ivermectin at a dose of 0.2 micrograms per gram of body weight.
- Sive et al (2000) states that MarOxy solution can be used to good effect.
- Thiabendazole (at 0.1g/L water) has also been suggested as a possible treatment by Xenopus laevis frog colony care (2003) (note: this substance has been observed to be highly irritating to the frog's skin when administered at an improper dose).

4.10.5 Injury to skin

In the (hopefully) rare event of injury, either caused by other frogs or during handling or procedures, healing (of skin abrasions for instance) can be aided by bathing the individual in a mild saline solution (NASCO, 2003).

4.10.6 Responses to acute noxious stimuli

The behaviour of the frog may indicate that the animal is finding a procedure noxious or painful. Green (2003) observes that behavioural responses of *Xenopus laevis* frogs to aversive stimuli include:

- startle reactions (sudden movements);
- activity to physically avoid the stimuli (including 'escape' behaviours); wiping or rubbing of the irritated area.

4.10.7 Disturbance caused by noise

The potential of laboratory equipment and human activity to disturb or cause stress to these animals must be considered. The hearing range of these frogs is 200-3900 Hz, with optima at 600 Hz and 1400-1800 Hz (Elepfandt 1996(b))

---

personnel may therefore wish to reconsider the acceptability of this substance, and if still used, then give special regard to how the water treated with this substance is disposed of.

and they are sensitive to surrounding vibrations (through the lateral line system). The range of hearing for healthy young humans is 40 to 20,000 hertz (Blakemore & Jennett 2001). Although hearing ability does decrease with age, personnel should be sensitive to most of the range of environmental noises/vibrations that these frogs can perceive. They should therefore be in a position to act to minimise them, for example, by limiting the disturbance caused by air conditioning systems, industrial vacuum cleaners or radios.

- **A good understanding of frog health, including diseases, symptoms and treatments, is necessary to minimise suffering or death.**
- **Frogs should be regularly monitored for signs of ill health.**
- **Personnel should be well informed regarding any pain or distress frogs may experience as a result of experimental procedures. They should be aware of the responses frogs are likely to show, what the humane endpoints of the project are, and what to do when they are reached.**
- **Many diseases that *Xenopus* are susceptible to are caused or exacerbated by stress. The importance of providing the most optimal environmental and care conditions for the frogs is therefore paramount.**

As this section has only dealt with the small number of the most common diseases and infections found in *Xenopus* frogs, those with a more specific interest or expertise may wish to consult the following resources for more detailed information:

- **The Biology of *Xenopus* - Tinsley, R.C. & Kobel, H.R. (1996)**
  Oxford University Press, Oxford (UK)
  In particular:
  Pages 233-261 - Parasites of Xenopus

- **Laboratory Animal Medicine (2002)** (Second edition)
  American College of Laboratory Animal Medicine Series
  In particular:
  Chapter 17 - Biology and Diseases of Amphibians

- **Amphibian Medicine and Captive Husbandry - Wright, K. & Whitaker, B.R. (2001)**
  Krieger Publishing Company, Florida (USA)

5 Scientific procedures

There are a number of procedures commonly carried out on *Xenopus laevis* frogs. These include egg harvesting, oocyte collection and the induction of anaesthesia. All of these have the potential to cause pain, suffering or distress and the opportunities for refinement therefore need to be explored. This section therefore sets out background information on the purpose of some common procedures, together with recommendations for refinements that will help minimise and ideally avoid suffering.

In this context, it is important to note that amphibian behaviour in relation to pain perception is difficult to recognise and largely undefined. For this reason, specific clinical signs or objective means of pain assessment in *Xenopus laevis* frogs have not yet been determined (Green, 2003). However, the current understanding of pain in non-mammalian vertebrates, based on recent anatomic, physiological, and biochemical studies, suggests pain perception in amphibians is likely to be analogous to that in mammals (CCAC, 2004; Green, 2003). In particular, amphibians have nociceptive mechanisms analogous to those found in mammals, so it is justifiable to give amphibians the benefit of the doubt and assume that they are aware of and can suffer as a result of pain. Invasive, potentially painful procedures (such as surgical collection of oocytes) should therefore be subject to appropriate ethical review, and accompanied by both anaesthesia and post-operative care including appropriate analgesia (Council of Europe, 2003; CCAC, 2004).

5.1 Egg harvesting

Researchers, and particularly those in the fields of developmental biology, embryology or genetics, generate a high demand for a constant supply of *Xenopus* eggs. There are a number of techniques that may commonly be associated with the procurement of these eggs. The main ones are:

- natural mating;
- induction of ovulation;
- the ‘squeezing’ of females.

Nowadays, only a small proportion of the fertilised eggs used in experimental studies are obtained through natural mating using a live male.

5.1.1 Natural mating

Natural mating (i.e. placing a female with a live male frog to fertilise the eggs) is a way of obtaining many different stages of embryos at once, with minimal stress to the frogs (Sive et al, 2000).

When a female is receptive, she shows little preference for individuals and instead will be attracted towards any advertising male (Tobias et al, 1998). This is because under natural conditions competition between males for access to the relatively scarce receptive females results in the establishment of a hierarchy where subordinates rarely advertise vocally (Tobias et al, 2004).

Amplexus occurs where the male and female frogs clasp together. This can last for 12 hours or longer (Sive et al, 2000) with the male releasing sperm as the female lays her eggs (which are laid singly).

Oogenesis (the formation, development and maturation of eggs) is a continuous process under favourable environmental conditions and, given suitable temperatures, ovary production is determined primarily by food supply (Tinsley and Kobel, 1996). This means that wild individuals may have well-developed ovaries at almost any time of the year if there is an abundance of prey.

5.1.2 Induction of ovulation and mating behaviour

Leaving reproduction to occur naturally in the laboratory affects both when, and how many, eggs are produced. However, the hormone human chorionic gonadotrophin (hCG) can be used to overcome this, enabling laboratories to have access to a near constant supply of eggs all year.

Female *Xenopus laevis* frogs can be induced to ovulate by injecting them with hCG (usually at a level of 500 I.U). Ovulation generally occurs within 36 hours following injection (NASCO, 2003).

Males too can be stimulated to perform mating behaviour by injecting 50 units of hCG into the dorsal sac, a few days before being placed with a female. Readiness for mating is indicated in the males by the production of advertisement calls (in the form of a rapid series of 'clicks') and the appearance of a dark pigment on the front legs.

5.1.3 Squeezing/milking the eggs from the female frog

Applying *gentle* pressure with the hand to the ventral and lateral sides of the belly mimics male behaviour during amplexus and stimulates hormonally primed females to expel eggs. Egg-laying should begin within a minute of beginning to massage the belly. The frog may either not move, or she may push the eggs out in a couple of vigorous bursts (Sive et al, 2000).

The back of the frog should *not* be rubbed. Some eggs may be obtained in this way but as there is little subcutaneous fat on its back, the animal is very likely to bruise and may succumb to a secondary bacterial infection as a result of disturbance to the epidermis (Sive et al, 2000). After induction of ovulation, the cloaca becomes red and swollen and is probably rather sensitive, so personnel should avoid touching it during subsequent handling.

‘Squeezing’ or ‘milking’ of females speeds up the egg laying process, which normally takes several hours. Obtaining a batch of eggs in this way also means that they can all be fertilised at approximately the same time by mixing them with the ground up testes of a previously euthanased male frog. Simultaneous fertilisation is often required when studying egg development.

However, the above technique has negative impacts on the frogs because it requires greater and more prolonged handling of the females, as well as euthanasing the male frogs to obtain their testes (although the ground testicles of one male can serve multiple females). Furthermore, it has been stated that **the stress of the induction of ovulation and subsequent egg collection is the most common cause of disease outbreaks in frogs** (Sive et al, 2000). To minimise the risk of disease, these authors recommend that females should be kept in a bath of 20mM NaCl, supplemented with 5µg/ml gentamycin during egg laying and for 12 hours afterwards. It is then imperative to keep frogs in isolation in clean water for 12-24 hours after egg collection and to monitor them for signs of illness.

- **There is less potential for frogs to suffer when eggs are obtained as a result of natural ovulation, egg laying and mating. The requirement for 'inducing' these behaviours must be justified on scientific grounds.**
- **It is imperative that only those individuals experienced and trained in the 'squeezing' procedure attempt this process. Harm and bruising can be caused to the frog through incorrect or too heavily applied pressure. This is in addition to any stress caused through possible unsuitable handling techniques.**

5.1.4 Egg quality

Obtaining good quality eggs from the animals is important, not only for helping realise research objectives, but also to keep the number of animals used to a minimum.

The number of eggs laid is less important than the quality of eggs; for example, a hundred good-quality eggs is far preferable to thousands of poor quality eggs (Sive et al, 2000). Since the majority of *Xenopus* eggs produced at research establishments are for experimentation not reproduction, 'quality' is stated to refer to the ability of the eggs to remain viable after experimental manipulation, such as centrifugation and microinjection (DeNardo, 1995). For most of the academic areas in which these eggs are utilised (e.g. developmental biology, anatomy, genetics studies) it is particularly important that a high percentage (i.e. > 80%) of the eggs are fertilised successfully, that the eggs undergo a clean and even first cleavage, and that they remain normally developed at gastrulation.

DeNardo (1995) observes that 'it is currently unknown what factors influence the quality of eggs; however, numerous husbandry parameters are often manipulated in an attempt to improve egg quality. Such factors include diet, lighting, water salinity, water flow, frequency of cleaning, tank size, type of hormonal stimulation, frequency of egg collection, and age of females'.

Some establishments obtain better quality eggs than others. Since egg quality can have a direct effect on the standard of scientific research produced, it is important that the sharing and publication of information regarding factors affecting egg quality is improved.

5.1.5 Frequency of egg collection

Many laboratories induce or naturally mate their females three or four times a year as might happen in the wild, though others induce their females to produce eggs more frequently. Constant breeding and egg production places physiological pressure on females mated regularly (e.g. every four to six weeks) and so this might affect the length of time females may be considered to be productive.

Rest periods of two to three (Sive et al, 2000) or four months (Xenopus frog colony care, 2003) are suggested as being required between ovulations. Possibly, frogs used less intensively (e.g. only every three months) may continue to provide eggs of good quality for a far longer time. Dawson et al (1993), however, note that the period between laying must not be too great, as frogs who are not bred from for four to six months, tend to deposit a higher percentage of necrotic eggs and the general embryo quality tends to be poorer.

5.1.6 Age of females

It is possible for animals to reach sexual maturity within one year of hatching (Measey, 2004). However, initial batches of eggs from such young animals may be of poor quality and therefore unusable (anon., *pers.comm*). For reproduction purposes, animals aged between 2 and 5 years are commonly used (Van Zutphen, 2001; LASA, 2001).

It has been suggested that frogs may only be useful for egg production purposes for one or two years (e.g. Dawson et al, 1993) though sometimes longer. Some 'older' females may have atretic (degenerating) eggs and cannot be used for breeding (Kelly n.d.). Nevertheless, a number of laboratories successfully keep and use their female *Xenopus* over a period of many years (e.g. 6 - 8 years, *anon, pers.comm*). Xenopus frog colony care (2003) states that frogs can be induced to lay repeatedly for several years providing they are healthy and have suitable rest periods.

- **It is better to use a larger number of animals to produce a given number and quality of eggs than to use fewer animals more intensively. Minimising levels of individual animal suffering is more important than simply reducing the numbers of animals used.**
- **Good environmental record keeping is essential for *Xenopus* colonies, since there are many factors that influence egg quality and their mechanisms and interactions are poorly understood.**

- **It is unlikely good quality eggs will be obtained from female *Xenopus* frogs before they are at least 18 months to 24 months of age.**
- **As long as the animal remains healthy and egg quality is high, frogs can continue to be used. There is no upper age limit.**
- **A minimum interval of 8 – 10 weeks[12] is recommended between episodes of induced egg laying in these frogs.**

5.2 Oocyte collection

One of the major uses of *Xenopus laevis* frogs in biomedical research is to obtain oocytes (immature eggs not yet capable of being fertilised) for research within molecular biology, biophysics, ontogenesis (Elsner et al, 2000) and in mRNA expression applications.

5.2.1 Surgery to obtain oocytes

The process of oocyte collection is more invasive than the process of egg collection, as it requires surgical removal of ovarian tissue under anaesthesia and the collection of developing stage I to VI oocytes from within the body. Although all stages of eggs are present at any given time, investigators typically require healthy looking mature stage VI oocytes, which generally account for around 30% of a clutch (Green, 2002). Oocyte removal for a scientific purpose by laparotomy surgery (undertaken following injection 48 hours prior with low level hCG) with recovery can only be done in the UK under Home Office licence. As a fully mature female *Xenopus* can contain as many as 30,000 oocytes (LASA, 2001) which researchers may want to take over a period of time, animals may be subjected to repeated episodes of surgery during their lifetime (Green, 2003).

There is a consensus that multiple episodes of surgery with survival on animals should be discouraged, but exceptions are often granted for oocyte removal from *Xenopus* (e.g. University of Arizona, 2001). The rationale for this is that surgery is usually rapid (normally around 10 minutes or less), the oocytes are located in the immediate area of the incision and thus the other visceral organs should be undisturbed, and because the animals are observed to apparently quickly return to normal feeding and activity. However, in the absence of effective behavioural indicators of pain in *Xenopus,* it is important not to assume that there is no surgical trauma. When deciding whether to undertake multiple surgeries on a single animal or subject a larger number of animals to fewer occasions of invasive surgery, the total number of animals used must be considered relative to the pain or distress experienced by an

---

[12] This recommendation (and also the interval recommended between episodes of surgery for oocyte collection - see next page) is based on the minimum periods suggested in the relevant literature and on discussions with personnel using and caring for frogs on how the impact of these procedures can be reduced. They are designed to be the minimum advisable in order to allow the individual animal sufficient time for recovery. The period should be extended if possible.

individual animal (NIH, 2001). **The welfare, experiences and potential suffering of each individual frog must be considered.**

The number of times a frog is subjected to surgery and recovery for oocyte collection varies among studies and between research establishments around the world. Frogs sometimes have oocytes collected up to *six* times (Elsner et al, 2000; University of California, 2003; University of Arizona, 2001[13], NIH, 2001). If repeat surgery is undertaken, the use of individual frogs is generally rotated so that the interval between episodes of surgery on each animal is maximised (NIH, 2001).

In the UK, the wellbeing of frogs is better protected through greater restrictions on the frequency of surgery. Surgery is usually restricted to two occasions per frog, with the second being undertaken under terminal anaesthesia. Specific scientific justification must be presented for allowing a frog to undergo and recover from laparotomy surgery under general anaesthesia on more than one occasion.

Many establishments seek to avoid causing suffering to the animals through surgical trauma, and undertake oocyte collection only once per animal under terminal anaesthesia.

- **If oocytes are to be taken from a frog on more than one occasion, it is recommended that the second collection takes place under terminal anaesthesia.**
- **Where specific scientific justification has been presented for collecting oocytes (with recovery from general anaesthesia) from the same frog on more than two occasions, a minimum interval of 12 weeks is recommended between episodes of surgery.**

i) Post-operative care

The University of Arizona (2001) suggests that post-operative care after surgery should, as a minimum, include singly housing animals and monitoring for 24-48 hours. During this time the frogs should be closely and regularly observed, but if possible, be left as undisturbed as possible. After this time, it is stated most *Xenopus* swim well enough to be returned to regular housing (Green, 2003). Skin sutures and wound clips, if non-absorbable, must be removed two to three weeks after surgery (Green, 2003).

- **As much serious consideration must be applied to surgery on *Xenopus* as to any other species – the justification and necessity for surgical procedures should be critically evaluated and the provision of good post-operative care is essential.**

[13] The University of Arizona (2001) sets a limit of six surgeries per animal (three on alternating sides with the sixth surgery being a terminal procedure) and stipulates that animals should be given no less than one month to recover between surgeries.

5.2.2 Oocyte quality

Since procedures that can cause stress and discomfort are required to obtain oocytes, it is essential to maximise oocyte quality so that the frogs do not suffer needlessly. Some laboratories undergo periods where oocytes produced are consistently of poor quality and the reason is often hard to determine.

Poor quality oocytes may be characterised by any of the following (Waterman et al, 2001):

- discoloured or speckled yolks;
- bursting oocytes (white, bloated eggs);
- stringy coats and attachment to other oocytes;
- small size;
- dirty jelly coats with debris adhering to the surface.

Possible causes can be parasitic disease in the colony, water quality[14], water temperature or contamination with bacteria such as *Pseudomonas fluorescens* via contact of the ovary with the frogs' skin during surgical removal.

Elsner et al (2000) emphasise the importance of ensuring pre-operative skin antisepsis, which reduces the development of marbled spots and premature collapse of electric resting potential in oocytes. Pre-operative skin antisepsis should be established using *very dilute* povidone-iodine solution, sterile drape and instruments, and careful consideration of the antibiotic supplement in the Barth medium.

5.3 <u>Blood collection</u>

Blood collection in amphibians is often difficult because of their low body weight and poor accessibility of vessels (University of Arizona, 2001). The clipping of the toe web of frogs with a sharp fingernail clipper to collect a few drops to 0.5ml of blood is considered an acceptable method by some establishments (e.g. University of Arizona, 2001). However, it is unclear how commonly this procedure takes place, or to what extent it causes the frog pain and distress. As a precaution, local anaesthetic should be used for this procedure (Wolfensohn & Lloyd, 2003).

Cardiac puncture under general anaesthesia is usually employed where a greater volume of blood needs to be obtained. This should only be used as a terminal method. Death after exsanguination should be ensured by the administration of an overdose of an anaesthetic or other physical method before the animal recovers.

[14] see specifically Godfrey & Sanders (2004) study on the effect of general water hardness on oocyte and embryo firmness and successful development

5.4 Injections

Injections are normally given to frogs via the dorsal lymph sac because substances administered via this route should be rapidly absorbed into the blood (University of Arizona, 2001). This is also the common site for injection of hormones to stimulate breeding in *Xenopus*.

Intramuscular injections are generally administered into the thigh muscles, while intraperitoneal injections may be given while the frog is placed on its back in the hand with the head directed downward (Wolfensohn & Lloyd, 2003). Inserting the needle into the groin area lessens the likelihood of puncturing any internal organs (University of Arizona, 2001).

Further information on refinement of the administration of substances, including by injection, can be found in Morton et al (2001).

5.5 Analgesia and anaesthesia

5.5.1 Analgesia

Little research has been undertaken into the efficacy of analgesic agents in amphibians. Extrapolating doses of analgesic agents from studies using other species can be dangerous and there are concerns over potential adverse side effects that could have a negative impact on the animals' health or welfare or that could affect oocyte or egg production and quality (Green, 2001; Wright & Whitaker, 2001).

There are few recommendations in the literature for appropriate analgesia, and where suggestions are made, little detail is provided on how successfully the agents have been used and how their efficacy and potential side-effects have been measured. For example, it has been suggested that xylazine is a potent and appropriate analgesic in 'frogs', being very effective for up to 24 hours duration when delivered at a dose of 10 mg/kg (University of Minnesota, 2003). However, there is no assurance given that it is suitable for all species. Elsewhere, CCAC (2004) guidelines suggest that 2% lidocaine applied to the surgical site might be an effective analgesic for some procedures. Given the lack of additional guidance provided, the potential use of either of these substances for providing pain relief in *Xenopus laevis* frogs requires further analysis.

There is no evidence that whole body cooling of frogs reduces pain or is clinically efficacious (AVMA, 2001). Hypothermia should not be used as a method of analgesia.

- **It is essential to recognise that amphibians need, and should have, appropriate perioperative pain relief.**
- **Relevant animal care and use committees should regularly review and discuss the possibility for trials of potentially useful agents. Trials should take note of changes in behaviour and egg quality in response to the use of different analgesic agents.**

More research is necessary to determine appropriate analgesic agents and doses for *Xenopus* frogs, and to disseminate relevant information on suitable protocols.

5.5.2 Anaesthesia

Amphibian anatomy and physiology gives rise to some special considerations in relation to anaesthetising *Xenopus* frogs. Some key points are set out below.

- **Considerations**

- *Xenopus laevis* frogs respire through both the lungs and the skin. They usually rely on lung respiration for the majority of normal gaseous exchange, but this can be affected during periods of anaesthesia. It is therefore essential to ensure that cutaneous respiration (i.e. through the skin) is not also interrupted. The animal should be kept moist whilst under anaesthesia (Johnson, 1991) by placing wet tissue in contact with the skin (Council of Europe, 2004).

- Fasting of frogs prior to anaesthesia is recommended as frogs who have been disturbed may regurgitate food, which fouls the water and increases the chance of inhalation of foreign material while the animal is being anaesthetised (Smith and Stump, 2000).

- Since amphibians are ectotherms, the environmental temperature during anaesthesia will affect the frog's metabolism. This, in turn, influences the rate of absorption and excretion of the anaesthetic agent and its subsequent effectiveness.

- Successful anaesthesia in *Xenopus laevis* is judged by loss of righting reflexes and respiratory effort with subsequent slowing and halting of gular (throat) movements (LASA, 2001; University of Arizona, 2001).

- The length of time required for recovery from anaesthesia depends on the anaesthetic agent, frog's life stage, environmental temperature and depth of anaesthesia. Frogs should be propped on wet tissue or foam rubber to prevent drowning during recovery (LASA 2001). They should not be returned to their tank until completely recovered.

Comments regarding suggested anaesthetic agents

MS-222® [15](tricaine methane sulphonate) is the most common and convenient anaesthetic used for *Xenopus laevis* [16] (Halliday, 1999; CCAC, 2004). It can

---

[15] Argent Laboratory Redmond, WA

[16] There are some concerns that MS 222 affects *Xenopus* oocytes by inducing maturation, however, according to the current literature these concerns have not been properly evaluated or verified and so MS 222 use should not be avoided (Martin 1995).

be administered by injection (50-150mg/kg) or immersion (300-500 mg/L water) (Wolfensohn & Lloyd (2003). In the latter case, induction should take around 5 minutes, though others suggest it could up to 20-30 minutes (LASA 2001). MS-222 is acidic when dissolved in water, and so it should be buffered with 50ml of 0.5 M $NaHCO_3$/L (sodium bicarbonate) to a pH of between 7 and 8 (O'Rourke 2002).

A frog should recover from MS-222 within 15 to 30 minutes after being rinsed in clean water (Wolfensohn & Lloyd 2003), though some individuals may take longer.

Most establishments are content with the efficacy of immersion in MS-222. However, the induction of anaesthesia in frogs may sometimes take up to half an hour at recommended concentrations (Smith & Stump 2000, LASA 2001), so attempts have been made to develop alternative methods.

Isoflurane - the topical application of liquid isoflurane has been suggested by Smith & Stump (2000) as a viable alternative method. A single application of isoflurane (dose range 0.03 - 0.06 mL/g) applied to an absorptive pad with impermeable backing so that the pad is saturated, then the saturated side is placed in contact with the skin. This provides a working anaesthetic time averaging 6 minutes that Smith and Stump deem sufficient for short procedures such as obtaining oocytes.

Bubbling isoflurane into the water has also been attempted, but the frogs showed repeated and vigorous escape behaviours (interpreted as a sign of distress) and did not reach a suitable depth of anaesthesia (Smith & Stump 2000). Administering isoflurane in this way should therefore *not* be attempted on welfare grounds. Intracoelomic or subcutaneous injection of isoflurane can be fatal to *Xenopus* or can lead to prolonged recovery times of up to 15 hours. Given these results, these methods too should *not* be utilised, whilst the humaneness of applying isoflurane via an absorptive pad should be further scrutinised.

Ketamine or **Telazol**® (tiletamine/zolazepam) should be restricted to minor procedures (e.g. radiography), as animals sedated with these drugs may remain sensitive to pain, even at high doses (Wolfensohn & Lloyd 2003). University of Arizona (2001) suggested that the surgical use of these drugs should therefore be limited to pre-anaesthetic use only.

Hypothermia is not supported by available published articles as a clinically efficacious method of anaesthesia in this species (Martin 1995). As there are other, more appropriate protocols, hypothermia should never be used for this purpose.

- **At the time of writing, MS-222 appears to be the anaesthetic agent of choice in most circumstances.**
- **Current in-house protocols for *Xenopus* anaesthesia or analgesia may not be the most appropriate or effective. It is very important to ensure that the literature is regularly reviewed so that protocols can be updated to take account of new and better agents, dosing regimes or routes.**

5.6 Euthanasia

5.6.1 The principle

The word euthanasia means "a gentle or good death" and should be regarded as an act of humane killing with the minimum of pain, fear and distress (European Commission 1996). Killing an animal has the potential to cause substantial pain and distress if it is done incompetently or by using an unsuitable method. Whoever carries out euthanasia should therefore be appropriately trained, competent and willing to use the approved method deemed to cause the minimum stress or pain to the animal.

When deciding upon the method of euthanasia, consideration should be given to the following points:

- There must be minimal psychological stress on the animal (and any others who may be present – it may be necessary to remove the animal to be euthanased, although this will need to be balanced against any stress caused by such movement)
- Loss of consciousness must ensue as rapidly as possible
- Death, following loss of consciousness, must be as rapid as possible
- Death must occur without causing pain
- The method must be reliable and the animal must not regain consciousness
- There must be minimal psychological stress to personnel and any observers
- It must be safe for personnel carrying out the procedure
- It must be compatible with the requirements of the experiment
- It must be compatible with any requirement to carry out further studies on the tissues
- It should be simple to carry out, with little room for error

(Note: *there may be conflicts between some of these points in practice - the most appropriate method should be determined on a case by case basis, giving animal welfare top priority*).

5.6.2 Methods for euthanasia of *Xenopus laevis* frogs

Acceptable methods for the euthanasia of amphibians have been listed (e.g. Home Office 1997) as either:

- overdose of an anaesthetic using a route and an anaesthetic agent appropriate for the size and species of animal;
- concussion of the brain by striking the cranium - in amphibians up to 1kg - with destruction of the brain before the return of consciousness

Figure 8: Key points from the literature relating to methods that have been recommended for the euthanasia of *Xenopus laevis* frogs

Injectable agents

- Injection of tricaine methane sulfonate (MS 222) either intravenously or intraperitoneally. Where MS 222 is used as an injectable agent, 200-300mg/kg of a 1% buffered solution has been recommended (e.g. Stanford University 2003).
- Sodium pentobarbital (60-100 mg/kg) may be injected into the dorsal lymph sacs or intracoelomically (Sanders 2004(b), CCAC 2004).
- Pentobarbitone must not be given intramuscularly as it is very irritant to the tissue and this will cause the animal pain (Wolfensohn and Lloyd 2003).

Immersion methods

- Potential stress for an animal (i.e. through handling and injection) can be reduced if drugs are administered by dissolving them in the water in which the amphibians are placed (European Commission 1997) NB. some agents may be irritant, e.g. Isoflurane.
- The most common and preferred method for euthanasia in Xenopus laevis frogs is immersion in an overdose of MS 222. This method appears to be one of the least stressful to the animals involved (Wright & Whitaker 2001). A concentration of >3g/litre (Schultz 2003) or 0.2% solution should be used for at least three hours (Halliday 2003, LASA 2001). The agent is absorbed into the animal through the skin. To prevent the frog suffering pain or irritation from this agent (which is low in pH), MS 222 should be buffered to near pH 7 with sodium bicarbonate. As the immersion time needed to assure death can range from 20 minutes to three hours, many researchers may use MS222 as an anaesthetic followed by a physical method of euthanasia (Stanford University 2003).
- Benzocaine hydrochloride is water-soluble and has been used directly for euthanasia – a bath with concentration ≥250mg/L (for at least 10 mins) has been suggested (AVMA 2001).
- Death after using a chemical method should be confirmed by a physical method (European Commission 1996, DeNardo 1995).

Concussion methods

- Concussion can be an effective and humane way of stunning amphibians if carried out by a person who is well trained in the method (European Commission 1997)
- In the UK, the Home Office (1997) authorises ‘concussion of the brain by striking the cranium’, with the destruction of the brain before the return of consciousness, but advises caution when using this method. The ‘Code of Practice for the Humane Killing of Animals under Schedule 1 to ASPA 1986' (Home Office 1987) states that the 'brain of amphibians, reptiles and fishes is extremely tolerant to hypoxia. It cannot be assumed therefore that the effects of concussion will be irreversible, or that even subsequent decapitation would necessarily destroy brain function in the time to avoid the return of sentience. In the case of amphibians, reptiles and fishes, if the brain is not destroyed by the initial blow, there must be no delay in destroying the brain by a penetrating probe or by a blow sufficient to cause a severe brain contusion with fracture of the cranial bones’.

5.6.3 Unacceptable methods

Decapitation or double-pithing *if used in isolation* is no longer approved by many ethical committees (e.g. University of Arizona) on welfare grounds. Decapitation, by itself, does not produce immediate unconsciousness within the severed heads of amphibians (Stewart, in UFAW 1989) and should therefore only be used if the amphibian has been made unconscious first by other methods (European Commission 1997). To ensure brain death, subsequent double-pithing (insertion of a sharp needle through the base of the brain and the spinal cord) while the animal is still unconscious is advised.

Hypothermia should never be used either for the purposes of anaesthesia or euthanasia in amphibians (Schaeffer 1999, AVMA 2001). This is because cooling the body does not reduce the animal's ability to feel pain (ANZCCART 2001, AVMA 2001). Cooling followed by freezing of frogs for euthanasia could also cause suffering due to ice crystal formation, both on the skin and within the body (European Commission 1997).

Formaldehyde solution - amphibians, even in larval form, should not be killed either by placing them directly in formaldehyde solution (University of Arizona 2001).

Carbon dioxide - may cause irritation to the skin and induction takes too long

Ether - this is irritant to the mucous membranes, as well as being highly inflammable and potentially dangerous to humans

Chloroform - this is hepatoxic and carcinogenic and is dangerous to humans

Hyperthermia - this is slow and painful, so is inhumane

Exsanguination - may not render amphibians immediately unconscious, which causes suffering and distress

Strangulation - impractical, is slow and is inhumane

Volatile inhalational anaesthetics - these are slow to act and may be irritating to the skin (European Commission 1997)

Ketamine hydrochloride, chlorbutonol, methylpentynol, 2-phenoxyethanol, tertiary amyl alcohol, tribromoethanol, urethane are other agents not considered acceptable (European Commission 1997)

Chloral hydrate - immersion in a container holding 2-3mm of a 3% solution of chloral hydrate has been recommended by ANZCCART (2001). In contrast, the European Commission (1997) suggests this agent should *not* be used *by itself* on amphibians as it lacks analgesic effects, is slow to take effect, large volumes are required and it can cause distressing animal movements.

Where there are reservations regarding a method (whether due to the welfare of the frogs or the health and safety of personnel carrying out the procedure) alternative methods should be sought.

- **Euthanasia - a "good" death - is as important for amphibians as it is for mammals.**
- **It is essential to recognise that (i) brain activity can persist for significant periods of time in amphibians, even though they may show no behavioural responses, and (ii) the animals may be suffering if they are held in aversive chemical agents or have undergone physical damage.**
- **Wherever possible, non-aversive chemical means of euthanasia should be chosen over physical methods. This is because there is less perceived scope for error, it will generally cause less suffering to the animal and less distress to the personnel carrying it out.**
- **Immersion in buffered MS 222 solution (preferably followed by pithing to ensure death) is a straightforward method that should cause minimal stress to both the frog and the person performing the procedure. There should be exceptional justification given for using any other method.**

6 Training of animal care staff and users

The importance of personnel having a good understanding of the animals with whom they interact, cannot be underestimated. Persons not suitably trained are likely to harm animals when performing procedures such as capture, marking or squeezing for harvesting eggs. Inappropriate care or behaviour will have a direct impact on scientific validity, the achievable benefits of the work will be reduced and the potential harms increased (Orlans 2001).

The general principles of training in good laboratory animal care and use apply to amphibians but additional species specific training is essential. Personnel should have a detailed species-specific knowledge of the natural history, behaviour and requirements of the animals in their care. They should be up to date with the latest thinking and publications on best practice with regard to good housing and care and, where appropriate, with advances in the refinement of scientific procedures. A sound understanding of the importance and practical aspects of the prevention, recognition and alleviation of ill health, pain and stress is also essential.

Currently, many training courses for laboratory personnel cover amphibians in general. Although many laboratories may also provide some in-house training, it could be considered beneficial that those working with animals should undertake training specifically tailored to the species in their care and not simply amalgamated into general groups of animals e.g. courses on ‘carnivores’ or ‘amphibians’. As the use of *Xenopus* species is wide ranging and expanding, there is scope for such species- specific training to be useful.

Training for those persons caring for and using animals should be species-specific. More focused courses should be available addressing the behaviour and requirements of *Xenopus laevis.*

7 Conclusions

There is a widely held belief that *Xenopus laevis* frogs are hardy (Wolfensohn & Lloyd, 2003) and robust (The Wellcome Trust, 2003), capable of tolerating a range of environmental conditions and experimental procedures. There has historically been little investigation into the capacity of these animals to suffer and failure to appreciate that they are at times housed in sub-optimal environments.

As knowledge relating to *Xenopus laevis* increases so does the understanding of the physical and social complexity of these animals. The attitudes of contemporary animal technicians and those using the animals in research are shifting away from the traditional view that *Xenopus laevis* 'have no special housing requirements other than a tank of water' (i.e. Davys, 1986). There is a growing acceptance of the necessary requirement to first investigate, evaluate, and then implement the *most suitable* conditions relating even to the finest specifications and features of environmental design including tank material, tank size, degree of wall and floor transparency, lighting regime and spectrum, provision and type of hiding refuges, and group composition. Failure to meet species-specific needs can and will result in stress-induced disease, low growth rate and poor feed efficiency (AWIC, 2001). In *Xenopus* frogs this might subsequently lead to sub-optimal health and a decrease in the quantity and quality of eggs produced, thereby increasing animal usage and reducing welfare. With the expansion in our understanding of this species and of their requirements for good health and welfare, has come a broad desire to try to better define and then provide the most appropriate housing and care conditions.

The knowledge and experience of animal technicians, veterinarians and researchers using *Xenopus laevis* within laboratories is extensive, but this valuable resource is not currently always widely published, transferred or communicated in a co-ordinated way, either within the UK or further afield. There have been some useful attempts to pool knowledge, for example with the British Xenopus Group meetings and LASA workshops in the UK. However, these formal opportunities for information exchange are irregular and there are many people 'outside the loop', using and caring for these frogs, who need and want more information regarding perceived best practices.

Many key factors have been highlighted in this report, which need to be considered by those using and caring for these frogs. Existing guidelines and recommendations have been summarised, with perceived best practice highlighted where possible. Ultimately, further research, discussion and continued study must be encouraged in other areas where science based consensus is currently lacking so that there can be a properly evaluated basis for future recommendations. This report has argued, that from the perspectives of ethics, science and animal welfare, effort must be focused towards discovering and then implementing the *optimal* protocols for housing and care, not simply those that these animals can tolerate.

8 Summary of recommendations

Supply and transport

- Purpose-bred animals should be used in preference to animals taken from the wild - a researcher must be able to provide specific justification for the need to use wild-caught individuals.
- It is preferable for frogs to be bred at the establishment where they will be used - this will avoid any potential risks to health or welfare associated with their transport.
- Transport containers must have an adequate air supply.
- During transport frogs must be kept within acceptable temperature limits and measures should be taken to prevent desiccation.
- Transportation of frogs should be postponed if extreme weather conditions (e.g. extreme cold or heat) could threaten the health or welfare of the animals.
- Journey times should be the minimum practicable.
- Adverse weather, mechanical problems or industrial action can cause flight delays or cancellations and it is important to check and plan for such eventualities.
- Frogs being transported together in the same box should be at a similar stage of development, size and weight, and should not be overcrowded.
- An appropriately trained and competent person must check the health and welfare of frogs on arrival. Animals should then be closely monitored and, to avoid introducing disease, should be quarantined away from any existing resident colonies.
- To help prevent the potential spread of disease or bacteria, when animals are transferred from shipping containers to the new holding tanks, the sphagnum moss and foam cubes with which they were transported should not be transferred with them.
- A minimum quarantine period of 30 days is advised for all new animals entering an establishment with strong consideration given to extending this period to 90 days for those animals obtained directly from the wild.
- Routine husbandry procedures for frogs in quarantine should be undertaken after the resident colony has been attended to and must be carried out with separate dedicated equipment.
- Frogs should be observed daily for signs of ill health.

Housing and care

- Continuous 24-hour lighting must not be used.
- A lighting regime of between 12 and 14 hours light and 12 and 10 hours dark is recommended.
- Where artificial lighting is used, a gradual brightening/dimming period of around 20 - 30 minutes in the morning/evening should be incorporated.
- Any artificial lighting used should simulate outdoor sunlight levels, with consideration given to including the ultraviolet (UV) part of the spectrum.

- Tanks should not be openly exposed to direct sunlight or very bright artificial light and frogs should have access to darkened refuges.
- A water depth of at least 8 inches is recommended for housing adult *Xenopus laevis* frogs. This is based on current evidence and knowledge, and should allow them to perform most normal behaviours.
- Water temperature should not be maintained below 16ºC or above 24ºC, with strong consideration given to providing a relatively controlled temperature at a point between 18 and 22ºC.
- Abrupt significant fluctuations of either room or water temperature should be avoided.
- Maintenance of a stable water pH between 6.5 and 8.5 is advised.
- Water quality and pH level should be routinely monitored. Contingency plans should be made in case of system breakdown or other emergency.
- When devising water quality assurance schemes for frog housing, a water expert knowledgeable of the local water treatment procedures should be consulted.
- Establishments using individual standing water tanks should review their current water changing protocols. Although most establishments change the water in these tanks at least three times a week, and maybe even everyday, the benefit of such frequent water changes in view of the increased disturbance and stress caused to the animals has yet to be comprehensively evaluated.
- If exposing frogs in tanks to constantly running water, the flow rate should be the minimum required, in order to reduce the potential for causing the animals distress.
- Cleaning strategies should be designed to minimise disturbance and distress to the frogs. Disinfectants should be used with extreme caution.
- Opaque containers or those made of darkened plastic are preferable to completely transparent ones – in particular, the bottom of the container should not be permeable to light.
- Containers must prevent escape and should allow space for sufficient volume and depth of water and for enrichment such as refuges to be added.
- Careful consideration should be given to whether identification of individuals is necessary, and is so, then to the method of identification used, particularly where the methods are invasive and likely to cause a degree of trauma.
- Non-invasive methods of identification such as using group cards or photographs of the dorsal markings of the frogs are the methods of choice.
- Group housing of these frogs in relatively stable groups is advisable.
- Overcrowding should be avoided.
- It is important that animals kept together in the same tank are of similar size.
- Handling should be kept to a minimum and every precaution to avoid stressing or injuring the animals should be taken.
- Personnel should consider taking a precautionary approach and wear gloves (non-powdered) whilst removing frogs from the water with soft-mesh non-abrasive nets.

- Unnecessary movements of objects or hands over the tank should be avoided.
- Frogs and their surrounding water should remain undisturbed for a 3-5 hour period after feeding.
- Feeding of frogs should preferably take place towards the end of the day - in this way, frogs can be observed to be eating adequately and then be left undisturbed to digest their meal.
- Frogs must be provided with refuges to hide in or under.
- A good understanding of frog health, including diseases, symptoms and treatments, is necessary to minimise suffering or death.
- Frogs should be regularly monitored for signs of ill health.
- Personnel should be well informed regarding any pain or distress frogs may experience as a result of experimental procedures. They should be aware of the responses frogs are likely to show, what the humane endpoints of the project are, and what to do when they are reached.

Scientific procedures

- There is less potential for frogs to suffer when eggs are obtained as a result of natural ovulation, egg laying and mating. The requirement for 'inducing' these behaviours must be justified on scientific grounds.
- It is imperative that only those individuals experienced and trained in the 'squeezing' procedure attempt this process. Harm and bruising can be caused to the frog through incorrect or too heavily applied pressure. This is in addition to any stress caused through possible unsuitable handling techniques.
- It is better to use a larger number of animals to produce a given number and quality of eggs than to use fewer animals more intensively. Minimising levels of individual animal suffering is more important than simply reducing the numbers of animals used.
- Good environmental record keeping is essential for *Xenopus* colonies, since there are many factors that influence egg quality and their mechanisms and interactions are far from fully understood.
- It is unlikely good quality eggs will be obtained from female *Xenopus laevis* frogs before they are at least 18 to 24 months of age.
- As long as the animal remains healthy and egg quality is high, frogs can continue to be used. There is no upper age limit.
- A minimum interval of 8 – 10 weeks is recommended between episodes of induced egg laying in these frogs.
- If oocytes are to be taken from a frog on more than one occasion, it is recommended that the second collection takes place under terminal anaesthesia.
- Where specific scientific justification has been presented for collecting oocytes (with recovery from general anaesthesia) from the same frog on more than two occasions, a minimum interval of 12 weeks is recommended between episodes of surgery.
- As much serious consideration must be applied to surgery on *Xenopus* as to any other species – the justification and necessity for surgical procedures should be critically evaluated and good post-operative care is essential.

- It is essential to recognise that amphibians need, and should have, appropriate perioperative pain relief.
- Relevant animal care and use committees should regularly review and discuss the possibility for trials of potentially useful analgesic agents. Trials should take note of changes in behaviour and egg quality in response to the use of different agents.
- At the time of writing, MS-222 appears to be the anaesthetic agent of choice in most circumstances.
- Current in-house protocols for *Xenopus* anaesthesia or analgesia may not be the most appropriate of effective. It is very important to ensure that the literature is regularly reviewed so that protocols can be updated to take account of new and better agents, dosing regimes or routes.
- Euthanasia – a ‘good’ death, is as important for amphibians as it is for mammals.
- It is essential to recognise that (i) brain activity can persist for significant periods of time in amphibians, even though they may show no behavioural responses, and (ii) the animals may be suffering if they are held in aversive chemical agents or have undergone physical damage.
- Wherever possible, non-aversive chemical means of euthanasia should be chosen over physical methods. This is because there is less perceived scope for error, it will generally cause less suffering to the animal and less distress to the personnel carrying it out.
- Immersion in buffered MS-222 solution (preferably followed by pithing to ensure death) is a straightforward method that should cause minimal stress to both frog and the person performing the procedure. There should be exceptional justification given for using any other method.

Training of animal care staff and users

- Training for those persons caring for and using animals should be species-specific. More focused courses should be available addressing the behaviour and requirements of *Xenopus laevis.*

9 Future areas for focus

The report has highlighted some of the main areas where further information to determine best practice, or decisions based on what is already known, are required. The most important issues are listed below:

- **Better information on *Xenopus laevis* use.** Government statistics detailing the use of animals in scientific procedures each year should list figures for amphibians at species level so a clearer understanding of the scale of use can be obtained.

- **Health considerations regarding the supply of animals.** Comprehensive data on the comparative health of wild-caught versus purpose-bred *Xenopus laevis* frogs throughout the duration of their use in a laboratory setting should be recorded and critically evaluated. Currently, many of the comparisons made regarding egg production and quality, and health, are anecdotal.

- **Volume of water to be provided.** There is wide variation in the volume of water recommended per frog. More discussion and research is needed to determine best practice, with due regard to the behavioural needs of the animals.

- **Optimal temperature.** More research and discussion is required to determine the optimal temperature for keeping *Xenopus laevis.*

- **Control over microhabitat.** Allowing an individual animal some control over the immediate microhabitat is desirable. Current animal housing design should be reviewed to determine if measures can be taken (such as the inclusion of temperature gradients) within the constraints of the laboratory environment to allow more scope for this.

- **Nocturnal behaviour.** Establishments should review current lighting, feeding and tank cleaning protocols to determine whether there are alternative regimes which may better cater for the nocturnal nature of these animals.

- **Environmental complexity.** Further discussion and research is required into the relative preferences of *Xenopus* frogs for different objects in the surrounding environment.

- **Pain relief.** Greater efforts must be made to determine appropriate (safe and effective) analgesic agents and doses for *Xenopus* frogs and to disseminate information on suitable protocols.

10 References

Alexander, S.S. & Bellerby, C.W. (1938) 'Experimental studies on the sexual cycle of the South African clawed toad (*Xenopus laevis*). I. Journal of Experimental Biology **15** p74-81

Altafaj, A., Kolar, A., de Lapuente, J., Borràs, M. & Visa, J. (2004) 'Refinement in embryo production and maintenance of *Xenopus laevis* in the Parc Cientific de Barcelona' FELASA Symposium: 14-17 June 2004 - Nantes, France

American Veterinary Medical Association (AVMA) (2001) '2000 Report of the AVMA Panel on Euthanasia' Journal of the American Veterinary Medical Association Vol.**218**, No.5, March 1 2001

Animal Welfare Information Center (AWIC) (2001) 'Information resources on amphibians, fish & reptiles used in biomedical research' AWIC Resource Series no.10 - US Department of Agriculture

Australian and New Zealand Council for the Care of Animals in Research and Teaching (ANZCCART) (2001) 'Euthanasia of animals used for scientific purposes' (2nd Edition) Editor- J.S. Reilly; ANZCCART: Adelaide

Beck, A. (1994) 'Information and care sheet for the African Clawed Frog' http://www.geocities.com/ptimlin/xenopus.html *(accessed 02/07/2004*)

Bellerby, C.W. & Hogben, L. (1938) 'Experimental studies on the sexual cycle of the South African clawed toad (*Xenopus laevis*). III. Journal of Experimental Biology **15** p91-100

Blakemore, C. & Jennett, S. (eds) (2001) 'The Oxford Companion to The Body' Oxford University Press, Oxford

British *Xenopus* Group (2001) 'A LASA workshop to Explore Refinements in the Housing, Health and Husbandry of *Xenopus laevis*' http://template.bio.warwick.ac.uk/staff/hwoodland/HRW4.HTM *(accessed 02/07/2004)*

Brown, M. & Nixon, R.M. (2004) 'Enrichment for the captive environment - The *Xenopus laevis*' Animal Technology and Welfare Vol. **3**, No.2, p87-95

Canadian Council for Animal Care - CCAC (1994) 'Guide to the Care and Use of Experimental Animals –Volume II, Chapter 2'

Canadian Council for Animal Care - CCAC (2004) 'Species-specific recommendations - amphibians and reptiles'

Canadian Council for Animal Care – CCAC (2004) 'CCAC Survey of Animal Use – 2001' Resource Vol. **27** (1): Winter 2003-2004

Clark, R.D. (1972) 'The effect of toe-clipping on survival in Fowler's toad (*Bufo woodhousei fowleri*)' Copeia **1972** p185-185

Council of Europe (1968) 'European Convention on the Protection of Animals during International Transport : CETS 65 Council of Europe: Strasbourg

Council of Europe (2003) 'Revision of Appendix A of the Convention ETS123 – Species specific provisions for Amphibians : Background information for the proposals presented by the Group of Experts on Amphibians and Reptiles (Part B) (draft – 7th meeting of the working party)' Council of Europe: Strasbourg

Council of Europe (2004) 'Draft Appendix A – of the European Convention for the Protection of Vertebrate Animals used for experimental and other Scientific Purposes (ETS No.123) (8th meeting of the working party)' Council of Europe: Strasbourg

Crawshaw, G.J. (1991) 'Medicine and Diseases of Amphibians' in The care and use of amphibians, reptiles and fish in research – proceedings from a SCAW/LSUSVM - sponsored conference' (eds) Schaeffer, D.O., Kleinow, K.M. & Krulisch, L. (1991); New Orleans, US

Cunningham, A.A., Sainsbury, A.W. & Cooper, J.E. (1996) 'Diagnosis and treatment of a parasitic dermatitis in a laboratory colony of African clawed frogs (*Xenopus laevis*)' The Veterinary Record **138**, p640-642

Davys, J.S. (1986) 'The breeding of *Xenopus laevis* on a large scale in the laboratory' Animal Technology Vol. **37**, no.3, p217-223

Dawson, D.A, Schultz, T.W. & Shroeder, E.C. (1992) 'Laboratory care and breeding of the African Clawed Frog' Lab Animal **21**(4) p31-36

DeNardo, D. (1995) 'Amphibians as laboratory animals' ILAR Journal **V37 (4)**

Deuchar, E.M. (1975) '*Xenopus*: the South African Clawed Frog' John Wiley and Sons, London

Elepfandt, A. (1996(a)**)** 'Sensory perception and the lateral line system in the clawed frog' p97-120; in 'Biology of *Xenopus*' Eds Tinsley & Kobel: Oxford University Press, Oxford

Elepfandt, A. (1996(b)**)** 'Underwater acoustics and hearing in the clawed frog, *Xenopus*' p177-193; in 'Biology of *Xenopus*' Eds Tinsley & Kobel: Oxford University Press, Oxford

Elepfandt, A. (2004) Oral presentation 'Natural history of *Xenopus laevis'* at Best Practice in *Xenopus* care meeting: 11 February 2004, University of Sheffield, UK. [Joint meeting by the UK Medical Research Council's Centre for Best Practice for Animals in Research (CBPAR) and the Laboratory Animal Science Association (LASA)]

Elsner, H.A, Honck, H.H, Willmann, F, Kreienkamp, H.J, & Iglauer, F. (2000) 'Poor quality of oocytes from *Xenopus laevis* used in laboratory experiments: Prevention by use of antiseptic surgical technique and antibiotic supplementation' Comparative Medicine Vol. **50**, no.2, p206-211

European Commission (1996) 'Recommendations for euthanasia of experimental animals: Part 1' Laboratory Animals **30**(4) p293-316

European Commission (1997) 'Recommendations for euthanasia of experimental animals: Part 2' Laboratory Animals **31**(1) p1-32

European Commission (2005) 'Fourth report from the Commission to the Council and the European Parliament on the statistics on the number of animals used for experimental and other scientific purposes in the member states of the European union' EC, Brussels

Fineberg, E. (2003) 'Electrical pen tattooing as a means of identification for *Xenopus laevis* and *Xenopus tropicalis*' Animal Technology and Welfare - April 2003, p3-8

Gampper, T. (2002) 'The Natural History of the Upland Clawed Frog' http://www.anapsid.org/xenopus.html. *(accessed 02/07/2004)*

Garvey, N. (2000) '*Xenopus laevis* - African Clawed Frog' http://animaldiversity.ummz.umich.edu/accounts/xenopus/x._laevis$narrative.html. *(accessed 02/07/2004)*

Godfrey, E.W. & Sanders, G.E. (2004) 'Effect of water hardness on oocyte quality and embryo development in the African Clawed Frog (*Xenopus laevis*)' Comparative Medicine Vol.**54**, no.2, p140-145

Golay, N. & Durrer, H. (1994) 'Inflammation due to toe clipping in natterjack toads (*Bufo calamita*)' Amphibia-Reptilia, **15** p81-96

Goldin, A.L. (1992) 'Maintenance of *Xenopus laevis* and oocyte injection' Methods in Enzymology **207**, p266-279

Green, S.L., Moorhead, R.C, & Bouley, D.M. (2003) 'Thermal shock in a colony of South African clawed frogs (*Xenopus laevis*)' Veterinary Record **152**, no.11, p336-337

Green, S.L. (2003) 'Are post-operative analgesics needed for South African Clawed Frogs *(Xenopus laevis*) after surgical harvest of oocytes' Comparative Medicine **53** (3) p12-15

Green, S.L. (2002) 'Factors affecting oogenesis in the South African Clawed Frog (*Xenopus laevis*) Comparative Medicine **52** (4) p307-312

Gresens, J. & Cravens, A. (2003) 'Amphibian identity crisis: Telling who's who in an axolotl colony' Contemporary Topics in Laboratory Animal Science Vol. **42** no.4, p117: AALAS, Memphis- US

Gurdon, J.B, Laskey, R.A, & Reeves, O.R. (1975) 'The developmental capacity of nuclei transplanted from keratinized skin cells of adult frogs' Journal of Embryology and Experimental Morphology **34**, p93-112

Gurdon, J.B, (1996) 'Introductory comments –*Xenopus* as a laboratory animal' p3-6; in 'Biology of Xenopus' Eds Tinsley & Kobel: Oxford University Press, Oxford

Halliday, T. (1999) 'Amphibians' p90-102; in 'The UFAW handbook on the Care and Management of Laboratory Animals – Volume 2 – amphibious and aquatic vertebrates and advanced invertebrates' eds Poole & English: Blackwell Science Ltd, Oxford

Hayes, M.P., Jennings, M.R. & Mellen, J.D. (1998) 'Enrichment for amphibians and reptiles' p205-235; in 'Second Nature – Environmental enrichment for captive animals' Eds Shepherdson, Mellen & Hutchins: Smithsonian Institute, Washington

Hilken, G., Dimigen, J. & Iglauer, F. (1995) 'Growth of *Xenopus laevis* under different laboratory rearing conditions' Laboratory Animals **29**, p152-162

Home Office (1997) 'Code of Practice for the Humane Killing of Animals under Schedule 1 to the Animals (Scientific Procedures) Act 1986' The Stationery Office, London

Home Office (2000) 'Guidance on the Operation of the Animals (Scientific Procedures) Act 1986' The Stationery Office, London

Home Office (2004) 'Statistics of Scientific Procedures on Living Animals: Great Britain 2003' The Stationery Office, Norwich

Hoogstraten-Miller, S. & Dunham, D. (1997) 'Practical identification methods for African Clawed Frogs (*Xenopus laevis*)' Lab Animal Vol. **26** (7), p36-38

Huxsoll, D.L (1991) 'Introduction', in The care and use of amphibians, reptiles and fish in research – proceedings from a SCAW/LSUSVM - sponsored conference' (eds) Schaeffer, D.O., Kleinow, K.M. & Krulisch, L. (1991); New Orleans, US

Johnson, J.H. (1991) 'Anaesthesia, analgesia and euthanasia in reptiles and amphibians', in The care and use of amphibians, reptiles and fish in research – proceedings from a SCAW/LSUSVM - sponsored conference' (eds) Schaeffer, D.O., Kleinow, K.M. & Krulisch, L. (1991); New Orleans, US

Kaplan, M.L. (1993) 'An enriched environment for the African Clawed Frog' Lab Animal **22**(5) p25-27

Kelly, D.B. (n.d.) 'The underwater songs of *Xenopus laevis'*
http://www.columbia.edu/cu/biology/faculty/kelley/webessay/ *(accessed 02/07/2004)*

Kreger, M.D (2002) 'Comfortable quarters for amphibians and reptiles in research institutions : p109-114' in 'Comfortable Quarters for Laboratory Animals' Reinhardt,V. & Reinhardt, A. (eds). Animal Welfare Institute, Washington DC, US

Laboratory Animal Science Association (LASA) (2001) 'Good Practice Guidelines – Xenopus Husbandry' (John Cadera) LASA; Staffordshire

Laboratory Animal Science Association (LASA) (2005) 'Guidance on the transport of laboratory animals - report of the Transport Working Group established by the Laboratory Animal Science Association' Laboratory Animals **39**, 1-39

McCarthy, M.A. & Parris, K.M. (2004) 'Clarifying the effect of toe clipping on frogs with Bayesian statistics' Journal of Applied Ecology **41**, 780-786

Mahony, M. (1994) 'Physiological and behavioural assessment of well-being, pain and distress in amphibians used in research' in 'Improving the Well-being of Animals in the Research Environment' – conference proceedings: (Eds) Baker, R.M., Jenkin, G. & Mellor, D.J. - ANZZCART - Australia

Major, N. & Wassersug, R.J. (1998) 'Survey of current techniques in the care and maintenance of the African Clawed Frog (*Xenopuis laevis*)' Contemporary Topics Vol. **37** no.5, p 57-60

Mattison, C. (1998) 'Frogs and toads of the world' Blandford; London

Martin, B.J. (1995) 'Evaluation of hypothermia for anaesthesia in reptiles and amphibians' ILAR Journal Online, Volume 37 (4) 1995: Fish, Amphibians and Reptiles

Mathers, K. (2004) Oral presentation 'A novel method for the identification of *Xenopus laevis'* at Best Practice in *Xenopus* care meeting: 11 February 2004, University of Sheffield, UK. [Joint meeting by the UK Medical Research Council's Centre for Best Practice for Animals in Research (CBPAR) and the Laboratory Animal Science Association (LASA)]

Measey, G.J. (1998) 'Diet of feral *Xenopus laevis* (Daudin) in South Wales, UK' Journal of Zoology (London) **246**, 287-298

Measey, G.J. (2001) 'Growth and ageing of feral *Xenopus laevis* (Daudin) in South Wales, UK' Journal of Zoology (London) **254**, 547-555

Measey J. (2004) 'Xenopus laevis' Global Invasive Species Database http://www.issg.org/database/welcome/ *(accessed 02/07/2004)*

Mellor, D.J., Beausoleil, N.J. & Stafford, K.J. (2004) ' Marking amphibians, reptiles and marine mammals: animal welfare, practicalities and public perceptions in New Zealand' Department of Conservation - Massey University, New Zealand

Morton, D.B., Jennings, M., Buckwell, A., Ewbank, R., Godfrey, C., Holgate, B., Inglis, I., James, R., Page, C., Sharman, I., Verschoyle, R., Westall, L. & Wilson A.B. (2001) 'Refining procedures for the administration of substances - Report of the BVVAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement' Laboratory Animals **35**, p1-41

Mrozek, M., Fischer, R., Trendelenburg, M. & Zillmann, U. (1995) 'Microchip implant system used for animal identification in laboratory rabbits, guineapigs, woodchucks and in amphibians' Laboratory Animals **29**, p339-344

NASCO (2003) 'NASCO On-line catalogs' http://www.enasco.com/prod/Static?page=xenopus *(accessed 20/07/2004)*

National Animal Ethics Advisory Committee (NAEAC) (2003) 'NAEAC Annual Report - 2002' Ministry of Agriculture and Forestry, Wellington, New Zealand.

National Institutes of Health (NIH) - Office of Animal Care and Use (Animal Research Advisory Committee) (2001) 'Policy on oocyte harvesting in *Xenopus'* http://oacu.od.nih.gov/ARAC/oocyte.pdf *(accessed 20/07/2004)*

Nieuwkoop, P.D. & Faber, J. (1994) 'Normal table of *Xenopus laevis* (Daudin)' http://www-cbd.ups-tlse.fr/organismes/nieuwkoop/nieuwkoop.html *(accessed 20/07/2004)*

Olsson, I.A., Nevison, C.M., Patterson-Kane, E.G., Sherwin, C.M., Van de Weerd, H.A. & Wűrbel, H. (2003) 'Understanding behaviour: the relevance of ethological approaches in laboratory animal science' Applied Animal Behaviour Science **81** p245-264

Orlans, F.B. (2001) 'Ethical themes of national regulations governing animal experiments –an international perspective' in 'Applied Ethics in Animal Research - philosophy, regulation and laboratory applications' (eds) Gluck, J.P., DiPasquale, T. & Orlans, F.B.: Purdue University Press - Indiana, US

O'Rourke, D.P. (2002) 'Reptiles and amphibians as laboratory animals' Lab Animal **31** (6) p43-47

Phillips, R.J. (1979) 'The care and induced breeding of *Xenopus laevis'* Journal of the Institute of Animal Technicians Vol. **30**, no 1, p11-16

Poole, T. (1997) 'Happy animals make good science' Laboratory Animals **31**, 116-124

Sanders. G. (2004(a)**)** Oral presentation '*Xenopus* husbandry and care: an American perspective' at Best Practice in *Xenopus* care meeting: 11 February 2004, University of Sheffield, UK. [Joint meeting by the UK Medical Research Council's Centre for Best Practice for Animals in Research (CBPAR) and the Laboratory Animal Science Association (LASA)]

Sanders. G. (2004(b)**)** Oral presentation 'Water quality for *Xenopus*: Assessment and significance' at Best Practice in *Xenopus* care meeting: 11 February 2004, University of Sheffield, UK. [Joint meeting by the UK Medical Research Council's Centre for Best Practice for Animals in Research (CBPAR) and the Laboratory Animal Science Association (LASA)]

Schaeffer, D.O. (1999) 'Breeding, maintenance and use of non-mammalian species – care of laboratory reptiles and amphibians' Proceedings of the International XII ICLAS and VII FELASA Joint meeting 26-28 May 1999, Palma de Mallorca, Spain

Schultz, T.W & Dawson, D.A (2003) 'Housing and husbandry of *Xenopus* for oocyte production' Lab Animal Vol. **32**, No. 2, p34-39

Shepherson,D.J. (1998) 'Introduction' p1-11 in 'Second Nature – Environmental enrichment for captive animals' Eds Shepherdson, Mellen & Hutchins: Smithsonian Institute, Washington

Sive, H.L., Grainger, R.M. & Harland,R.M. (2000) 'Early development of *Xenopus laevis* – a laboratory manual' Cold Spring Harbor Laboratory Press: New York

Smith, J.M. & Stump, K.C (2000) 'Isoflurane anaesthesia in the African Clawed Frog' Contemporary Topics Vol. **39**, no.6 p39-42

Stanford University (2003) ' Department of Comparative Medicine resource manual - Guidelines for anaesthesia, analgesia and tranquilization in amphibians': Dept of Comparative Medicine Veterinary Service Center (VSC) http://compmed.stanford.edu/animal_care/guidelines.html#anes *(accessed 26/02/2004)*

The Berlin Workshop (1994) 'The Accomodation of laboratory animals in accordance with animal welfare requirements – Proceedings of an international workshop 17-19 May 1993' Eds P.O'Donoghue

The Wellcome Trust (2003) 'Wellcome News' Issue **36** (Q3): London

Tinsley, R.C. & Kobel, H.R. (1996) 'The Biology of *Xenopus*' Oxford University Press, Oxford

Tinsley, R.C. & McCoid, M.J. (1996) 'Feral populations of *Xenopus* outside Africa' p81-94; in 'Biology of Xenopus' Eds Tinsley & Kobel: Oxford University Press, Oxford

Tinsley, pers.comm. - Correspondence between B.Reed and R.Tinsley, Feb 2005.

Tobias, M.L., Viswanathan, S. & Kelley, D.B. (1998) 'Rapping, a female receptive call, initiates male-female duets in the South African clawed frog' Proceedings of the National Academy of Sciences, USA, **95** p1870-1875

Tobias, M.L., Barnard, J., O'Hagan, R., Horng, S.H., Rand, M. & Kelley, D.B. (2004) 'Vocal communication between male *Xenopus laevis*' Animal Behaviour **67** p353-365

Tyler, M.J. (1999) 'Frogs and toads as experimental animals' ANZCCART news Vol. **12**, no.1: Australia

United States Department of Agriculture (USDA) (2002) 'Animal Welfare Report – Fiscal Year 2001' USDA - Animal and Plant Health Inspection Service, USA.

United States Geological Survey (2003) 'Non-indigenous aquatic species database – African Clawed frog' http://nas.er.usgs.gov/queries/SpFactSheet.asp?speciesID=67 *(accessed 20/07/2004)*

Universities Federation for Animal Welfare / World Society for the Protection of Animals (1989) 'Euthanasia of amphibians and reptiles' UFAW/WSPA: Potters Bar/ London

University of Arizona (2001) - Institutional Animal Care and Use Committees (IACUC) guidelines on 'Care and handling of *Xenopus laevis'* http://www.ahsc.arizona.edu/uac/iacuc/xenopus/xenopus.shtml *(accessed 04/06/2003)*

University of California (2000) 'ARC Policies and Guidelines – *Xenopus spp*. husbandry' http://www.oprs.ucla.edu/animal/xenopus.htm *(accessed 05/09/2003)*

University of Minnesota (2003) 'Guidelines for the Use of Anesthetics, Analgesics and Tranquilizers in Laboratory Animals' Research Animal Resource: http://www.ahc.umn.edu/rar/anesthesia.html#General *(accessed 20/07/2004)*

University of Washington (2002) 'UW policy for frog oocyte harvest' IACUC (approved July 18, 2002). http://depts.washington.edu/compmed/iacuc/policies/index.html. *(accessed 22/02/2005)*

van Zutphen, L.F.M., Baumans, V. & Beynon, A.C. (2001) 'Principles of laboratory animal science' Elsevier, Amsterdam

Verhoeff-De-Fremery, R. & Vervoordeldonk, F.J.M. (1982) 'Skin autografts as markers in the toad (*Xenopus laevis*)' Laboratory Animals **16**, p156-8

Videler, J.J. & Jorna , J.T. (1985) 'Functions of the sliding pelvis in Xenopus laevis' Copeia 1985 (1): p251-254

Waterman, C. et al (2001) 'Xenopus harvesting and extract preparation' http://www.scripps.edu/cb/waterman/ *(accessed 25/07/2003)*

Williams, D. (1991) 'Chapter 18 - Amphibians' pg 261-271; in 'Manual of exotic pets' Eds Benyon, P.H. & Cooper, J.E: BSAVA, Cheltenham

Wilson, R.S., James, R.S. & Van Damme, R. (2002) 'Trade-offs between speed and endurance in the frog *Xenopus laevis*: a multi-level approach' The Journal of Experimental Biology **205**, p1145-1152

Wilson, R.S., James, R.S. & Johnston, I.A. (2000) 'Thermal acclimation of locomotor performance in tadpoles and adults of the aquatic frog *Xenopus laevis'* Journal of Comparative Physiology B: Biochemical, Systematic, and Environmental Physiology Vol. **170,** no. 2, p117-124

Wolfensohn, S. & Lloyd, M. (2003) 'Handbook of laboratory animal management and welfare - 3rd edition' Blackwell Science, Oxford

Woodland, H.R. (2003) '*Xenopus* Early Development Group' http://www.bio.warwick.ac.uk/res/frame.asp?ID=51 *(accessed 20/07/2004)*

World Small Animal Veterinary Association (WSAVA) (1999) 'Microchip Implantation Sites Update - October 1999' http://www.wsava.org/Site1099.htm *(accessed 20/07/2004)*

Wray, S. & Wilkie, D.R. (1995) 'The relationship between plasma urea levels and some muscle trimethylamine levels in *Xenopus laevis:* A ^{31}P and ^{14}N nuclear magnetic resonance study' The Journal of Experimental Biology **198** p373-378

Wright, K. & Whitaker B.R. (2001) 'Amphibian medicine and captive husbandry' Krieger Publishing Company: Florida, USA

Xenbase (2003) 'Introduction to *Xenopus*' http://www.xenbase.org/intro.html *(accessed 20/07/2004)*

XenopusExpress (2003) '*Xenopus* husbandry' http://www.xenopus.com/ *(accessed 09/07/2003)*

Xenopus Zoo (2003) '*Xenopus* health' http://xenopuszoo.50megs.com/catalog.html *(accessed 05/09/2003)*

***Xenopus laevis* frog colony care (2003)** http://www.xlaevis.com/ *(accessed 02/07/2004)*