

Egg Incubation and Collection of Painted Turtle Embryos

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INTRODUCTION

This protocol describes how to incubate painted turtle eggs and collect embryos for gene expression analysis. The same basic protocol can be used to incubate eggs and collect embryos from other turtle species by modifying the incubation temperature to suit the particular developmental needs of each species.

RELATED INFORMATION

A companion article, **The Painted Turtle, *Chrysemys picta*: A Model System for Vertebrate Evolution, Ecology, and Human Health** (Valenzuela 2009) describes how the painted turtle is becoming an emerging vertebrate model for ecological, physiological, evolutionary, and genomic studies.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <!**>**, and recipes for reagents marked with <R>.

Reagents

Chrysemys picta eggs

Eggs should be as fresh as possible; eggs ovoposited the night before are best.

Incubation substrate (fine play sand or medium grade vermiculite)

RNA^{later} (Applied Biosystems)

RNAse-away (Sigma-Aldrich) or equivalent surface decontaminant

Equipment

Balance ($\geq 3000g$ capacity and 1 g readability or better)

Dissecting tools (fine scissors and fine forceps; spatula for smaller embryos as described in Step 18)

Incubation boxes (plastic boxes with lids)

Incubators (set to desired temperatures, see Step 7; $\pm 0.5^{\circ}\text{C}$ accuracy or better)

<!**>**Liquid nitrogen (optional; see Step 23)

Pencil (No. 2) or indelible ink pen (e.g., Sharpie)

Petri dishes

Squirt bottle

Vials for embryo collection (2-, 10-, 15-, and 50-mL, depending on size of embryos and dissected tissues)

METHOD

Egg Incubation

1. Maintain eggs in the same orientation as they were found in the nest (if an egg has developed the “white spot,” keep that on the top at all times).
2. Carefully clean mud from the eggs, if necessary.
Use H₂O with extreme care, because too much H₂O can damage embryos.
3. Gently label each egg with a unique ID using a soft pencil or indelible ink pen.
4. Moisten the incubation substrate by adding 4% H₂O content to the sand, or by adding 1.11 g of H₂O per 1 g of medium to fine vermiculite to obtain approximately –150 kPa (Morris et al. 1983).
5. Fill the incubation boxes halfway to two-thirds full with moist substrate.
6. Using a random number generator, assign eggs from different nests randomly to different boxes, positions within boxes, and incubators.
7. Incubate the eggs at the desired temperature or temperatures.
Constant 26°C produces 100% males, 31°C produces 100% females, and ~28°C-28.5°C (depending on population of origin) produces ~50% of each sex.
8. Bury the eggs in rows in the moist incubation substrate. Keep a written record of the box number and position within the box to which each egg was assigned.
Eggs can also be buried just halfway, in order to observe the top of the egg. Note, however, that the conditions the egg/embryo experience in the air inside the box will differ from the conditions within the substrate. Do not allow the number or the position of boxes to obstruct air circulation.
9. Weigh the box with the eggs and the lid and write down the date and weight on the lid.
10. Weigh the box again at least weekly, open the box to allow gas exchange, and replace any lost H₂O using a squirt bottle.
11. Rotate the boxes within each incubator every couple of days, so that each box experiences different positions within the incubator (left, right, upper, lower, back, front, and so on).
See Troubleshooting.
12. Open the boxes daily during the last quarter of the incubation period to replace the air inside the box with fresh air.
This step is important to ensure that enough oxygen is available for the developing embryos.

Embryo Collection

13. Using a random number generator, randomly choose eggs to be collected from each temperature treatment at each sampling time.
14. On the sampling day, weigh the box with the lid and replace any lost H₂O using the squirt bottle. Remove the eggs to be sampled from each box. Weigh the box again with the remaining eggs and the lid and write down the date and new weight on the lid, so that when replacing lost moisture from that point on, this new value will be used as the baseline.
15. Rinse the sampled eggs thoroughly with tap H₂O to remove debris from the eggshell.
From this point on, work in an area and with tools that are RNase free.
16. Place each egg in a clean Petri dish or equivalent.
17. Holding the egg so that the embryo remains at the top of the egg, make a clean incision with sharp small scissors around the equator of the egg.
18. Collect the embryo without yolk. Specifically, remove the yolk sac of each larger embryo using a fine forceps to grab them from the neck. For smaller embryos, use a spatula to spoon out only the embryo.

19. If the embryo is too small to proceed as described above (e.g., day 5 at 31°C or up to day 20 at 26°C), then cut around the shell area where the embryo is attached and immerse the shell with the embryo attached into a vial with five volumes of RNAlater.
20. Dissect target organs from larger embryos (e.g. stage 12 and later) and place them in a vial of RNAlater. Immerse each embryo or dissected tissue in at least five volumes of RNAlater.
21. If whole embryos are to be preserved, cut open the abdominal cavity of larger embryos (e.g., stage 12 and later) to ensure that the RNAlater bathes the internal organs.
22. Incubate the samples in RNAlater for 30 min at room temperature or overnight at 4°C.
23. Store the embryos at –20°C until they are to be used for RNA extraction and processing. Alternatively, flash-freeze the embryos and tissue samples in liquid nitrogen and store them at –80°C until RNA extraction and processing.

TROUBLESHOOTING

Problem: Incubating eggs become infected with fungus.

[Step 11]

Solution: To prevent fungal infection of incubating eggs, consider the following.

1. Sterilize the incubation substrate, water, and containers prior to use.
2. Reduce handling to a minimum.
3. Remove any eggs lacking signs of proper development (e.g., absence of white spot after a couple of days in the incubator, lack of blood vessels when candled for older eggs).

REFERENCES

Morris KA, Packard GC, Boardman TJ, Paukstis GL, Packard MJ. 1983. Effect of the hydric environment on growth of embryonic snapping turtles (*Chelydra serpentina*). *Herpetologica* **39** 272–285.

Valenzuela N. 2009. The painted turtle, *Chrysemys picta*: A model system for vertebrate evolution, ecology, and human health. *Cold Spring Harb Protoc* (this issue). doi: 10.1101/pdb.emo124.