Development Of Extra-Embryonic Membranes And Fluid Compartments.

Glenn K. Baggott

Seventy years ago the Cambridge embryologist Joseph Needham coined the term "cleidoic" to describe the special characteristics of the avian egg (Needham, 1931). He pointed out that the avian egg was essentially closed because nearly all the materials needed for the development of the embryo are contained within the shell. Sufficient water, nutrients and energy (in this case fats) for tissue growth and maintenance are provided. Only oxygen (and heat) is required from the environment. Whereas Needham's perceptive analysis of the nature of the avian egg has been vindicated, the implications of this egg design for the fashioning of embryonic tissue from the fertilised ovum are still to be fully explored. One of the primary difficulties is in the supply of water to the growing mass of embryonic and extra-embryonic tissues. The latter consist of membranes and fluid compartments that develop outside of the body of the true embryo and fulfil a pivotal role in normal development. This review describes the importance of water in development of the extra-embryonic compartments of the egg in order to describe how there is a resolution of the water problem.

Development of the Extra-Embryonic Membranes

It is difficult to give an account of extra-embryonic fluid compartments without reference to the embryological development of the extra-embryonic membranes. This topic is briefly summarised below for the domestic fowl (*Gallus gallus* var. *domesticus*), to which the days of incubation indicated refer, based upon the account in Romanoff (1960) and Mossmann (1987). It is also hard to describe the development each membrane without reference to another, but as far as is possible, a self-contained account of the development of each extra-embryonic membrane is provided. For the fowl embryo, the spatial relationships of the extra-embryonic membranes and fluid compartments at around a third of the developmental period are illustrated in Figure 1.

Yolk sac membrane

The first of the extra-embryonic membranes to develop is the yolk sac membrane. Initially recognised as the *area vitellina*, this is the outer area of the blastoderm, consisting of three cell layers, which are a continuation of the cell layers of the embryonic disc. There is an ectodermal layer adjacent to the vitelline membranes, an endodermal layer adjacent to the yolk and in between a layer of mesoderm cells. The mesoderm is split into two by a cavity, the extra-embryonic coelom, and only the mesoderm next to the endoderm develops blood vessels (called the vascular mesoderm). It is these two cell layers that form the definitive wall of the yolk sac. The vitelline membranes enclose the yolk sac until day 4 of incubation. Contact of embryonic tissue with the vitelline membranes alters their structure (Jensen, 1969), probably facilitating their rupture when yolk sac volume increases at this time. This causes the yolk to lose its spherical shape and the embryonic-yolk structure adopts the shape of the egg. The top of this structure is bounded by the yolk sac membrane and the lower by the vitelline membrane, which slips down to the pole of the yolk sac opposite to the embryo. In this way the partition of the yolk sac from the albumen (the so-called yolk sac umbilicus) is maintained and at this "vegetal" pole, the yolk sac membrane remains incomplete until day 17.

The yolk sac membrane passes the equator of the yolk sac at 5-7 days and achieves its maximum area around 10-11 days finally surrounding yolk at 14-15 days. From day 12 the yolk sac membrane changes into a flabby three-lobed mass. After day 10-11 the area of the yolk sac membrane actually decreases, although the

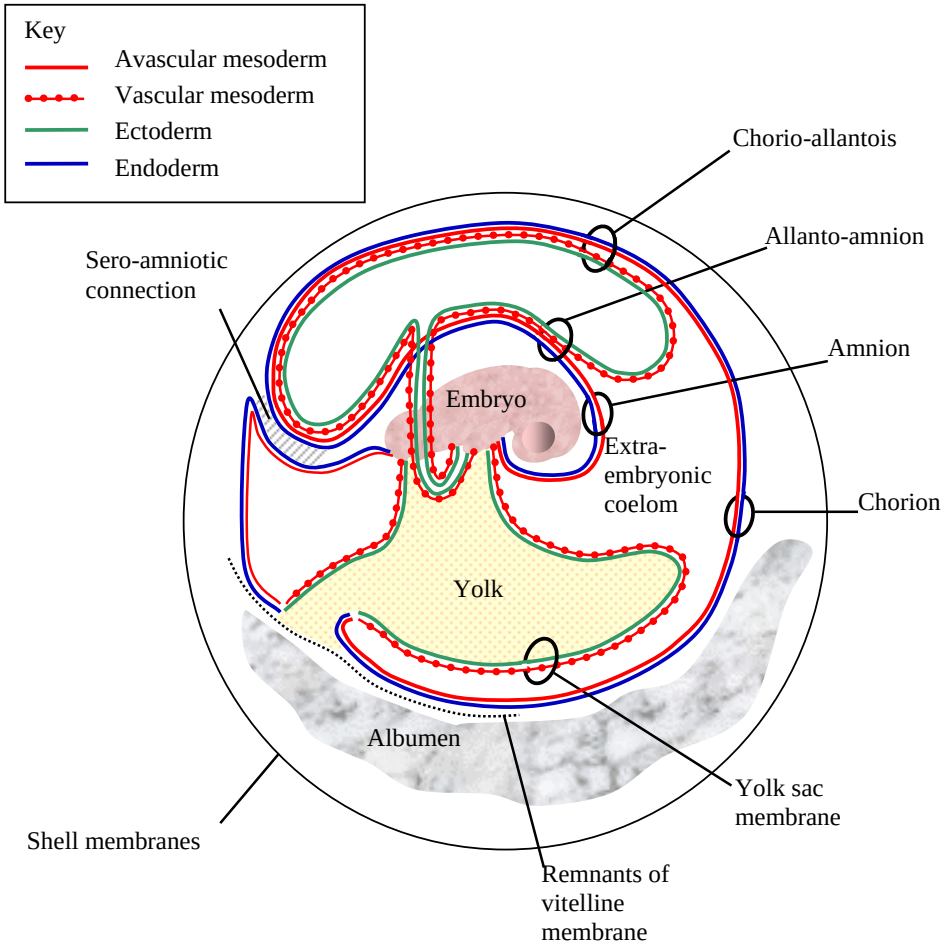

Figure 1. A diagrammatic representation of the extra-embryonic membranes and fluid compartments for the chick embryo around a third of the way through incubation. Note that the sizes of these structures, and their development relative to each other, have been modified to clarify types of cells present in each of the membranes.

membrane is still increasing in mass and reaches its peak weight at around 15 days (Table 1). This is due to a decrease in the size of the yolk sac as yolk is absorbed.

The primary blood system of the yolk sac membrane, the *area vasculosa*, is evident by day 2-3 of incubation when the vitelline arteries that carry blood from the heart to the periphery of the blastoderm become clearly visible (Figure 2). At the same time a vein develops at the margin of the blastoderm, the *sinus terminalis*. The blood in the peripheral capillary vessels empties into this vein, which carries blood anteriorly to the anterior vitelline vein, which then conveys blood back to the heart. A secondary blood system of new veins starts to appear whilst the primary blood system is

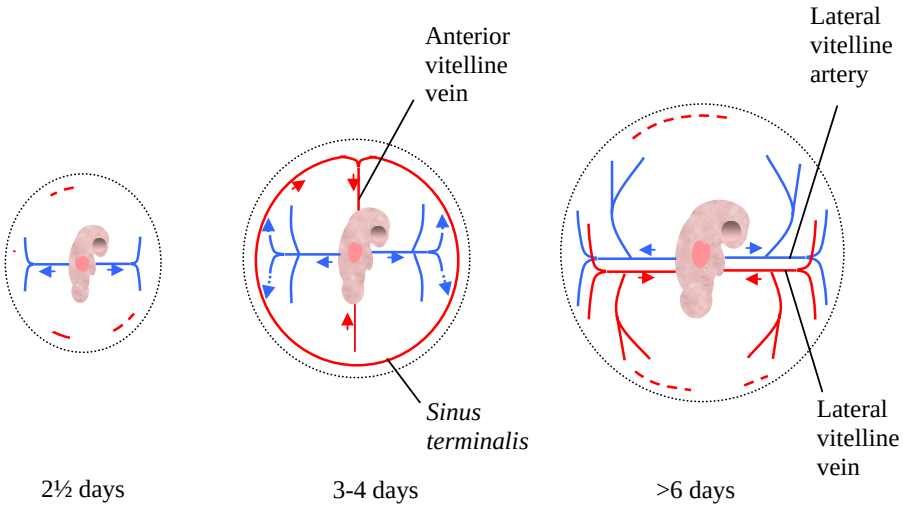

Figure 2. Development of the primary and secondary blood systems of the *area vasculosa*. Blood becomes oxygenated in the peripheral areas of the area, the vitelline veins carry oxygenated blood towards the embryo, and vitelline arteries carry deoxygenated blood away from the embryonic tissue.

still developing. By day 5, the lateral vitelline veins have grown parallel to the vitelline arteries, and in the peripheral capillaries, in which blood was formerly conveyed in a peripheral direction into the *sinus terminalis*, the flow is reversed. Blood now flows from periphery of the blastoderm to heart via the lateral vitelline veins, a more direct route. By day 6 the *sinus terminalis* starts to regress (Figure 2).

Amnion

The amnion is formed from a layer of ectoderm and underlying avascular (does not develop blood vessels) mesoderm immediately adjacent to the embryo. The flat tissue rises up to form folds over both the head and tail of the developing embryo and by day 4 of incubation the folds fuse together over the embryo to enclose the amniotic sac. Consequently, the inner layer of the amnion is ectoderm, and the outer one is avascular mesoderm. Muscle cells appear within this mesoderm so that the amnion becomes contractile by day 5, but the blood vessels of the amnion arise from the subsequent fusion of this avascular mesoderm with vascular mesoderm from the allantois. On day 12 a duct develops between the albumen sac and the amniotic sac; this sero-amniotic connection (Figure 1) is the point where the head and tail folds of the amnion met on day 4 of incubation. This duct allows the movement of albumen proteins into amniotic fluid where they are swallowed ending up in the yolk sac.

Chorion

The chorion develops from tissue continuous with the amnion and away from the embryo. It is comprised of an outside layer of ectoderm adjacent to the inner shell membrane and an inner avascular mesoderm, which lines the extra-embryonic coelom. Eventually this mesoderm fuses with vascular mesoderm of allantois, so supplying the blood vessels and forming the chorio-allantois. By day 11 the allantois has completely lined the chorion and the chorio-allantois covers 98% of the area of the eggshell membranes, thereby acting as the primary respiratory surface for the embryo during the second half of incubation.

Allantois

At day 2 the allantois is a small bud of endodermal cells and by day 4 it is visible as a free sac growing out from the primitive hindgut of the embryo. Its inner side is endoderm and the outer surface is vascular mesoderm. It expands into, and eventually fills, the extra-embryonic coelom, so that by day 6 its vascular mesoderm is fused both with the chorion, to form chorioallantois, and with the avascular mesoderm of the amnion. Hence, the allantois supplies the blood system for both the chorio-allantois and the amnion. The allantois is a fluid-filled and is a repository for kidney excretions that first appear by day 5 and enter via the allantoic duct from the cloacal region of the hindgut.

The Water Problem

Observations of many avian species have established that with a total water loss during incubation of about 20% of the initial egg mass, the water concentration of the hatchling (plus spare yolk) is very similar to the initial concentration in the egg at lay (Ar, 1991). This is because fat provides the energy source for embryonic growth and maintenance, and for this substrate the amount of water consumed is replaced by almost exactly the same amount of water produced by metabolism (Ar & Rahn, 1980). Thus the design of the avian egg produces "constant hydration", such that the chick has an appropriate tissue water content at hatching (Ar, 1991). So what then is the nature of the "water problem"?

At ovulation the ovum consists of a single cell containing large amounts of yolk, a store of nutrients and energy. In the upper part of the oviduct a second protein membrane is laid down over the primary vitelline membrane that contains the fertilised ovum (Bellairs, 1991). Subsequently, within the oviduct, albumen is added around the fertilised ovum, to act as a source of water, and finally the shell membranes and the shell are added around the albumen prior to oviposition (Gilbert, 1971). By the time of lay the ovum has undergone cell division to produce an embryo (Bellairs, 1991), which is physically separated from the water in the albumen by the vitelline membranes. The problem, then, is to ensure that this source of water is made accessible to the growing mass of tissue. Resolution of this difficulty is crucial for growth of the embryo.

At lay, 60% of the water in the egg is located in the albumen, which itself comprises 88% water (Romanoff, 1968). The tissues of the embryo contain about 70% water (Romanoff, 1968), and water must also be supplied to the extra-embryonic tissues. Most of the albumen water is transferred during the first half of the incubation period (Figure 3): about 76% albumen water has disappeared by day 10 of incubation (Table 1). During this period the total amount of solids in the albumen hardly changes at all (Table 1), indicating that the water is removed preferentially. By contrast, at lay the yolk sac contains 49% water and the water content of yolk changes by only 2% over this 10 day period (Table 1).

Over the whole period of incubation 28.6 g of water disappears from albumen and 7.2 from the yolk; 24.7 g appears in embryonic tissue and 2.5 g in the yolk sac membrane (Table 1). However, up to day 10 of incubation the water content of the embryo increases by only 2.2 gms (Table 1). Thus, the largest increase in water content of the embryo is in the second half of incubation, but by 10 days 76% of the albumen water has been removed. So where has it gone, and how? The answers to these questions lie in the formation and depletion of three extra-embryonic fluid compartments in the egg: the sub-embryonic fluid, amniotic fluid and allantoic fluid.

Sub-embryonic fluid (SEF)

Apparently, therefore, water does not move directly to embryonic tissue from albumen: in fact, during embryonic development water appears and disappears from a number of separate fluid compartments. The first new compartment becomes evident around 2-3 days of incubation, when a fluid appears in the yolk sac beneath the embryo, the sub-embryonic fluid. Its water content reaches a maximum at day 6 of incubation, when it is more than 95% water, and decreases in mass thereafter (Figure 3; Table 1). There is a "critical period" for the production of SEF between days 3-7 of incubation: if eggs are not turned during this period SEF volume is decreased and the formation of other fluid

Table 1. The mass of water and solids (dry mass) of the embryo of the domestic fowl for selected days during development (from Romanoff, 1968). The maxima are indicated in bold: either the maximum absolute water or solids content, or the maximum change, increase or decrease, in water or solids content (Δ water or Δ solids).

	water (g)	Δ water (g)	solids (g)	Δ solids (g)
Albumen				
Day 0	**29.9**		**4.1**	
Day 10	7.3	**– 22.6**	3.6	–0.5
Day 21	1.4	– 5.9	0.2	**–3.4**
Yolk sac				
Day 0	9.4		**9.9**	
Day 10	**9.6**	+ 0.2	8.8	–1.1
Day 21	2.2	**– 7.4**	5.1	**–3.7**
Embryo				
Day 1	0.00014		0.00006	
Day 10	2.2	+2.2	0.2	+0.2
Day 21	**24.7**	**+22.5**	**6.5**	**+6.3**
Yolk sac membrane				
Day 10	1.6		0.06	
Day 15	**3.8**	**+2.2**	**0.41**	**+0.35**
Day 21	2.5	–1.3	0.28	-0.13
Sub-embryonic fluid				
Day 2	0.6		0.02	
Day 6	**12.9**	**+12.3**	0.5	+0.5
Day 10	6.3	–6.6	**1.1**	**+0.6**
Amniotic fluid				
Day 7	1.2		0.01	
Day 10	2.8	**+1.6**	0.03	+0.02
Day 13	**3.7**	+0.9	0.13	**+0.10**
Day 19	1.6	–2.1	**0.15**	+0.02
Allantoic fluid				
Day 7	1.5		0.01	
Day 10	4.1	**+2.6**	0.05	+0.04
Day 13	**6.1**	+2.0	0.09	**+0.05**
Day 19	1.2	–4.9	**0.10**	+0.01

compartments and embryonic growth is retarded (Deeming, 1989a, 1989b). An absence of egg turning also reduces the growth of the *area vasculosa* over the yolk sac (Deeming, 1989c), and this is most likely the cause of the reduced amount of SEF found in unturned eggs (Latter & Baggott, 2001). The endoderm cells of the *area vasculosa* that face the yolk are specialised for the transport of water and sodium ions from albumen to yolk sac (Babiker & Baggott, 1995; Latter & Baggott, 2000), so with fewer cells less SEF will be produced. It is assumed that water from the SEF is distributed to the growing tissue, embryonic and extra-embryonic, by the blood system (derived from the vascular mesoderm), although nothing is known about this process. The transport of water into the yolk sac has an additional benefit: the yolk becomes lighter than albumen in which it is immersed and floats towards the upper surface of the egg, so placing

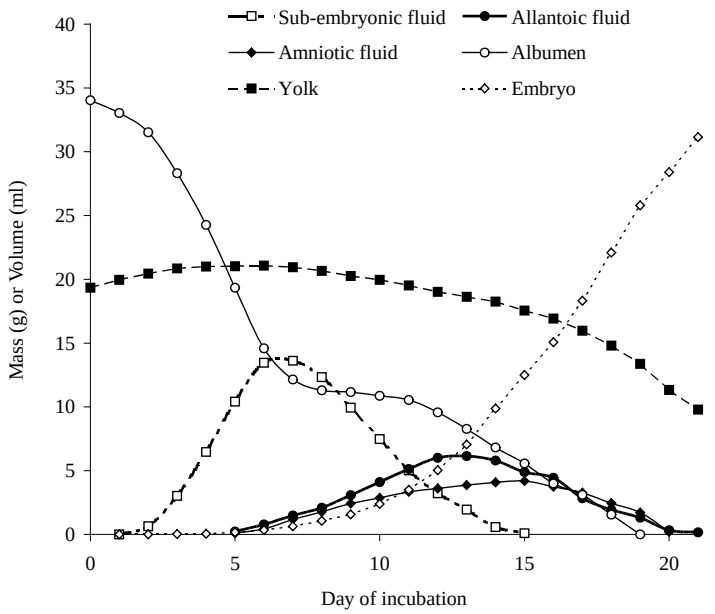

Figure 3. The pattern of changes in the mass of the embryo, yolk and albumen, and in the volume of the fluid compartments, of the developing fowl egg. Data from Romanoff (1968).

the *area vasculosa* just beneath the shell membranes (Babiker & Baggott, 1992). As the *area vasculosa* is also a respiratory organ (Lomholt, 1984), this process improves the access of the embryo to the air. In unturned eggs the yolk sac floats less and sits deeper in the albumen (Babiker & Baggott, 1992), an outcome that may contribute to the greater embryonic mortality of unturned eggs.

Amniotic fluid

Amniotic fluid appears later in development than SEF and achieves peak water content at day 13 of incubation; solids accumulate slowly throughout development (Figure 3; Table 1). Amniotic fluid has a unique ionic composition (high in chloride ions), which is responsible for the inflow of water into the amnion (Faber *et al*., 1973). Amniotic fluid volume is, apparently, unaffected by the hydration state of the egg (Faber *et al*., 1973; Ar, 1991); it is thought that the main function of the fluid is mechanical protection of the embryo. An absence of egg turning substantially decreases the mass of amniotic fluid from days 12-18 of incubation (Deeming, 1989a). This is thought to be caused by less albumen entering the amniotic fluid *via* the sero-amniotic connection, probably due to the altered spatial relationship between albumen, amnion and allantois in unturned eggs (Deeming, 1991). Additionally, by mid-incubation the albumen volume of unturned eggs is greater than that of turned eggs, which may also impede the transfer of albumen through the sero-amniotic connection (Deeming, 1991; Babiker & Baggott, 1992).

Allantoic fluid

Like amniotic fluid, the maximum allantoic fluid water content is found on day 13 with largest increase in water content preceding this (Figure 3; Table 1). The source of this fluid is blood filtered by the embryonic kidney. As for amniotic fluid, a lack of egg turning decreases the mass of this fluid compartment, but for allantoic fluid this reduction was detectable only on day 12 (Deeming, 1989a). Solids form only a small proportion of the allantoic fluid (Table 1), but can be important in determining its composition and

properties. Excretory nitrogen, as ammonia, urea and uric acid, are present in allantoic fluid at day 5, but it is predominantly the uric acid content of this fluid that increases during development (Fisher & Eakin, 1957).

It has been claimed that the rate at which water is absorbed from allantoic fluid varies with the degree of embryonic desiccation, thus producing a stable water and ion content of the embryo and fluid compartments (Hoyt, 1979). However, it is well established that embryos subjected to higher water losses during incubation have a lower tissue water content and a smaller allantoic fluid volume (Vleck, 1991). Also, investigators have repeatedly emphasised the potential for alteration of allantoic fluid composition by ion and fluid transport across the allantoic membrane into the blood. However, as pointed out by Murphy (1997), changes in allantoic fluid volume can be explained adequately only if urine inflow to the allantois, and fluid re-absorption *via* the allantoic membrane, are both measured. Certainly, ions (and water) are transported across the membrane from allantoic fluid (Vleck, 1991), and this process, as well as a reduction in urinary output from the kidney into the allantois, is most probably enhanced by the action of hormones (Murphy, 1997). However, the relative importance of these two processes in determining the volume of allantoic fluid remains uncertain. Moreover, the ionic composition of the allantoic fluid is determined by at least three factors: the capacity of the allantoic membrane to reabsorb water and ions, the composition of urine flowing in from the kidney, and the interactions between excretory uric acid and ions in the fluid.

For example, by 15 day of incubation, calcium, sodium and sulphate ions are all reabsorbed into the blood by kidney, so less are added to the allantoic fluid (Clark *et al.*, 1993). However, uric acid in the allantoic fluid also can change the ion composition of the fluid, as solid uric acid sequesters sodium ions: under dry incubation conditions uric acid excretion is increased, allantoic fluid contains more solid uric acid and more sodium ions are removed from the fluid, so increasing the potential for water re-absorption into the blood across the allantoic membrane (Bradfield & Baggott, 1993a, 1993b). The solid uric acid (with faecal material, meconium) is discarded in the eggshell at the time of hatching (Romanoff, 1968).

Conclusion

As is now clear, a number of fundamental aspects concerning the production of the fluid compartments of the avian egg remain to be explored. We are a little closer in understanding what factors, hormonal and environmental, influence the development of the *area vasculosa*, but still lack a complete account for a relatively simple environmental variable such as egg turning. We now know that both water inflow from the kidney and outflow, across allantoic membrane, determines allantoic fluid volume, but we are unsure of the relative importance of these processes, especially with regard to the hydration status of the embryo. Even more fundamentally, we have little understanding of the mechanisms whereby fluid moves between SEF, amnion, allantois and embryo. From this long list, the role of the *area vasculosa* in moving water from SEF to embryo would appear to be the process most amenable to experimental analysis.