PILAB COURSE NOTES

Amphibian bolt-on course for personal licence holders

Learning outcomes

The learning outcomes for the course are taken from the Home Office modular framework for personal licence applicant training, mainly the species-specific module (PILA, EU 3-5, and 7-8). Learning outcomes in *italics* are covered in the general parts of the course, those in ***bold*** identify the species-specific elements to be covered in the species-specific bolt-on course. The course will also cover some species-specific information relevant to modules PILB (EU20) and K (EU 6)

EU Module 3.1: Basic and appropriate biology
Learning Outcomes
3.1.1. Describe basic anatomy, physiology, reproduction and behaviour of the relevant species.
3.1.2. Recognize and describe life events that have the potential to cause suffering including sourcing, transport, housing, husbandry, handling and procedures (on a basic level).
3.1.3. Indicate how good welfare can promote good science: e.g. explain how the failure to attend to biological and behavioural needs may affect the outcome of procedures.
3.1.4. Indicate how husbandry and care may influence experimental outcome and the number of animals needed e.g. example where the place in the room influences the outcome, hence randomisation.
3.1.5. Describe the dietary requirements of the relevant animal species and explain how these can be met.
3.1.6. Describe the importance of providing an enriched environment (appropriate to both the species and the science) including social housing and opportunities for exercise, resting and sleeping.
3.1.7. When relevant to the species, recognise that there are different strains, and that these can have different characteristics which can affect both welfare and science.
3.1.8. When relevant to the species, recognise that alterations to the genome can affect the phenotype in unexpected and subtle ways, and the importance of monitoring such animals very carefully.
3.1.9. Maintain and interpret accurate, comprehensive records of animals held in the animal facility, including the wellbeing of the animals.

EU Module 3.2: Basic and appropriate biology – species specific (practical)
3.2.1. Be able to approach, handle/pick up and restrain an animal and return it to its cage/pen in a calm, confident and empathetic manner such that the animal is not distressed or caused harm.

EU Module 4: Animal care, health and management
Learning Outcomes
4.1. Describe suitable routines and husbandry practices for the maintenance, care and welfare for a range of animals used in research, to include small laboratory species and large animal species where appropriate.
4.2. Describe suitable environmental and housing conditions for laboratory animals, how conditions are monitored and identify the consequences for the animal resulting from inappropriate environmental conditions.
4.3. Recognise that changes to or disruption of circadian or photoperiod can affect animals.
4.4. Describe the biological consequences of acclimatisation, habituation and training

4.5. Describe how the animal facility is organized to maintain an appropriate health status for the animals and the scientific procedures.
4.6. Describe how to provide water and an appropriate diet for laboratory animals including the sourcing, storage and presentation of suitable foodstuffs and water
4.7. List the methods, and demonstrate an understanding of appropriate, safe and humane handling, sexing and restraint of one or more named species for common scientific procedures.
4.8. Name different methods for marking individual animals and state an advantage and disadvantage for each method.
4.9. List potential disease risks in the animal facility, including specific predisposing factors which may be relevant. Name methods available for maintaining appropriate health status (including use of barriers, different containment levels use of sentinels as relevant to the species).
4.10. Describe appropriate breeding programmes
4.11. Describe how genetically altered animals can be used for scientific research and the importance of monitoring such animals very carefully.
4.12. List the correct procedures for ensuring health, welfare and care of animals during their transport.
4.13. List potential human health hazards associated with contact with laboratory animals (including allergy, injury, infection, zoonosis) and how these can be prevented.

EU Module 5: Recognition of pain, suffering and distress
Learning Outcomes
5.1. Recognise normal or desirable behaviour and appearance of the individuals in the context of species, environment and physiological status.
5.2. Recognise abnormal behaviour and signs of discomfort, pain, suffering, or distress, as well as signs of positive well-being and principles of how pain, suffering and distress can be managed.
5.3. Discuss factors to be considered and methods available for assessing and recording the welfare of animals e.g. score sheets.
5.4. Describe what a humane end point is. Identify criteria to be used to set humane endpoints. Define action to be taken when a humane endpoint is reached and consider possible options for refining methods to finish at an earlier endpoint.
5.5. Describe the severity classifications included in the Directive and give examples of each category; explain cumulative severity and the effect this may have on the severity classification.
5.6. Describe the circumstances when anaesthesia or analgesia may be necessary to minimise pain, suffering, distress or lasting harm

EU Module 7: Minimally invasive procedures without anaesthesia
Learning Outcomes
7.1. Describe appropriate methods and principles to be followed when handling animals (including methods of manual restraint and use of restricted environments).
7.2. Describe the biological impact of procedures and restraint on physiology.
7.3. Describe refinement opportunities for procedures and restraint e.g. through training (using positive re-enforcement), habituation and socialisation of animals.
7.4. Describe techniques/procedures including, for example, injection, sampling and dosing techniques (routes/volumes/frequency), dietary modification, gavage, tissue biopsy, behavioural tests, use of metabolic cages.
7.5. Describe how to perform minor techniques and relate appropriate sample volumes and sampling frequencies for the relevant species.
7.6. Describe the need for rigour and consistency in conducting scientific procedures and the correct recording and handling of samples.
7.7. Describe appropriate methods for the assessment of the welfare of animals with respect to the severity of procedures and know what appropriate action to take.
7.8. Recognize that refinement is an on-going process and know where to find relevant, up-to-date, information.
7.9. Describe the biological consequences of transport, acclimatization, husbandry conditions and experimental procedures on the species concerned and describe how these can be minimised.

EU Module 8: Minimally invasive procedures without anaesthesia

Learning Outcomes

8.1. Select and explain the best methods for common procedures (such as blood sampling and application of substances) including route/volume/ frequency as appropriate.

8.2. Demonstrate that s/he can handle and restrain the animal in the best position for the technique.

8.3. Perform minor techniques under supervision, in a manner that does not inflict unnecessary pain, suffering, distress or lasting harm.

Supplementary information

Module K/EU Module 6: Humane methods of killing

Learning Outcomes

6.1.1. Describe the principles of humane killing (e.g. what constitutes 'a good death')

6.1.2. Describe the different methods by which the relevant animals are allowed to be killed, the influence different methods can have on scientific outcomes, and how to select the most appropriate method.

6.1.3. Explain why someone competent to kill animals should be available at all times (whether care staff or person carrying out procedures)

PILB/EU Module 20: Anaesthesia for minor procedures

Learning Outcomes

20.1. Define sedation, local and general anaesthesia

20.2. Identify the three components of the triad of anaesthesia and understand that different anaesthetic agents produce these to different degrees.

20.3. Define balanced anaesthesia and indicate that this is best achieved by using drugs in combinations to achieve all components of the anaesthetic triad to an acceptable degree

20.4. Relate why and when sedation or anaesthesia might be used for restraint.

20.5. List the factors to be considered in pre-anaesthetic evaluation of animals - how to perform a basic health check, consider physiological or pathological status of the model they are working with and how these may influence the choice of anaesthetic agent.

20.6. Discuss the relative merits / drawbacks and principles of selection of different agents and their application, including calculation of doses, in relevant species, including injectable and volatile agents (or dissolved agents in the case of aquatic species), including local anaesthesia regimes

20.7. Indicate the importance of minimising stress prior to anaesthesia in reducing the likelihood of complications due to anaesthesia.

20.8. Recognise when premedication is beneficial to incorporate into an anaesthetic regime.

20.9. Describe and demonstrate the correct set-up, operation and maintenance of anaesthetic equipment appropriate to the species concerned.

20.10. Evaluate and appreciate the different levels and planes of anaesthesia (voluntary excitement, involuntary excitement, surgical anaesthesia (light, medium & deep), excessively deep).

20.11. List the factors indicating that an animal is suitably anaesthetized (stable and of appropriate depth) to enable procedures to be undertaken and what actions should be taken if an adverse event occurs. This will include basic "hands on" and "observational" anaesthetic monitoring techniques, including assessment of reflexes appropriate for species.

20.12. Describe methods of optimising post anaesthetic recovery (e.g. heat blankets, analgesia, reversal agents, access to food and water, environmental conditions) to ensure a smooth and rapid recovery from anaesthesia.

20.13. Demonstrate an understanding of safe/good working practices with regard to use,

INTRODUCTION

Amphibia are aquatic, semi-terrestrial or terrestrial tetrapods, and there are about as many amphibian species as mammals. Amphibia are ectothermic, their body temperature being determined by the environment, although they use behaviour and evaporation to control temperature to some extent. Their metabolic rate falls at low temperatures, allowing them to survive on very little food. Amphibians are unique among vertebrates in that the juvenile and adult forms are different: fish-like tadpoles undergo metamorphosis into the tetrapod juvenile form.

There are three orders of amphibian: Apoda, which have no legs and resemble worms or snakes; Urodeles, which includes newts and salamanders, and Anurans, which includes frogs and toads. The Anurans are tail-less apart from larval stages, with well-developed hind limbs adapted for jumping. The head and trunk are fused and the toes are webbed. The most common amphibians used in the laboratory are the anurans *Xenopus laevis* (the African clawed frog) and the smaller *Xenopus tropicalis* (the western clawed frog). *Xenopus* are used particularly in studies of developmental biology because they have a large and easily manipulated embryo, with a single pair of adults producing many thousands of offspring with limited genetic variability. *Xenopus* oocytes are used in molecular biology and studies of DNA replication and repair. The first vertebrate to be cloned was an African clawed frog, and genetic modification of *Xenopus* embryos is now relatively commonplace. Some amphibians can regenerate lost limbs as adults whilst all can regenerate tissues as larvae.

Xenopus general: biology and behaviour

For detailed information on the biology of *Xenopus* species see Tinsley RC and Kobel HR (Eds) (1996). The biology of *Xenopus*. Oxford: The Zoological Society of London, Clarendon Press.

Xenopus have smooth, greenish-grey skin on their backs with a creamy white underside. Albino varieties also occur. They shed their skin regularly and often eat their own shedded skin. Amphibian skin is moist, delicate and covered in mucus. The skin acts as a barrier to infection and secretes chemicals with protective functions. The skin secretions are toxic to deter predators, and stressed animals will produce excess secretions. These can be irritant to the mucous membranes of the handler.

Amphibians do not drink, absorbing moisture through their skin, although they also lose water by this route. They cannot concentrate their urine and are susceptible to dehydration in dry environments.

In the wild, like most amphibians, social behaviour of *Xenopus* is mainly restricted to the mating season. However, group housing is advisable, as it improves feeding and reduces fear responses. Group feeding promotes feeding, inducing all animals to feed. At very low stocking densities food is frequently not eaten.

Water depth and quality

Wild Xenopus live in deep water, and water that is too shallow may lead to stress. The minimum required depth is between 6 and 12.5 cm depending on the size of the animal, but for optimum welfare a depth of 30 or even 50 cm may be preferable. *Xenopus* can jump, so tanks should have well-fitting lids, up to 20 cm above water surface to prevent trauma if the animals do jump.

Frogs are very sensitive to toxic substances and traces of hand lotions or other chemicals on the hands of handlers may kill the animals. Personnel should always wear impervious gloves to handle frogs or change their water.

Xenopus can adapt to a wide range of temperatures, but spawning seems to be optimal at 20◦C for *X. laevis* and 25◦C for *X. tropicalis.* All aspects of the physiology of *Xenopus* are affected by temperature, for example immunosuppression occurs below 18◦C in *X. laevis* and they become less able to

withstand stress as temperature falls. At higher temperatures more food is needed as the metabolic rate rises. The optimum is approximately 18–20◦C for *X. laevis*, and is higher for *X. tropicalis* (25–26◦C). Tadpoles prefer slightly warmer temperatures.

XENOPUS LAEVIS

Xenopus laevis are nocturnal, secretive animals originating from sub-Saharan Africa. *X. laevis* occurs in the cooler parts of a wide range of habitats from South Africa to Sudan. Wild *Xenopus* are found in stagnant or slow flowing water, where the water is cloudy and affords some protection from predators. They can be found at very high densities in such areas, especially when the weather is dry. *X. laevis* are robust, and animals can survive and continue to spawn for 15–20 years or more, reaching a length of 12 cm. *X. laevis* are tetraploid, so there are extra copies of many genes that may or may not be functional.

Xenopus are mainly nocturnal hunters and can eat a variety of food. *Xenopus* have three claws on each hind foot, from which their name is derived: *Xenopus* means 'strange foot'. They use the claws to tear up large food pieces, then feed by using their hands to push the food into their mouths, where they use a pump mechanism to suck the food in. They are tongueless, toothless and completely aquatic. They have powerful legs and are strong swimmers.

They have no ears, but sense movement and vibration via lateral lines running down the length of their body, which unusually for frogs are maintained into adulthood. They communicate using sound and use this to maintain dominance hierarchies. Males call to attract females, and females call back in reply. Calling is heard particularly in the evenings.

Xenopus breathe air and respire mainly through the lungs as well as through the skin. Frogs don't have ribs, so air is pumped into the lungs by the muscles in the mouth. Larval stages respire via gills which degenerate after metamorphosis. It is important to note that adult *Xenopus* can drown.

Xenopus are cannibalistic, which has to be taken into account when rearing them.

***Xenopus* behaviour**

In the laboratory *Xenopus* are often seen hanging seemingly immobile for long periods, either at the water surface with nostrils protruding, especially *X. tropicalis*, or on the bottom of the tank. They can adjust their buoyancy, so they can hang like this without effort. Normal healthy frogs have neutral buoyancy.

They move towards food immediately it is introduced. Frogs will react to movement above the tank and try to swim away when disturbed – any animal that fails to try to escape is probably unwell. Once caught, they struggle to escape.

Life cycle

Development of frogs is temperature dependent, and growth is also density dependent. Frogs undergo metamorphosis approximately 60 days after fertilisation. Figure 1 shows frog development, and figure 2 shows internal anatomy.

Figure 1. Frog development.

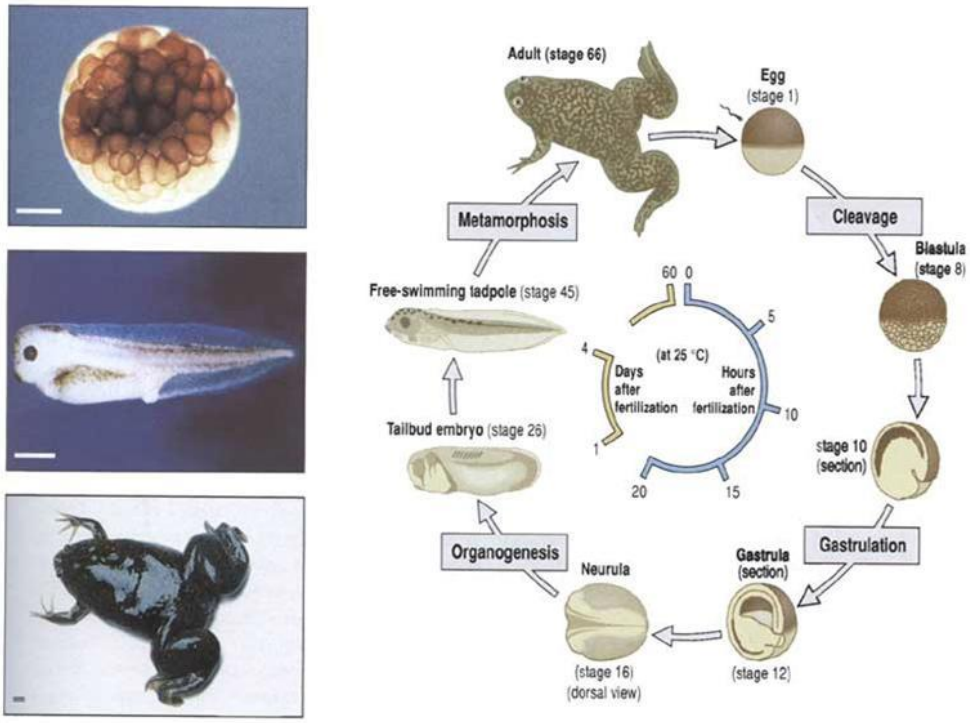
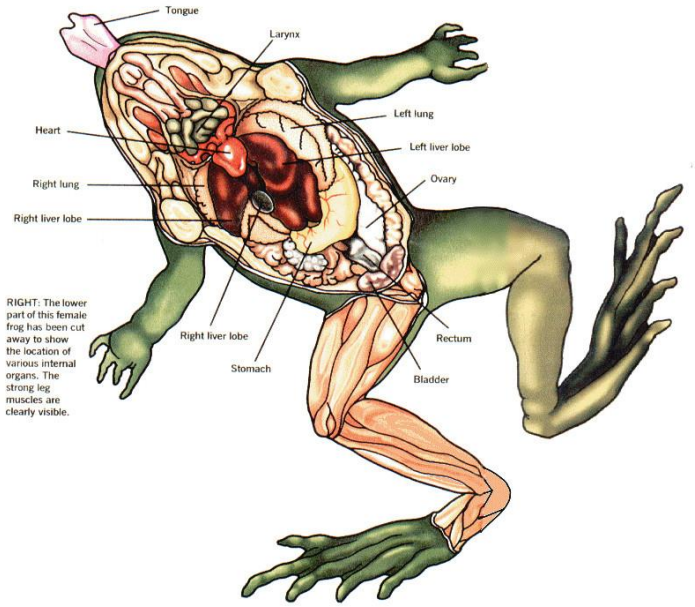


Figure 2. *Xenopus* anatomy



Holding conditions

Laboratory housing for *Xenopus* needs to balance the conflict between their natural environment (deep, murky, still water with vegetation or shade to provide cover and protection from predators) and the experimental requirement for easy maintenance and observation, and access to the animals. In addition, there is often no agreed optimum husbandry method. Recommendations for stocking

density for *X. laevis* vary from 2 litres per animal to 12 litres per animal, and 0.6–0.8 litres per animal for *X. tropicalis.*

The most critical factor in *Xenopus* husbandry is water quality, and water-quality parameters should be checked frequently. Table 2 lists appropriate water-quality standards.

Adults

Xenopus can be kept in 'fill and dump' static water systems, where water is changed periodically (e.g. twice weekly), or recirculating systems, which provide a constant flow of sanitised, aerated water with controlled temperature and composition. Regurgitation of food is common if *Xenopus* are disturbed too soon after eating, so water changes in fill and dump systems should be made before or 3–5 h after feeding. The flow rate in recirculating systems must be low, to produce minimal disturbance to the water.

Water should be dechlorinated, by leaving it to stand or by the addition of treatments to remove it. In self-contained water treatment systems, salts may be added to maintain conductivity.

Water quality, particularly ammonia, nitrite, nitrate and the pH level, should be regularly monitored. Ammonia is excreted by amphibians and its concentration will depend on the water handling regime. The level should be kept below 5 mg/l, depending on the pH (potential harm increases as the pH rises).

Changes in temperature need to be gradual to avoid stress, so when changing tanks, frogs must go into clean water of a similar temperature, and handling should be minimal.

Table 1: water quality parameters suitable for *Xenopus*

Temperature (^{o}C)	18-20 (25 for *X. tropicalis*)
Dissolved oxygen (mg/l)	>5
Carbon dioxide (mg/l)	<5
Ammonia (mg/l)	<0.2
Nitrate (mg/l)	<0.3
Chlorine (mg/l)	<3.8
pH	6.5-8.5
Alkalinity (mg/l)	150-250
Conductivity (uS)	4000-1200 (1000 for X. tropicalis)
Salinity (g/l)	0.6

Biological filters

In recirculating systems, the water is passed through a series of mechanical and biological filters designed to remove organic matter and toxic compounds before typically being exposed to ultraviolet light, to kill unwanted microorganisms. Mechanical filters need regular maintenance. Biological filters consist of chambers containing a substrate with a large surface area, and these become colonised with microorganisms that detoxify the water. Biological filters need several weeks to mature, so newly set-up racks cannot be used to hold animals for the first few weeks and then must be stocked gradually to allow the filter time to adapt.

Light

A photoperiod 12 h/12 h or 14 h/10 h light/dark is appropriate. For breeding, 'daylight' fluorescent tubes should be used to provide sufficient ultraviolet light to maintain vitamin D levels. Some facilities provide 'moonlight' at night.

Environmental enrichment

Tanks for *Xenopus* should ideally have dark sides, being lit from above. Refuges and cover should be provided, using plastic pipes or vegetation.

Cleaning regime

Tanks need regular cleaning to remove any food debris, which contaminates the water, or algae which can reduce visibility. Regular cleaning facilitates health checks.

Feeding *Xenopus*

Adults

As juveniles and adults, *Xenopus* are carnivorous scavengers and will eat both dead and live prey and may cannibalise their own tadpoles. They feed under water, detecting prey by movement, odour and sight. *Xenopus* are unusual in that they will feed communally on large prey. In nature, group feeding on large items can trigger a 'frenzy', which encourages all individuals to eat.

During growth, small amounts of food can be offered up to 2-5 x daily, reducing slowly to 3 feeds per week in adults. High protein pellets such as NASCO frog pellets or trout pellets can be used. Feeding requirements depend on temperature and light level. Animals should be fed little and often, giving just enough food to be consumed within 30–60 min, but so all animals are fed to satiation to avoid competition. Any remaining food should be removed after 30 -60 minutes to prevent contamination of water.

If animals are not thriving, then meat supplements such as strips of beef heart or mealworms can encourage animals to eat.

Tadpoles and froglets

Tadpoles and froglets can be held at higher temperatures than adults, to speed development and growth. The speed of metamorphosis is related to water temperature, and metamorphosis occurs at about 6–8 weeks.

Xenopus eggs hatch after 48 h, then larvae attach to a vertical surface until the yolk sac is absorbed. Tadpoles must be kept in clean, aerated water free from chemical contaminants, which is changed frequently. Tadpoles are filter feeders, eating phytoplankton, so care should be taken with water changes – Initially there may be very little or no water flow, otherwise it will remove the food.

Healthy tadpoles hang, head down at a 45◦ angle in the water and use their tails to generate a current in the water to brings their food to them.

There will naturally be substantial losses from every growing clutch of tadpoles. Dead or diseased larvae should be removed as soon as possible.

Xenopus tadpoles are quite large compared to other species and may need to be separated and re-grouped according to size several times before they metamorphose, to improve growth rates and limit cannibalism.

Tadpoles should be fed once they open their mouths, at around stage 42, at 5–10 days in *X. laevis* and 3–4 days in *X. tropicalis*. They may be fed algae, drops of strained vegetable baby food (green beans or peas), liquified fish food or commercial powdered tadpole food. More food is added only once the previous food has been consumed (e.g. if feeding algae, when the water goes clear). It is easy to overfeed tadpoles.

Once metamorphosis has begun, tadpoles can be supplemented with fine fish flakes. Pellets can be added as soon as the animals are large enough.

Recognising healthy and unhealthy frogs

There are no clear signs of pain and distress, so welfare assessment can be difficult in *Xenopus*. The skin should be smooth, glossy and slippery with a slight covering of mucus and minimal shedding of skin.

Healthy *Xenopus* should not be bloated and should swim normally. They should respond to the arrival of food.

Xenopus should be pear shaped, and females tend to be more rounded.

Unhealthy frogs may be bloated, and inactive when being captured. They may have dry, rough or ulcerated skin, with excess mucus or skin shedding apparent.

Skinny frogs with prominent bones and lack of muscles or abdominal content may be unwell. Body weight is very variable and depends on hydration status, so body condition may be a better indicator of health.

Frogs showing no signs of interest in food are very likely to be unwell.

Frog diseases

Numerous diseases can affect *Xenopus*. These are often subclinical and may only become apparent under stress, causing a sudden appearance of signs.

Batrachochytrium dendrobatidis (Bd or Chytridiomycosis) is a fungal infection found in all laboratory *Xenopus laevis* colonies at a low level. It does not affect *X. laevis* but affects wild amphibian populations and can cause disease in stressed *X. tropicalis*. Testing for this organism can be done at the European *Xenopus* Resource Centre (EXRC; see www.port.ac.uk/research/exrc/ for details). It is barely detectable in healthy *X laevis,* and detection is cyclical even using a sensitive molecular assay. It is not known if this affects experiments.

Mycobacterium – two species (*M. marinum* and *M. liflandi*) can destroy frog colonies. This is more of a problem for *X. tropicalis* than *X. laevis.* Care – these can infect humans and people with poor immune systems should not work with the frogs. Some mycobacteria are found in tap water and it may be impossible to keep them out.

Ranavirus – this can be found in *X. laevis* colonies but is asymptomatic.

Nematodes occasionally infect the skin of *Xenopus*. This can be treated, but may then leave the frogs immunocompromised and vulnerable to the other infections.

Red leg is a septicaemia caused by a variety of commensal organisms normally found in the environment, often including *Aeromonas spp*. This can kill frogs that are stressed or injured by attacking them opportunistically. Occasional success has been reported with broad spectrum antibiotic treatment or immersion in saline.

Dehydration is a major cause of morbidity in *Xenopus*, and desiccation must be avoided. The skin is delicate and prone to traumatic injury, which can predispose to infection.

Disease prevention and biosecurity

Always source the healthiest animals available, remembering that long journeys will stress them.

Animals should be quarantined on arrival, although this may not identify some organisms like mycobacteria or nematodes, that can remain dormant for long periods.

Reducing the pathogen load on a colony and keeping the frogs well fed in good quality water will allow their natural resilience to keep the animals healthy.

Colonies can be set up with 'clean' eggs, resulting in a population with a lower pathogen load. This appears worthwhile especially for *X. tropicalis*, embryo quality becomes more consistent.

Tank systems can be sterilised at intervals, however this can be a long process.

If a frog becomes ill, seek advice from the NVS immediately. Further advice can be obtained from the EXRC in Portsmouth. Generally sick frogs should be removed from the colony as soon as possible. Some diseases can be treated simply by removing them from stress and placing in clean water, or immersing in buffered saline. Sick frogs need frequent observation and should be offered a range of palatable foodstuffs. If recovery does not occur quickly then culling is the best option.

Handling

To prevent cross infection, separate equipment should be used for each set of connected tanks (i.e. one tank of fill and dump).

There is some evidence that *X. tropicalis* react poorly to handling with gloves, but hands need to be clean and cool. It can help to run them under the cold tap prior to handling the frogs.

Slippery skin makes handling amphibians difficult. Their powerful hind legs and claws mean they can escape readily and damage the handler. There is also the possibility of contracting a zoonotic infection so frogs should never be handled without gloves if the handler has an open skin wound. Latex, powder free gloves are reportedly the best ones to use.

Approach the animal gently. Place one hand over the back of the animal with fingers orientated towards the feet. Place the index finger between the frog's hindlegs and use the thumb and other fingers to grasp it around the flanks. Grip the animal firmly and remove from water. It may be necessary to improve grip by holding animals with soft moist paper towel or cloth. A soft net is the best method to transfer frogs between tanks. Scoop the frog out of the water, keeping a hand over the net to prevent the frog from jumping out. Frogs must be kept moist at all times.

Figure 3. Handling frogs

Identification

X. laevis have unique patterns on their backs, and photographs can be used to differentiate between them. This is more difficult in *X. tropicalis*, for which the markings are less distinct. In both species the pattern of the blood vessels in the toe webs can be used for identification. Microchip transponders can also be implanted subcutaneously or into the dorsal lymph sac.

Ink spots tattooed onto the belly can persist for several years. More invasive methods such as freeze branding or toe clipping are not recommended.

Sexing *Xenopus*

Male *Xenopus* are usually smaller than females, with slimmer bodies and legs. Females have hip-like bulges above their rear legs. Both males and females have a cloaca, through which the digestive, urinary tract and reproductive systems pass. The cloaca empties through a single opening called the vent. Females have a more pronounced cloaca due to larger ventral flaps, called anal papillae, located immediately above their cloacas.

Males have black, spiny nuptial pads which are rough to the touch on the inner arms, and enlarged thumbs to help grip the females during breeding.

Techniques

Administration of substances is often via the dorsal lymph sac. This is a subcutaneous lymph space that connects with the vascular system. Compounds given by this route are rapidly absorbed into the circulation, so this is the usual site for intravenous injection. Insert needle at a shallow angle in the skin of the upper thigh and towards back into the lymph space, or just beneath the skin between the dorsal lateral line stripes. See figure 4.

Intramuscular injections can be given into the thigh muscles.

Intraperitoneal injections are given with the frog held on its back. The needle is inserted into the groin area at a shallow angle.

Blood collection in amphibians is often difficult. Small samples can be collected from the toe web. Larger samples may be collected by cardiac puncture post mortem or under terminal anaesthesia. See https://www.youtube.com/watch?v=p4xJo7dqrgU

Use of hormones for inducing egg production

X. laevis usually takes 1–2 years to reach sexual maturity, but it can take only 6 months post-metamorphosis in the laboratory.

Wild *X. laevis* are seasonal breeders, spawning from early spring to late summer. Spawning in females and mating (amplexus) in males is usually induced in the laboratory by administering one or two injections of human chorionic gonadotropin (HCG), 48 h apart.

Adults should be in breeding condition (females well-fed and plump, and males with black nuptial pads on the insides of the arms).

The afternoon before the eggs are needed, females receive 100 iu HCG. For natural mating, males receive 50 iu HCG.

On the day eggs are needed, females should receive a second dose of 200 units and males 100 units.

For natural mating put male and female together after the second injection into a spawning tank with a perforated plastic base to protect the eggs. The male grips the female during a mating embrace,

(amplexus), then releases sperm over freshly laid eggs. The pair should be left undisturbed in a darkened tank until mating is over. Fertilisation is external.

Alternatively, shortly after the second injection, gravid females can be massaged for about 10 seconds to extrude eggs (figure 4). The female should have a red cloaca and be held as shown with gentle pressure applied to the abdomen. If no eggs are produced, further pressure should NOT be applied. Egg collection can be repeated at >30 minute intervals through the day until no further eggs are produced.

Figure 4. Dorsal lymph sac injection

Figure 5. Egg harvest

Fertilisation can be done *in vitro* if required, by covering eggs promptly with testicular extract obtained post mortem from hormone-treated males. Frozen sperm can also be used.

Females in good condition can be bred once per month, although an interval of 2–3 months is typical. Females that have not been bred for a period of 4–6 months may produce poor-quality eggs. Males can be bred more frequently. Such us of frogs constitutes re-use, and appropriate project licence authority is needed for this. Frogs should be re used only if they are in good health as judged by body weight and condition, skin condition, and body shape.

Euthanasia

Amphibians may be killed by overdose of anaesthetic, dislocation of the neck, concussion or electrical stunning. Buffered MS222 is recommended, at 2–3 g/l. Death can take up to 3 h to occur and should be confirmed by pithing or dislocation of the neck. Alternatively an overdose of pentobarbitone can be given into the dorsal lymph sac.

X. TROPICALIS

X. tropicalis originates from tropical forests of West Africa, suggesting they prefer shade and a constant temperature (25 - 26^{o}C). They are smaller than *X. laevis* and are less robust. They like to 'bask' on plastic water lilies (these must be made of suitable material).

X. tropicalis has a diploid genome which has been sequenced. It has a shorter generation time (<5 months), smaller size (4–6 cm body length) and a larger number of eggs per spawn compared with *X. laevis.* See Table 2 for biological data on *X. laevis* and *X. tropicalis.*

Feeding and rearing are similar to *X laevis*, other than differences in water parameters.

Table 2. *Xenopus* biological data

	X. laevis	***X. tropicalis***
Average size (cm)	Male 5–10	3.9–4.5
	Female 10–15	4.8–5-5
Clutch size	300–1000	1000–3000
Length to metamorphosis	2–3 months	1 month
Age tadpoles begin to feed	5–10 days	3 days
Lifespan (years)	15–20	25–30
Temperature range (◦C)	18-20	24-26
Feeding frequency (per week)	1–3	2–3
Heart rate/min At 2◦C	8	No data available
At 25◦C	40–60	
Generation time	1–2 years	4–6 months
Chromosome number	18	10

Breeding

Eggs hatch after 48 h, then larvae attach to a vertical surface until the yolk sac is absorbed.

X. tropicalis have a much shorter generation time than *X. laevis,* of only 4 months under ideql conditions. Females should be checked for health prior to breeding – they should be rotund - and males should have clear mating pads. Breeding can be induced by hormone injection into the dorsal lymph sac as for *X. laevis*. Prime with 10U of HCG for a female. On the morning eggs are needed inject the female with 100U. For natural mating, males should be treated in the same way. For *in vitro* fertilisation, eggs can be mixed with fresh or frozen sperm taken from a male after a schedule 1 kill. Finding *X. tropicalis* testes can be difficult.

To inject, females may need to be immobilised using the 'Burrito' method of wrapping in a wet paper towel (figure 5).

Figure 6. Burrito method for handling X. tropicalis

1. 
2. 
3. 
4. 

Larvae begin feeding from 3 days post-hatching.

Labs typically use 6 females and 2-3 males when doing an experiment, whereas for X. laevis only 2 females and one male are usually needed.

Anaesthesia

Before anaesthetising a frog, it should be checked to ensure it is in good health. The skin, body condition and activity levels should be assessed.

The absorption and excretion of anaesthetic agents is temperature-dependent. Tricaine methane sulphonate (MS222) is frequently used and is usually given by immersion. The animal is placed in a bath of appropriate concentration (0.2% buffered solution), and anaesthesia will be induced within 15–30 min. Anaesthesia is judged by loss of righting and toe pinch reflexes and respiratory effort. As the anaesthetic level deepens, abdominal respiration is lost, followed by slowing of throat movements, which stop as surgical anaesthesia is reached. Ketamine can be used to provide sedation for minor procedures.

Following anaesthesia, animals should be placed into a tank on tissue soaked in tank water – remember frogs can drown if placed into water when anaesthetised.

Table 3. Anaesthetic doses for amphibians.

Drug	Dose (mg/l)	Route	Notes
Tricaine methanesulphonate (MS 222)	Adults 500–2000 Tadpoles 200–500	Immersion	Buffer with $NaHCO_3$ to pH 7–7.5 Induction in 15–30 min Maintain by contact with MS 222 solution Recovery may take 3–6 h Keep animal moist and maintain at 22–26◦C
Ketamine	150–250	Intramuscular	Sedation only

Surgery

Surgery is often carried out on adult frogs, for example to collect oocytes from gravid females. The skin of frogs contains antimicrobial agents, but it is still necessary to conduct surgery under aseptic conditions, otherwise oocyte quality can be poor. The skin should be prepared prior to surgery by cleansing with dilute povidone iodine solution, which is well tolerated, the skin covered with a sterile occlusive drape, and surgery carried out using sterile instruments. Skin closure should be carried out using a monofilament suture material. Non-absorbable material may be preferable since soluble sutures often do not last long enough in aquatic environments to support wounds until fully healed. Sutures should be removed as soon as healing has occurred (10–14 days) to prevent reactions to the sutures. Note that reuse of frogs after oocyte collection may be subject to legal constraints.

Genetically altered animals

Transgenic and mutant *Xenopus* are now common. Genetic alterations can result in unknown phenotypes and they need observing carefully. Even if there is no immediately obvious adverse effect, there is the possibility of hidden harms or effects may appear later in life. A good record keeping system is essential to spot this type of event early.

Genetically Modified Organisms Contained Use Regulations require that these animals do not escape. Males and females should be kept separately. Rooms holding *Xenopus* should be secure - drains

should have mesh on them fine enough to prevent *Xenopus* eggs from passing through, and animals should be double packed for transport.

Unknowns

It needs to be remembered that there is a great deal that is not known about amphibia. For example, the best approaches to anaesthesia and analgesia in Xenopus, whether the widely used recirculating systems are really the best housing for Xenopus, the best approach to detecting stress in frogs, and how our current, limited Xenopus knowledge relate to other amphibia. Most current guidance is based on custom and practise, and as knowledge increases it is likely that changes to current practises wil be needed.

Summary

- It is important to treat Xenopus laevis and Xenopus tropicalis as significantly different animals.
- Keep your colony in good water conditions and well fed to minimise disease.
- Take care when re-using females to ensure they have recovered from the previous use.
- Remember to count (estimate) GA tadpole numbers for the HO returns.
- Help is available – contact the EXRC who can assist or find alternative sources of advice.