CHAPTER 44

Methods for Retrieving Crocodilian Embryos

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THE embryonic development of crocodilians has recently been reviewed in detail and a comprehensive embryonic staging guide, which appears applicable to all species, has been prepared (Ferguson 1985, see Chapter 45). These advances come at a time when interest in crocodilian embryos has been greatly enhanced. Temperature-dependent sex determination (Ferguson and Joanen 1982, 1983; see Webb *et al.* Chapter 50) and a relationship between egg incubation environment and post-hatching survivorship and growth (Hutton 1984; see Joanen *et al.* Chapter 51) have both been demonstrated in the last few years, and they are as significant to crocodilian farmers and managers as they are to theoretical zoologists. Crocodilian embryology has become economically important.

However, information on crocodilian eggs and embryos can also be used to: predict time of laying from clutches located in the field or in captivity (Magnusson and Taylor 1980; Webb *et al.* 1983a; Cox 1985; Ferguson 1985; Hall 1986); to predict time of hatching in either the field (Webb *et al.* 1983a) or in the laboratory (see Webb *et al.* Chapter 50); to determine when embryo mortality has occurred within a clutch (Webb *et al.* 1983b); and, to identify individual females that produce embryos with high proportions of developmental anomalies (unpublished data). All require a methodology for removing embryos from eggs with the minimum of damage.

In this chapter we briefly describe the methods we use to remove embryos of *Crocodylus johnstoni* and *C. porosus*. The extent of opaque band development on the eggshell is used as a general guide to embryo age, size and disposition within the egg, as with *Alligator mississippiensis* (Ferguson 1985) and as described in Chapter 43.

TRANSLUCENT EGGS

Crocodilian eggs are usually translucent at the time of laying and within 24 hours an opaque spot develops on the upper surface. Before opaque banding has started, eggs can be 'candled' with a strong light. If subembryonic fluid (Fig. 1) or an embryonic disc can be detected, then the egg is fertile, although the minute embryo may still be grossly abnormal or dead. Within translucent eggs, lack of subembryonic fluid indicates very early embryonic death or infertility.

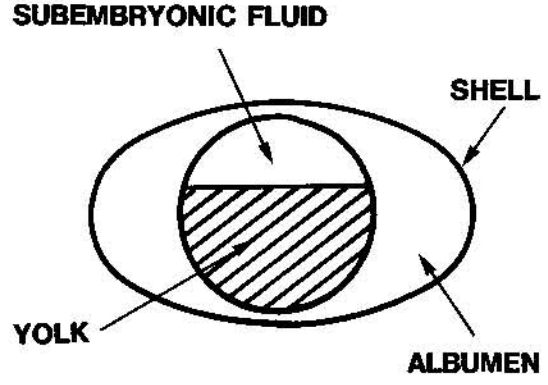

Fig. 1. 'Candling' translucent, fertile crocodile eggs will show subembryonic fluid, and sometimes an embryonic disc (not shown).

To retrieve embryos from translucent eggs, we carefully cut away the top of the eggshell with scissors (Fig. 2A), which exposes the top of the vitelline membrane beneath a layer of albumen (of much thicker consistency than the albumen of bird eggs). With oblique lighting, the small (5-7 mm diameter), translucent, embryonic disc is usually visible near the midline of the egg (Fig. 2B, C).

The best method we have found for removing and fixing these embryos, is to inflate the vitelline sac with fixative (usually buffered formalin or bouin) using a hypodermic needle inserted into the yolk beneath the embryo. The lower density fixative floats to the top of the yolk, expanding the vitelline membrane and fixing the embryo and its developing extraembryonic membranes *in situ* (Fig. 2D). The egg is then lowered into a container of fixative (on occasion a cloth or filter paper soaked in fixative has been laid across the exposed albumen), until the albumen hardens. A square of hardened, fixed albumen, with the vitelline membrane and embryo adhering to the inner surface, can then be excised with scissors. If necessary, the developing extraembryonic membranes and fixed embryo can be peeled off the vitelline membrane and albumen under fixative.

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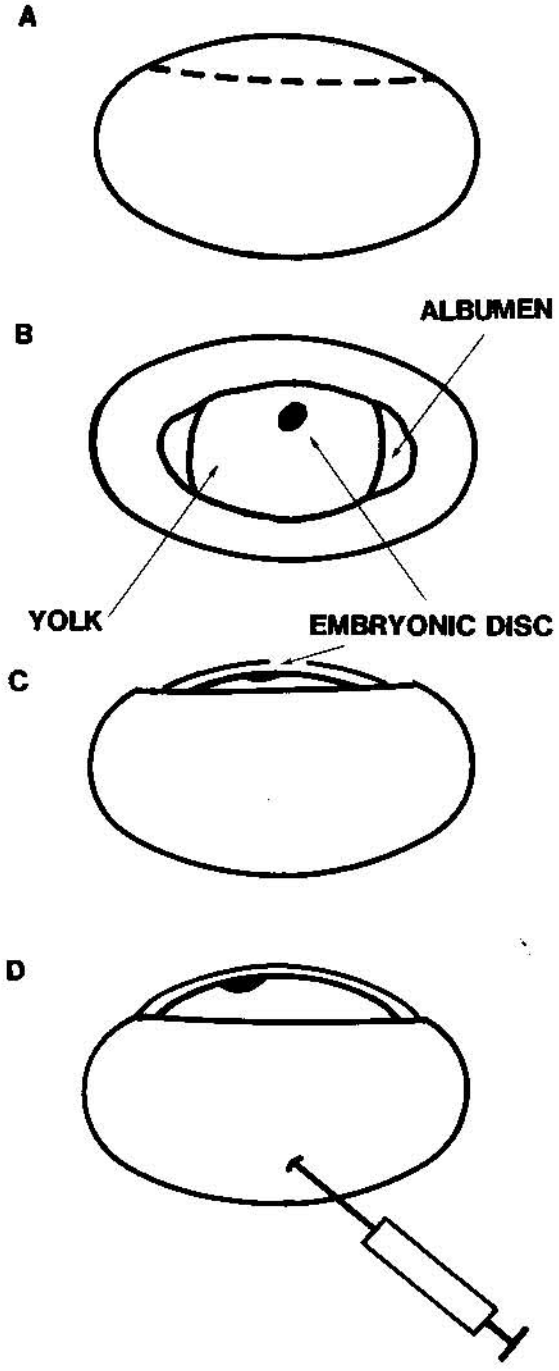

Fig. 2. Once the top of the eggshell is removed (A) and the yolk and embryonic disc are exposed (B, top view; C, side view), fixative is injected into the yolk sac, expanding it (D).

Live embryos can be removed from translucent eggs with a triangular shaped piece of card, however the thick albumen and small embryo make this a clumsier procedure than it is with bird eggs; embryos are often damaged, distorted or lost in the yolk contents. We use a piece of index filing card cut into the shape of a diamond about 2.0 cm wide and blacked with a felt pen. In the centre is a 5-7 mm hole. The layers of albumen are peeled back and the vitelline membrane is slit well to the side of the embryonic disc. The card is then inserted and raised such that the embryo lies in the hole. Scissors are then used to trim the vitelline membrane and remaining albumen around the edge of the diamond, such that it can be removed with the embryo spread out across the hole.

EGGS WITH AN OPAQUE SPOT

Once the opaque band begins to form (Fig. 3), the top of the shell surrounding the opaque spot can be cut away. The embryo will usually be directly beneath the opaque spot, in most cases adhering to the shell membrane within the area of opacity. On occasion the embryo may still be on the top of the vitelline membrane (as in Fig. 2D), but if so, it will be covered by a thin, dehydrated layer of albumen and can be treated as above. If adherent to the shell membrane, a square of shell and shell membrane containing the embryo can be cut and fixed whole, the embryo being easier to remove from the shell membrane after fixation.

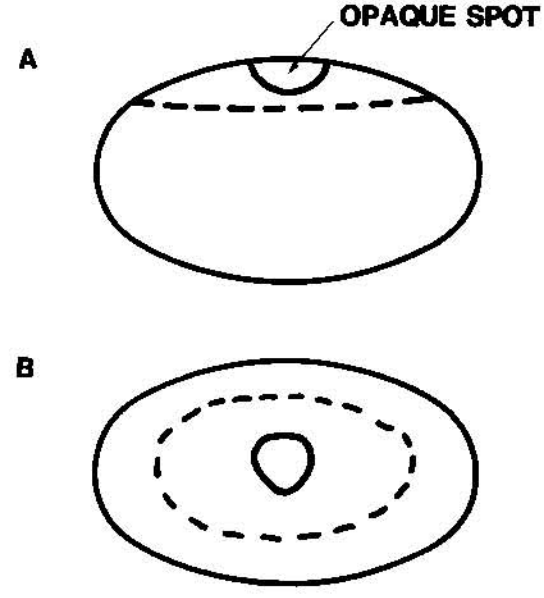

Fig. 3. In eggs with an opaque spot, the top part of the shell is removed. The embryo is usually attached to the shell membrane beneath the spot and a piece of shell containing the embryo can be removed and fixed. (A, side view; B, top view).

EGGS WITH A DEVELOPING OPAQUE BAND

When the arms of the opaque band begin to spread ventrally around the egg, the embryo is usually attached to the shell membrane at the top, with its developing vitelline circulation spreading within the area of opacity. Cutting a cap off the egg, as in Figure 3, can result in the embryo being cut. We remove a shell window to one side of the opacity (Fig. 4), so that the exact position of the embryo can be seen. The embryo is then removed and fixed on a piece of shell.

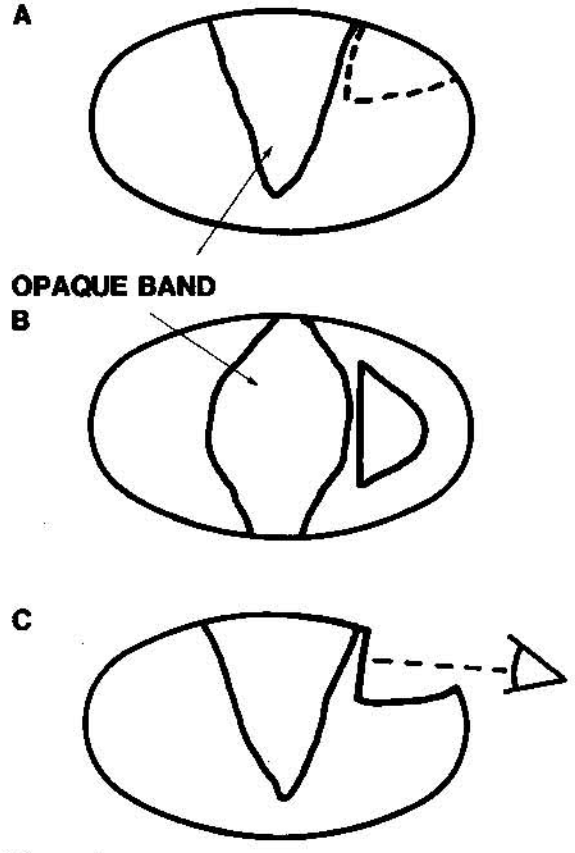

Figure 4.

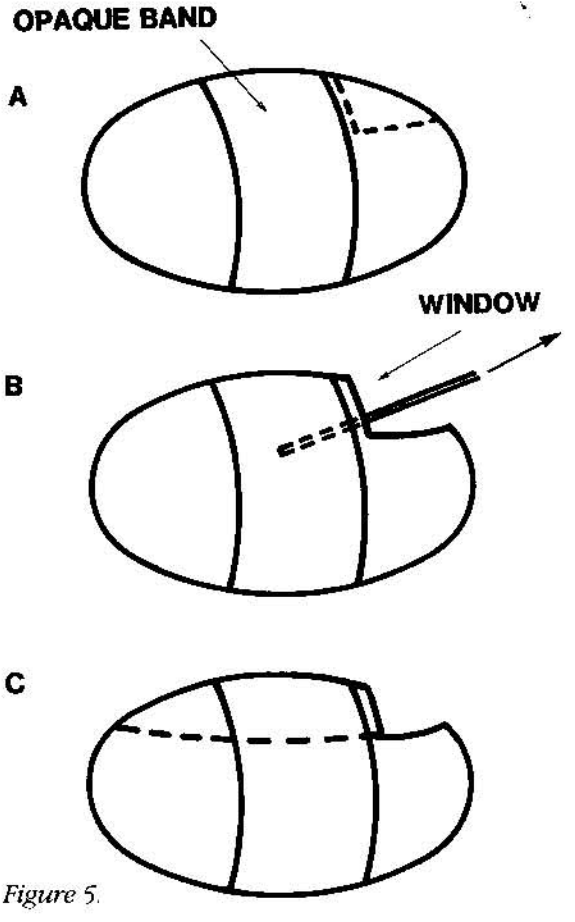

Figure 5.

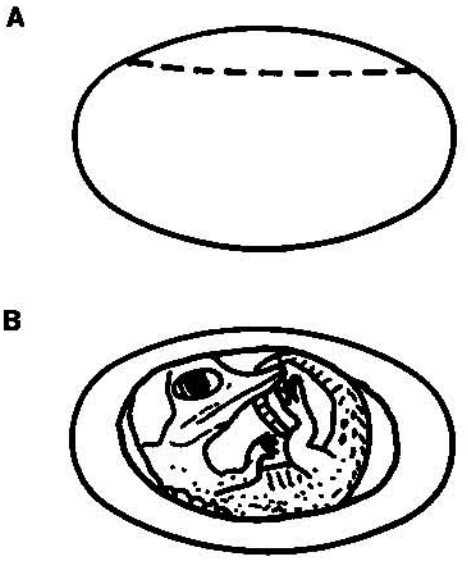

Figure 6.

Fig. 4. (Above left). After a 'window' is cut in the shell (A, side view; B, top view) so that the position of the embryo can be determined (C) prior to the remainder of the shell being cut away.

Fig. 5. (Below left). In eggs with a complete and even opaque band, a 'window' is cut to view the position of the embryo, then the egg fluids are removed with a pasteur pippette (A, B). The rest of the shell can then be cut away and the embryo removed.

Fig. 6. (Above). After the opaque band has covered the entire surface of the egg, removal of a piece of shell from the top is adequate to remove the embryo.

EGGS WITH AN EVEN BAND

This stage of shell opacity lasts a considerable time (see Webb *et al.* Chapter 43), and embryos may be either small and attached to the shell membrane, or large and lying on the yolk (which may be enclosed within a clearly defined extraembryonic yolk sac). We cut a window on one side of the opaque area on the upper surface (Fig. 5) and use a pasteur pippette to remove excess fluid (subembryonic fluid in younger embryos; allantoic and chorionic fluid in older ones). The position of the embryo can then be seen, and it can be removed by cutting a segment of shell and shell membrane (if the embryo is still attached), or with a small teaspoon, sometimes with holes drilled in it, if it is larger and lying on the yolk sac.

EGGS WITH A SPREADING BAND OR WHICH ARE COMPLETELY OPAQUE

Such eggs contain large embryos and the top of the shell can be removed (Fig. 6) as in translucent eggs. The chorioallantois will be severed, and will

bleed, as it underlies the area of opacity. While still within the egg, we usually inject larger embryos with a lethal dose of "Nembutal".

FIELD PRESERVATION OF EGGS

The method used by Ferguson (pers. comm.) to preserve whole eggs in the field, for later removal of embryos, has worked well for us. A small hole is made in both ends (poles) of the egg, and 20% buffered formalin is injected in one hole until albumen exudes from the other and the shell cracks with expansion. The whole egg is then submerged in the fixative.

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