Optimum egg incubation temperatures

The optimum temperature at which to incubate amphibian eggs depends on several factors including incubation range, incubation optimum, incubation fluctuation, and production parameters. Important cofactors affecting hatch rate are pH and oxygenation. Oxygen levels should be saturated and pH for most species at neutral of 7 (Figure 2).

In anurans Goncharov et al. 1989 showed that species varied widely in incubation range. However, over that range the hatch rate was constant (Figure 1).

Figures 1 and 2. From “Goncharov BF, Shubravy OI, Serbinova IA, Uteshev VK. 1989. The USSR programme for breeding amphibians, including rare and endangered species. International Year Book. 28:10-21.”

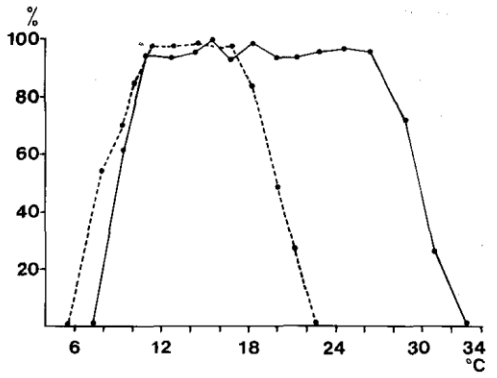


Fig. 1. Effect of temperature on the survival of embryos of *Bufo viridis* (solid line) and *B. verrucosissimus* (broken line).

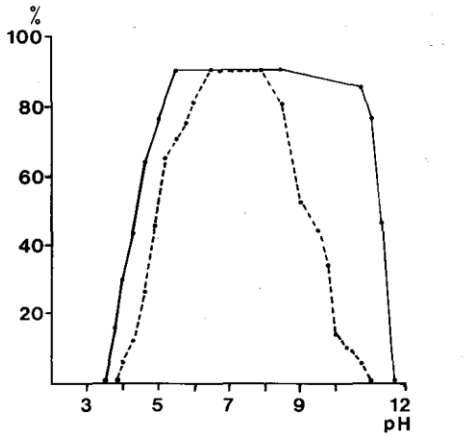


Fig. 2. Effect of pH on the survival of embryos of *Bufo viridis* (solid line) and *B. verrucosissimus* (broken line).

Optimum egg incubation temperatures

The optimum temperature at which to incubate amphibian eggs depends on several factors including incubation range, incubation optimum, incubation fluctuation, and production parameters. Important cofactors affecting hatch rate are pH and oxygenation. Oxygen levels should be saturated and pH for most species at neutral of 7 (Figure 2).

In anurans Goncharov et al. 1989 showed that species varied widely in incubation range. However, over that range the hatch rate was constant (Figure 1).

Figures 1 and 2. From “Goncharov BF, Shubravy OI, Serbinova IA, Uteshev VK. 1989. The USSR programme for breeding amphibians, including rare and endangered species. International Year Book. 28:10-21.”

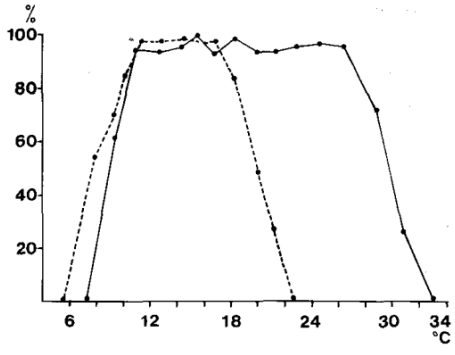


Fig. 1. Effect of temperature on the survival of embryos of *Bufo viridis* (solid line) and *B. verrucosissimus* (broken line).

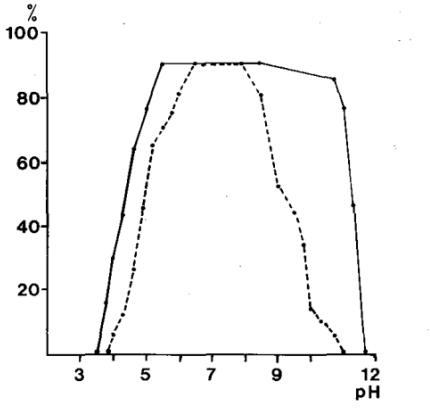


Fig. 2. Effect of pH on the survival of embryos of *Bufo viridis* (solid line) and *B. verrucosissimus* (broken line).

MS 222 (anesthetic) Sigma E 10521

The most useful anesthetic reagent for anurans is tricaine methanesulfonate (MS-222). At the doses recommended MS222 can be dissolved into distilled water (preferred where Ca^{2+} ions may interfere with subsequent treatments), or in physiological salines such as Simplified Amphibian Ringers (above). See methods.

Larvae: Larvae may be fully immobilized within 80 second at room temperature by immersion in a 0.05% to 0.03% (w/v) solution. Recovery occurs within 5-15 minutes upon return to normal medium, depending on the stage of development and length of exposure to the reagent. Some authors consider that exposure of developing embryos to MS-222 for as long as one hour does not result in abnormal development.

Adults: To achieve satisfactory and predictable anesthetization of anurans inject 1 ml MS-222 at 0.2% (w/v) per10 g of animal weight the dorsal lymph sac or intraperitoneally. If the animal is not completely anesthetized in 10 minutes, inject half the original dose. Once anesthetized the animal will be in deep anesthesia appropriate for surgical manipulation for about half an hour. Repeated doses can be used, and recovery is best achieved by placing the animals on moist paper or – if careful not to drown – running water over the animals undersurface. Anesthetized animals will drown if placed in deep water.

NB: If animals are to be euthanased use – double the dosage used from anesthetization – ie.1 ml MS-222 at 0.4% (w/v) per10 g of animal weight.

Counting eggs and larvae

Dr Robert Browne, AArk Research Officer, Royal Zoological Society of Antwerp, robert.browne@gmail.com

Special thanks to Jessica Seratt for her contribution to the computer analysis part of this document.

Techniques used to count eggs include the ‘DISPLACEMENT METHOD’, ‘IMAGE ANALYSIS’, and ‘DIRECT COUNTING’.

‘DISPLACEMENT METHOD’ This method is fast, yields immediate results for large numbers of eggs, enables field counts with replacement of eggs, and is reasonably accurate. In the displacement method a sub-sample of several hundred eggs are counted and their volume measured (usually in a measuring cylinder).

The volume of the total egg mass (including the sub-sample) is then measured and the total number of eggs calculated. Inaccuracies with this method can occur through differences in the gel and egg size between different parts of the spawn mass. This is particularly the case in some experimental situations where spawn masses may be of low quality with variable gel and egg quality.

SN = sub sample number.

SV = sub sample volume.

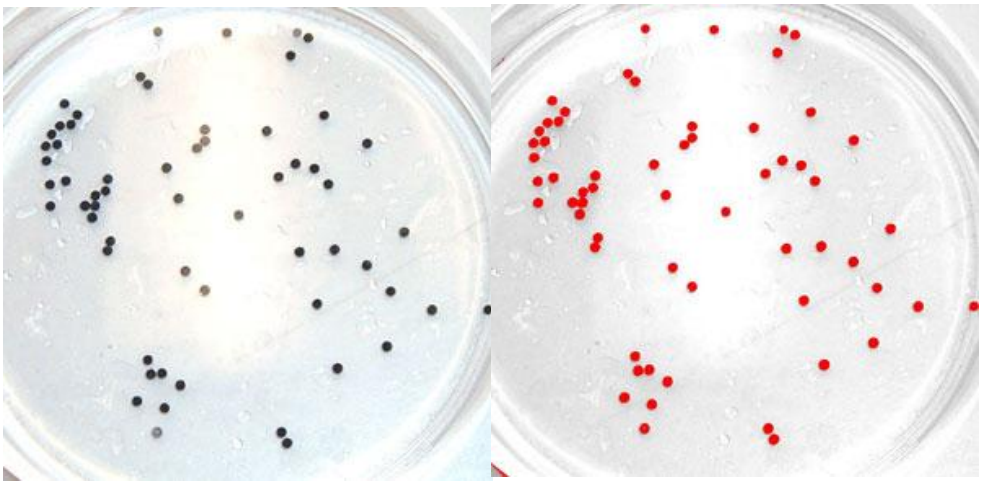
TV = total volume.

TN = total number

TN = TV/SV x SN

‘IMAGE ANALYSIS’ counts eggs from an image of the spread eggs against a contrasting background. Inaccuracies with this method can occur through overlapping eggs making counting difficult. Overlapping eggs are a particular problem where the eggs are in a globular mass rather than in strings. To some extent image analysis can also be used for larvae if they are in shallow trays. The number of eggs can also be counted and marked off digital images by hand.

The eggs must be separate. Separation of eggs is easier with partial removal of the gel coat. see “Gel removal and extract”. The use of the ImageJ™ program to automatically count eggs is described at the end of this document “Image analysis of object counts with ImageJ™”



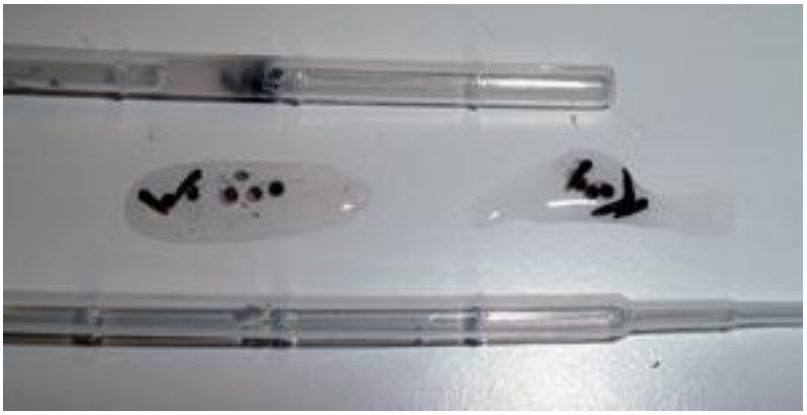
ImageJ™ program allows for easy counting of eggs. It automatically counts all objects it recognizes in the final red image (right).

See at documents end: Image analysis of object counts with ImageJ™

‘DIRECT COUNTING’ with pipettes by an experienced operator produces an exact count of eggs or larvae. However, direct counting can only be used when the eggs are dissociated (see **Reproduction technology** > Gel removal and extract), or when the larvae have separated from the gel naturally or naturally.

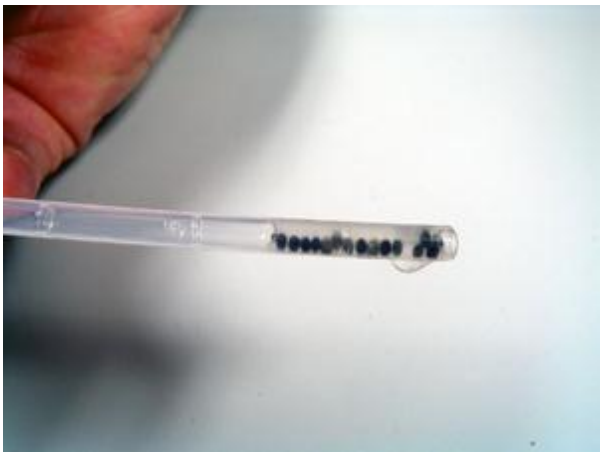
The end of a disposable plastic pipette is cut to leave the maximum open tube. Eggs or larvae - up to just below the diameter of the tube - can then be aspirated and counted and placed in a

new container. This method is often used to count when separating larvae from infertile eggs to prevent poor water quality causing mortalities.



Left: A complete disposable pipette (below) and one with the end removed (above) with toad eggs (middle left) and larvae (middle right). Plastic disposable pipettes are graduated in diameter at the ends. The pipette is generally best cut off to give best adjusted to about 1.5 the diameter of the eggs or larvae. Toad eggs are shown in the middle left and larvae in the middle right.

Right: The 15 eggs can easily be counted as they are transferred into a new container. Similarly larvae or small swim ups can also be counted by this method.



Left: A petri dish of mainly infertile toad eggs and some larvae about 48hrs after spawning. As the larvae develop and hatch they excrete enzymes that dissolve the egg gel. This also enables the infertile eggs to release their yolk into the water creating an ideal environment for bacterial growth.

Right: To prevent hypoxic and unsanitary water the larvae must be moved to a new container with fresh water. This is easily and quickly accomplished using a disposable plastic pipette.

Image analysis of object counts with ImageJ™

Special thanks to Jessica Seratt for her wonderful contribution to the computer analysis part of this document.

Using ADOBE Photoshop™ to get the best image for analysis.

1. Take an image with camera of eggs. White background for black eggs and black background for white eggs. The eggs must be separate. Separation of eggs is easier with partial removal of the gel coat. see “Gel removal and extract”
2. Open image in Adobe Photoshop™
3. Image > Mode > Greyscale
4. Image > Adjust > Brightness/Contrast. Adjust the contrast and brightness to maximize separation of the eggs from the background.
5. Delineate the area with eggs with the Elliptic or Rectangular marque
6. > Cut
7. > Create new file > OK
8. Paste

9. Image > Levels - Adjust to distinguish eggs
10. Filter > Noise > Despeckle
11. Save as tif for analysis in image analysis program.

Analysis of image with Image J™

1. Open in" ImageJ™" 1.33u: Wayne Rasband, National Institutes of Health, USA; http://rsb.info.nih.gov/ij/
2. process > sharper
3. Process > noise > despeckle
4. Image > adjust threshold – image goes red
5. Analyse > particles
6. Show "masks" , "display results", "summarise" > OK
7. The "Image J™" program then produces a count of objects and a summary of average size in pixels, and distribution of size. It also produces image of counted objects

Weighing tadpoles

Dr Robert K. Browne, AArk Research Officer, Royal Zoological Society of Antwerp, Belgium, robert.browne@gmail.com
Rachael Antwis, Royal Zoological Society of Antwerp, Belgium, rachael.antwis@googlemail.com

Where the variation in tadpole weight within replicate tanks is not required, or where tadpoles are too small to be easily weighed individually, tadpoles can be weighed 'on mass' very accurately with the minimum of disturbance or harm to their wellbeing. This technique is commonly used in studies of tadpole growth up until metamorphosis when individual weights must be taken.

Front leg emergence is the best stage to measure metamorphosis in anurans. The development of different organs in tadpoles during metamorphosis, and the relative rate of each metamorphosis stage, varies considerably between species.

Front leg emergence is the most defined stage of metamorphosis. The growth of the back legs from bud to final form occurs during the whole metamorphosis process. However, front leg emergence generally occurs over a 24hr period. Then most tail absorption occurs soon afterwards. However, the period of the final stage of tail absorption can be prolonged and vary greatly between species.

Depending on the size and number of tadpoles the balance should be accurate to at least 0.1g and preferably to 0.01g. The method described below is particularly valuable where very small tadpoles are weighed. With small tadpoles the weight of water can be considerable. As long as they are kept wet tadpoles can last out of water for minutes without harm. However, tadpoles are delicate and must be handled gently.

Sampling tadpoles

Right. Prepare the scales by placing a piece of absorbent paper towel several layers thick on plate. On top of this place a piece of fiberglass fly screen to prevent tadpoles adhering to the paper. A cylinder cut from a plastic bottle is then placed over the fly screen to restrain tadpoles. The balance is then zeroed.

Left. To sample tadpoles a net of appropriate size is used. Particularly for small tadpoles a white or light colored net should be used. Otherwise tadpoles may be left in the net. The net should be placed in a container with water. Then gently pour the water with the tadpoles through.

Right The tadpoles are quickly and gently poured onto the scale

Below. The weight is taken (**Wt1**).

The tadpoles are returned to their tray (at this stage they can be easily counted before adding water to the tray).

The weight of the wet paper, fly wire, plastic bottle is then taken (**Wt2**).