RED KITE
VETERINARY CONSULTANTS

Amphibia – bolt-on PILAB Module

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Introduction

- This course is delivered by The European Xenopus Resource Centre on behalf of Red Kite Veterinary Consultants Ltd, and fulfils the requirements for Home Office module PILA
- The course will also cover relevant aspects of modules PILB and K
- Learning outcomes for the course are in the species specific course notes, and also in the notes for candidates

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End of course assessment

- The course is accredited by the Scottish Accreditation Board (SAB), who will issue certificates to the successful candidates
- The end of course examination is set and marked by Red Kite for the SAB.
- Example exam questions can be found in the notes for candidates.
- Following successful completion, Red Kite will apply to the SAB for your training certificate.
- Once you have this, you may apply to the Home Office to add amphibia to your personal licence.

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Module PILA bolt on – Amphibia

Xenopus laevis and *Xenopus tropicalis*

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Why keep *Xenopus*?

- Embryos – for gene function, disease tools and developmental biology
- Eggs – for extracts to study cell cycle and cell transport
- Oocytes – to study ion channels and receptors

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We will treat *Xenopus laevis* and *Xenopus tropicalis* separately – their requirements are different

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Xenopus laevis in the wild

- Sub-saharan Africa – broad range of conditions.
- Stagnant or slow flowing water.
- Often at high densities especially during dry periods.
- Mainly nocturnal hunters – very wide variety of food including carrion which can be broken up by clawed, powerful hind limbs.
- Unusually for frogs they keep their lateral line as adults – detect movement in the water.
- Communicate using sound.
- Breathing is predominantly via well developed lungs not the skin – they can drown.
- They are cannibals – important for rearing strategies.

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Normal and Abnormal *Xenopus* behaviour

- Often still for long periods
- Move towards and eat food immediately on its introduction
- Frogs may stay at the bottom of the tank for periods or may prefer to "hang" below the surface
- *X. tropicalis* dwell on the surface more than *X. laevis*
- Any frog that does not swim away when you try to catch it is behaving abnormally
- Once caught, frogs will normally struggle to escape
- They usually react to movement above their tank
- Normally frogs have neutral buoyancy

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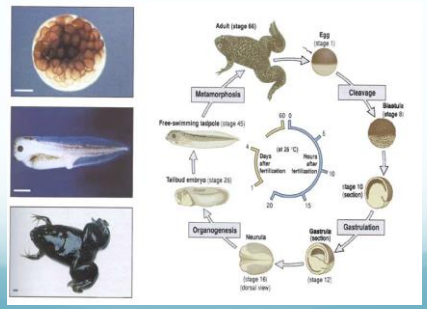
The *Xenopus* life cycle

- Remember development is temperature dependent and growth is also frog density dependent.



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Xenopus anatomy

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Basic adult holding conditions

- Clean(ish), high quality water – recirculating, fill and dump or flow through. Free of chlorine and chloramine at 18 – 20 C.
- Regular testing = pH, ammonium, nitrate, nitrite.
- Build up biological filters slowly – the microorganisms that deal with the waste take time to grow.
- 14 light to 10 dark hours, "moonlight" mimic used successfully.
- Plastic piping cut in half provides somewhere to hide for some frogs – enrichment that is easy to clean.
- Regular cleaning of tanks helps the frogs to be visible for health checks.

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Feeding adults

- In the wild the frogs react to movement of a wide range of food organisms (e.g. invertebrates, small fish) and groups can generate a feeding frenzy.
- Feeding – during growth small amounts 2-5 times daily. Can reduce slowly in adult animals to 3 feeds weekly.
- High protein pellets – NASCO frog pellets, trout pellets, we use Horizon XP23
- Feed little and often so nothing is left to pollute the water or if feeding less often remove/clean 30 mins after feeding (type of system drives this).
- If animals are not thriving then can give meat supplements (e.g. heart, pinks, mealworms but more difficult to control than pellets). Care - they can get fixated on these.

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Rearing tadpoles and froglets

- Can be at higher temperatures to speed development and growth.
- Take care with water changing – 0.1X MBS/MMR to system water exchange needs to be slow.
- Initially very little or no water flow, it will remove the food since they filter feed.
- Healthy tadpoles swim tails up and heads down.
- There will naturally be substantial losses – remove the dead as far as possible and as soon as possible.
- Keep dividing the animals by size to improve growth rates and later limit cannibalism.

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Feeding tadpoles and froglets

- Start feeding when the tadpoles start to open and close their mouths at around stage 42.
- Initially use Sera micron in tiny amounts, dipping 2 mm of the tip of a cocktail stick in the tub is a good starting amount for a 150mm Petri dish. Replace the food when the water begins to go clear (the algae in sera micron will make it green). It is easy to overfeed the tadpoles.
- Once the tail starts to re-absorb in metamorphosis begin to supplement the food with fish flake finely broken up.
- As soon as they are large enough start the froglets on small trout pellets together with the fish flakes.

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Characteristics of a healthy frog

- Smooth, "glossy" skin
- Minimal skin shedding
- No bloating, swim normally without stimulus
- Responds to the arrival of food
- Pear shaped – females noticeably rounder than males

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An unhealthy frog:

- Bloated
- Inactive if chased for capture
- Any skin lesions
- Shedding large quantities of skin
- Skinny
- Lack of interest in food
- No clear signs of pain or distress

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Main diseases of *Xenopus*

- Chytridiomycosis – the famous one. Found in all *X. laevis* colonies at very low levels if the frogs are healthy. Barely detectable and cyclical even using a sensitive molecular assay. We do not know if this affects experiments in any way – study underway to find out.
- Mycobacterium – two species (*M. marinum and M. liflandii*) can destroy frog colonies. More of a problem for *X. tropicalis* than *X. laevis*. Care – these can infect humans; 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 – found in *X. laevis* colonies but again asymptomatic.
- Nematodes – occasionally infect the skin of *Xenopus*, especially colonies where frogs were originally wild caught. Easy to treat but may then leave the frogs immunocompromised and vulnerable to the otherinfections.
- "Red leg"- a wide range of organisms normally found in the environment, often including *Pseudomonas* spp., that will kill frogs that are stressed or injured by attacking them opportunistically. Occasional success reported for wide spectrum antibiotic treatment.

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Biosecurity

- Source the most healthy animals you can remembering that long journeys will stress them.
- Quarantine – fine for obvious illnesses like "red leg" but we know that mycobacterium can remain undetectable for 2 years and nematode for 1 year.....
- 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. There are other actions that may help:
- Building a colony from "clean" eggs. Seems to be worthwhile for *X. tropicalis*, embryo quality becomes more consistent.
- Tank systems can be sterilised – one of ours which had mycobacterium has now been free for almost 5 years since sterilisation, but it is a long job!

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What if a frog becomes ill?

- Ask advice from your NVS, call here.
- Frog first aid: bloat can be treated by immersion in 1 X MBS
- Generally get the frog out of the colony immediately
- Regular observations, use a variety of food but if no recovery then culling is probably the best option rather than risking the remaining animals and to minimise suffering.

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Handling frogs

- Keep nets etc. for each set of connected tanks (i.e. one tank of fill and dump).
- Gloves – some evidence that *X. tropicalis* react poorly to handling with gloves but hands need to be clean and cool. Run them under the cold tap for a minute or two prior to handling the frogs.
- Remember the possibility of zoonosis – never handle frogs if you have an open wound.
- Use gloves if possible – latex powder free is reputedly best.

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Identifying frogs

- Previously a wide range of techniques were used to identify frogs: tatooing, toe clipping, beads on a string threaded through skin.
- For *X. laevis* this can be done by keeping a photographic record of the dorsal patterns on the animals. They are stable over many years and by careful choice of frogs in each tank then a colony of 1000s of frogs can be individually identified this way.
- For *X. tropicalis* individual identification is currently best achieved using tiny microchips.

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Sexing *Xenopus* sp.

- Females are normally substantially larger then males.
- Mature males have mating pads on the inside of their "forearms" which are dark and rough to the touch.
- Females are more pear shaped than males.
- Females have a pronounced cloaca.

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Techniques

- Administration of substances
  - Most injections are given into the dorsal lymph sac. Substances injected here are rapidly absorbed and this is similar to iv administration.
  - Intramuscular injection: muscles of the thigh.
  - Intraperitoneal injection: caudal ventral abdomen, with the needle at a shallow angle.
- Blood collection in amphibians is difficult
  - Small samples – toe web.
  - Large samples, by cardiac puncture post mortem or terminal anaesthesia.
  - https://www.youtube.com/watch?v=p4xJo7dqrgU

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Injecting with HCG

- Human chorionic gonadotrophin is used to induce egg laying in female frogs and mating (amplexus) in male frogs – do not use this if you are pregnant.
- Late in the afternoon before eggs/embryos are required prepare a 1 ml syringe with 600U HCG for a female for egg laying.
- For natural mating 50 units for a male on the day before the animals are put together then 100 units immediately prior to putting them together. For females 100 units and then 200 units are given at the same times as the male. We use "Chorulon" for our HCG which has to be supplied by your vet in the UK.
- Holding the frog as shown inject into the dorsal lymph sac.

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Producing eggs

- The female should have a red cloaca.
- Holding the frog as shown apply gentle pressure to massage the abdomen.
- Do not force the pressure if the frog is not laying.
- Alternatively lay the frog on foil and stroke down both sides of the abdomen.
- If a frog is not laying try a short (30 minute) exposure to natural sunlight.
- See our SOP.....

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Picture of frog "squeezing"

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In vitro fertilisation SOP

- Place hands in water to cool them down before handling frogs.
- The frogs are gently "squeezed".
- Eggs are dropped from a shallow height into labeled petri dishes (to monitor quality and quantity, and this information is entered on the frog database).
- Frogs should be gently squeezed for only about 10 seconds to prevent injury.
- One third of a testis is mashed in 1-2ml of 1 x mbs. Using a pair of broad forceps in an angled petri dish.
- This solution is taken up in a glass dropper pipette
- Eggs should be immediately covered with the testes mash using the dropper pipette and the dish shaken to distribute sperm to all the eggs.
- After about 5 minutes add 0.1 x MBS to the petri dish to cover the eggs.
- The eggs can be collected at half-hourly intervals throughout the day as required until squeezing becomes difficult.

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Re-use

- If on project licence – changed since 2013, must not have undergone a severe procedure or experienced severe pain, the new procedure must be mild of moderate, a vet must advise that the animal's health has been fully restored
- We check: **weight recovered**, skin condition, body shape
- Generally in your judgment is it a healthy frog and likely to lay (body shape)?
- Needs good record keeping and ID

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Schedule 1 killing

- Anaesthesia, dislocation of "neck" followed by destruction of the brain. This is a 2 stage process – it is critical to ensure the destruction of the brain, frogs are extremely robust.
- Currently recommend tricaine methanesuphonate/MS222 – wear gloves and take care, it may be carcinogenic.
- Make a 0.2% solution (2g in 1 litre) in water and neutralise it using bicarbonate (pH to 7.4 testing with pH paper). This will kill a frog but only after around 3 hours exposure.
- Immerse the frog for > 30 minutes
- Ensure it's completely unconscious – loss of righting reflex, loss of withdrawal response to toe pinch.
- Break "neck" using scissors
- Destroy brain using scissors to cut at anterior of brain case then straight down the middle – this has less chance of missing than pithing.

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Xenopus tropicalis

- In the wild they live in shady forest ponds in equatorial West Africa with water at 25-26°C, never found outside this.
- Smaller than *X. laevis* and less robust
- They like to come out and "bask" on plastic water lillies – ensure these do not leach chemicals into the water.
- Water temperature of 25°C and a conductivity of 1000uS appear optimum but there are few data.
- Feeding and rearing are as for *X. laevis* with the exception of water parameters.

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Breeding *X. tropicalis*

- Females should be rotund and males should have clear mating pads.
- Injection is into the dorsal lymph sac.
- Prime with 10U of HCG for a female from 1 – 3 days before use. On the morning eggs are needed, inject the female with 100U. For natural matings treat the males exactly the same.
- You may need to immobilise these small frogs using the "Burrito" method of wrapping in a wet paper towel (see picture)
- Labs typically use 6 females and 2-3 males when we want to do an experiment – only 2 female *X. laevis* for the same one.
- Finding *X. tropicalis* testes is sometimes a challenge!

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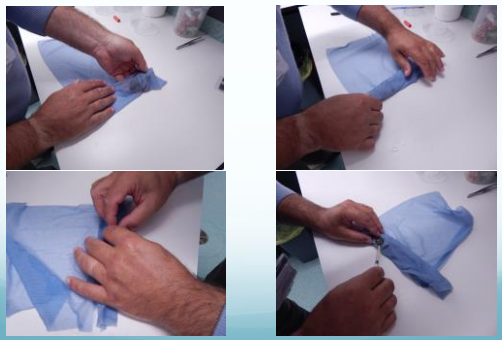
The Burrito method



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Anaesthesia of *X. tropicalis*

- Ensure the frog is in good condition: skin state, roundness and liveliness of the escape attempt are all good indicators.
- Use 0.2% neutralised MS222 (sodium bicarbonate and pH paper)
- Intramuscular injection of anaesthetics are also a possibility.
- Takes little time (around 5 minutes) to anaesthetise them for e.g. toe clipping.
- Check by loss of righting and toe pinch withdrawal.
- Recovery is on tank water-soaked tissue with frequent observation.

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Genetically altered frogs

- Transgenic and mutant *Xenopus* are now common.
- They need observing carefully – we have found one line that had no known deleterious transgene that dies as adults in some distress. A good record keeping system is essential to spot this type of event early.
- It is crucial that these animals do not escape: keep males and females separately, ensure all drains in a room have mesh on them smaller than a *Xenopus* egg, transport animals double packed.
- They need GMAG approval to house or make

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Things we don't know

- What are the best approaches to anaesthesia and analgesia in *Xenopus?*
- Are recirculating systems really the best housing for *Xenopus?*
- What is the best approach to detecting stress in frogs?
- How does our current, limited *Xenopus* knowledge relate to other amphibia?

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Summary

- 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 – if the EXRC staff can't answer questions we know people who can help.

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