

# Electrolyte and Water Balance of the Early Avian Embryo: Effects of Egg Turning

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## ABSTRACT

Formation of sub-embryonic fluid (SEF) is a key aspect of the physiology of the early avian embryo. Here we review the process of SEF formation and the factors which influence its composition and the rate of SEF production and depletion in the Japanese quail and domestic fowl. There is particular emphasis on the role of turning of the egg during incubation and we briefly consider the broader role of egg turning during avian incubation. The bulk of the review deals with the growth of the *area vasculosa* of the yolk sac membrane, the cellular processes of SEF formation, and the water and electrolyte physiology of the avian embryo during the first half of incubation. We conclude with a brief discussion of the areas for future investigation.

**Keywords:** sub-embryonic fluid, albumen, yolk, egg turning, water and electrolyte physiology, Japanese quail, domestic fowl

## 1. INTRODUCTION

The developmental period of the avian embryo can be split into two parts based around the extent of differentiation of the embryo. The first phase extends to approximately 50% of the incubation period and consists of morphogenesis and development of 95% of the tissues recognisable in an adult bird (Romanoff, 1960; Freeman and Vince, 1974). By the end of this differentiation phase the embryo resembles a bird, albeit a small one. The second phase of development, through to hatching, is one of growth and maturation of the existing tissues. Deeming and Ferguson (1991) suggested that these phases corresponded to the 'embryo' and 'foetus' stages of development in mammals. These stages of development are also characterised by differences in the pattern of extra-embryonic development. Hence, growth and expansion of the four extra-embryonic membranes (amnion, chorion, yolk sac membrane and allantois) occurs during the differentiation phase and these membranes are complete by the mid-point of incubation (Baggott, 2001). Similarly, there are changes in the fluid compartments within the egg such that sub-embryonic

fluid is formed and all but lost during the differentiation phase whereas the allantoic and amniotic sacs are expanding. There is a reduction in albumen volume during the first week of development but yolk volume remains largely unaffected during the first half of incubation (Romanoff and Romanoff, 1967). During the growth phase the extra-embryonic fluid compartments are drained and by hatching only the yolk sac remains although this has been retracted into the abdominal cavity of the neonate (Freeman and Vince, 1974).

Studies of the process of differentiation and growth of the embryo, and changes in the extra-embryonic components of the egg, often reflected descriptions of the normal condition as well as the result of conventional experimental manipulation of incubation temperature or humidity. However, during the 1980s lack of turning of the egg, incubated under otherwise normal conditions, was shown to have quite profound physiological effects on development of the embryo and its extra-embryonic environment. Failure to turn had significant effects on hatchability, growth rate of the embryo and extra-embryonic membranes, and rates

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of extra-embryonic fluid formation (see Deeming, 1991). In particular, lack of turning affected the production and composition of sub-embryonic fluid and growth of the *area vasculosa* of the yolk sac membrane. Hence, failure to turn eggs emerged as a new technique for manipulating embryonic development and allowed scope for further investigation of the normal development of the avian embryo.

This review describes the factors affecting the electrolyte and water balance of embryos of the domestic fowl (*Gallus gallus*) and Japanese quail (*Coturnix coturnix japonica*) during the first third of their incubation periods with the emphasis on changes in sub-embryonic fluid (SEF). As many of the techniques employed failure to turn eggs during incubation to investigate fluid dynamics, the overall effects of lack of turning on normal development are briefly described together with suggestions proposed for why there is a need to turn eggs during incubation. The bulk of the review concentrates on the interactions between the developing embryo and its extra-embryonic membranes, particularly the *area vasculosa*, with the yolk and albumen with respect to water and electrolyte regulation. As a conclusion, areas for further research in this field are briefly considered.

## 2. EGG TURNING AND THE AVIAN EMBRYO

A need to turn eggs during incubation to ensure good hatchability was recognised early in the development of artificial techniques for incubation and even in the earliest oven incubators eggs were turned by hand so as to mimic the behaviour of the bird. An early explanation was that turning by the bird allowed for re-distribution of the heat from the brood patch (de Reamur, 1751) is sometimes still suggested as being important (Caldwell and Cornwell, 1975). However, this is unlikely because turning is still required in force-draught incubators where temperature gradients do not normally exist in eggs (Drent, 1975). The long established and oft-quoted (Drent, 1975; Freeman and Vince, 1974; Skutch, 1976; Wilson, 1991) explanation for turning was that it prevents the embryo from adhering from the inner shell membrane (Dareste, 1891; Eycleshymer, 1906; New, 1957). An industry standard of turning once an hour through an arc of 90° (45° either side of the vertical) was established after considerable research during the 1930s–1950s into the frequency, angle and orientation of turning in artificial

incubators (reviewed by Lundy, 1969 and Deeming, 1991).

Such a situation did not suggest that any further investigation into egg turning would be profitable until the 1980s–1990s when the physiological effects of turning were investigated by examining development in unturned eggs of the domestic fowl and Japanese quail. A critical period for turning, between 3–7 days had been shown to be important by New (1957) and failure to turn during this critical period meant that hatchability and rates of embryonic growth were reduced even though the eggs were turned at all other times (Deeming, 1989a). It was also shown that turning after day 15 was not necessary (Wilson and Wilmering, 1988; Lourens and Deeming, 1999).

Early in development reduced levels of sub-embryonic fluid (SEF) were recorded in unturned eggs and the SEF that was present had a lower solid content (Deeming *et al.*, 1987; Wittman and Kaltner, 1988; Deeming, 1989a; Babiker and Baggott, 1992). Deeming (1989c) showed that removal of SEF from turned eggs led to a reduction in allantoic fluid at 12 days of incubation in the fowl but had little effect on amniotic fluid or embryonic growth.

Rate of growth of the *area vasculosa* of the yolk sac membrane during the first third of incubation was slower in unturned eggs (Deeming, 1989b). Later in development the pattern of growth of the chorio-allantoic membrane is disrupted in unturned eggs (Tullett and Deeming, 1987). Failure to turn eggs also affects the volume of the allantois and amnion and slowed the rate of embryonic growth (Tazawa, 1980; Tullett and Deeming, 1987; Deeming, 1989a, 1989b, 1989c, 1989d, 1991). For various reasons, the protein content of amniotic fluid is reduced in unturned eggs (Deeming, 1991) because less albumen proteins move through the sero-amniotic connection (Hirota, 1894; Deeming, 1991). Indeed unhatched, unturned eggs are characterised by residual albumen proteins in the bottom of the egg (Tullett and Deeming, 1987; Deeming, 1989a). Removal of albumen from 3-day-old, turned fowl eggs mimics the effects of failure to turn eggs with reduced allantoic and amniotic fluids at 12 and 14 days as well a reduction in embryonic growth rate during the second half of development (Deeming, 1989a).

Compared with control turned eggs, oxygen consumption and heart rate of unturned embryos were significantly lower during the second half of incubation (Tazawa, 1980; Pearson *et al.*, 1996). This

is probably a reflection of the retarded development of embryos in unturned eggs. By contrast, during actual turning events heart rate is usually increased (Vince *et al.*, 1979).

These results led Deeming (1991) to suggest that the physiological basis of egg turning lay in promoting access to the water and protein in the albumen is critical for normal development. This hypothesis predicted that bird eggs with differing amounts of albumen would require different rates of turning. Therefore, eggs producing altricial young, which have a high albumen content (relative to egg size), would require more turning than eggs from precocial species where relative albumen content is lower.

This hypothesis was recently tested by Deeming (2002) who collected rates of turning in nests from the literature together with hatchling maturity (developmental maturity), initial egg mass (IEM), incubation period ( $I_p$ ) and, where possible, details of egg composition for the species involved. For the 61 bird species represented, there were significant negative correlations with rate of egg turning (turns per hour during daylight) and IEM, and  $I_p$ . By contrast, as the developmental mode became more immature (*i.e.* more altricial) the rate of turning increased. Furthermore, for 27 species where egg composition was known there was a significant positive correlation between turning rate and the albumen composition of the egg. Deeming (2002) argued that this was strong support for the hypothesis that the physiological for egg turning was based around the need to ensure complete utilisation of the water and proteins in the albumen within the time scale of incubation.

Albumen plays two major roles during development of avian embryo. Firstly, albumen provides water and electrolytes for the embryo and, by the formation of SEF, the embryo is able to relocate these important nutrients within the egg. Secondly, albumen proteins are an important component of embryonic and post-natal nutrition. Turning has the most profound effects on the formation and composition of SEF and it was originally suggested by Deeming *et al.* (1987) that diffusion shells developed within the albumen, which caused a rate-limiting stage in the transfer of water and electrolytes from albumen into the yolk. This concept has stimulated considerable research into the factors affecting the rate of formation of SEF and this is reviewed hereafter. Given that SEF formation is intimately linked with growth of the *area vasculosa* this is considered first.

### 3. GROWTH OF THE AREA VASCULOSA

Early embryogenesis in amniotes is characterised by a parallel development of extra-embryonic membranes, which are extensions of the ectoderm of the body wall or the endoderm of the alimentary tract. The yolk sac develops as two distinct regions: the peripheral avascular *area vitellina*, and the central *area vasculosa*. Arising from the *area opaca* of the embryonic tissues the *area vitellina* is a bi-layer consisting of chorionic ectoderm overlying endoderm derived from the yolk sac membrane (Romanoff, 1960). Growth of the *area vitellina* is rapid with the vegetal pole being reached by day 4 of incubation in the fowl although its surface area continues to increase up to day 6 as it expands to accommodate the increasingly large volume of SEF (Grodzinski, 1934). It is movement of splanchnic mesoderm into the *area vitellina* that heralds development of the blood islands of Wolff, the first stages of development of the *area vasculosa* (Romanoff, 1960; Bellairs, 1963). Further expansion of the mesoderm leads to formations of distinct blood vessels and the area of vascularisation increases during the first half of development.

In eggs of the domestic fowl static incubation significantly impairs the expansion of the *area vasculosa*. Deeming (1989b) measured the expansion of the *area vasculosa* from 3 to 8 days of incubation in turned and unturned eggs. Static incubation did not affect the area measured on day 3, but by day 5 and all subsequent days a lack of turning had significantly reduced the expansion of the *area vasculosa*. Moreover, egg turning only during the critical period (days 3–7 of incubation) significantly increased the expansion of the *area vasculosa* when compared to unturned eggs. However, the size was smaller than in eggs turned throughout this period. Static incubation during this critical period in the domestic fowl had substantial effects later in embryonic development, including retarded formation of extra-embryonic fluids and reduced rates of embryonic growth (Deeming, 1989a)

Babiker and Baggott (1992) were unable to detect any effect of static incubation on the growth of the *area vasculosa* in eggs of the Japanese quail, from 36 to 108 hours of incubation. The reason for this remains unclear but may perhaps correlate with smaller egg size. However, Babiker (1991) found in this species that if the growth of the *area vasculosa* was restricted experimentally *in ovo* the amount of SEF produced

was reduced. After 24 hours of incubation a hole was cut in the shell and a 14-mm diameter glass ring was placed centrally over the blastoderm; the hole was then sealed with a glass coverslip and eggs incubated (unturned) for a further 48 hours. At 72 hours of incubation the control, sham-operated eggs without a ring had an *area vasculosa* with a mean diameter of 18.4 mm whilst the SEF mass was 0.945 g. The size of the *area vasculosa* and mass of SEF were typical for eggs after 60 hours of incubation, indicating that the treatment had retarded development by about 12 hours. However, eggs with a ring had a mean diameter of only 12.7 mm and the SEF mass was 0.672 g, values typical for eggs incubated for only 48 hours. Thus, although the experimental treatment retarded embryonic development, it is clear that the mass of fluid produced was directly related to the size of the *area vasculosa*.

Later in incubation development of other extra-embryonic membranes is also affected by turning. The size of the chorio-allantoic membrane (CAM) is smaller in unturned eggs at 19 days of incubation than in turned eggs (Tullett and Deeming, 1987). Ar and Sidis (2002) reported that the application of an artificial brood patch to fowl eggs for 12 and 24 h during the second half of the incubation caused a local reduction of *circa* 20% and 40%, respectively, in the number of blood vessels per unit area of CAM. These authors suggest this may also play a part in explaining the development of egg turning behaviour (see Deeming, 2002) as a requisite for normal embryonic development.

#### 4. FORMATION OF SUB-EMBRYONIC FLUID (SEF)

The appearance of a fluid within the yolk sac during early development could arise either from active processes, or could be due to passive water movement from albumen into the yolk. For the latter to occur the yolk must have a greater osmotic concentration than albumen, which is the case for the egg at lay (Bateman, 1932). However, osmotic equilibration of these two compartments in the fowl egg is achieved only slowly over long periods of storage at room temperature (Smith and Shepherd, 1931). In turned, unfertilised eggs of Japanese quail incubated for 144 hours at 37.6°C had increased the water content of yolk sac by 0.39 g (Babiker, 1991). By contrast, over the same period embryonated eggs of the Japanese quail

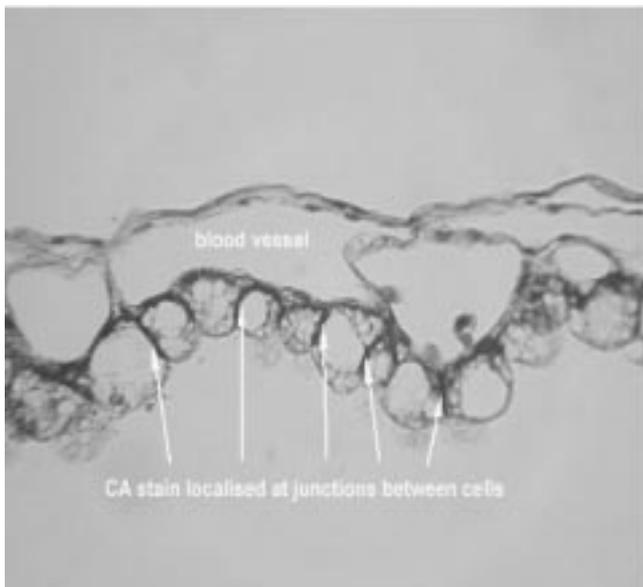
increased their SEF mass by an average of over 2.7 g (Babiker and Baggott, 1992). Clearly, the difference in osmotic concentration of albumen and yolk is at most a minor contributor to SEF accumulation within the yolk sac and most of the movement of water from albumen into the yolk sac must be ascribed to active processes.

New (1956) demonstrated unequivocally that the chick blastoderm secretes SEF: explants cultured solely on albumen produced SEF on their ventral (endodermal) surface. Babiker and Baggott (1995) and Latter and Baggott (2002) investigated the mechanisms determining fluid transport by Japanese quail blastoderms cultured on albumen. Babiker and Baggott (1995) found that SEF production was dependent upon sodium transport. When blastoderms, at 54 h and 72 h of incubation, were cultured on an albumen medium deficient in sodium SEF volume was diminished in proportion to the reduction in the sodium content of the medium. Even at the lowest albumen sodium concentration ( $[Na]$ ) of the medium,  $[Na]$  of SEF was maintained at about  $40 \text{ mM L}^{-1}$  greater than  $[Na]$  of the medium. This was not the case for potassium whose SEF concentration was always less than that of the medium.

In culture, ouabain, an inhibitor of the enzyme  $Na^+/K^+$ ATPase, substantially reduced SEF production, but only when added to the SEF (Latter and Baggott, 2000a; Latter and Baggott, 2002). Thus, a  $Na^+/K^+$ ATPase is apparently present on the plasma membranes of the endodermal cells facing the SEF. Earlier reports had identified a blastodermal  $Na^+/K^+$ ATPase in the chick embryo (Stern, 1982, 1991; Stern and Mackenzie, 1983), but this was located in the epiblast and found at an earlier stage of development. Latter and Baggott (2002) concluded that sodium is supplied to this ATPase by a passive sodium ion/hydrogen ion exchanger that is located on a plasma membrane facing the albumen. Amiloride, a known inhibitor of this exchanger (Benos, 1982), substantially reduced the volume of SEF produced, but only when added to albumen side of the blastoderm (Babiker and Baggott, 1995; Latter and Baggott, 2002). Thus, *in ovo* the accumulation of sodium in the SEF at concentrations in excess of those found in albumen (Howard, 1957; Babiker and Baggott, 1992, 1995) is a consequence of the movement of sodium from albumen into the transporting cell. This process is facilitated by a sodium ion / hydrogen ion exchanger on the albumen-facing side of the blastoderm, and is dependent upon the subsequent exit of sodium via a  $Na^+/K^+$ ATPase on

the SEF-facing side of the blastoderm. However, for this movement of sodium to be sustained protons must be supplied to the passive  $\text{Na}^+/\text{H}^+$  exchanger of the transporting cell.

Carbonic anhydrase, which catalyses the hydration of carbon dioxide so producing protons and bicarbonate ions (Maren, 1967), is also essential for production of SEF. Sulphonamide inhibition of this enzyme reduced SEF production in cultured quail blastoderms (Babiker and Baggott, 1995). Benzolamide proved to be the most potent inhibitor (Latter and Baggott, 2002). Of all sulphonamides this has the least ability to penetrate plasma membranes (Maren, 1982; Wistrand and Knuutila, 1989) and so Latter and Baggott (2002) concluded that in quail eggs SEF production was dependent primarily upon a membrane-associated isozyme of carbonic anhydrase. Indeed, histochemical staining for carbonic anhydrase confirmed this proposal, because carbonic anhydrase activity is localised to the lateral plasma membranes of the endodermal cells of the blastoderm (Figure 1; Latter and Baggott, 2002). This suggests that the predominant carbonic anhydrase isozyme of these

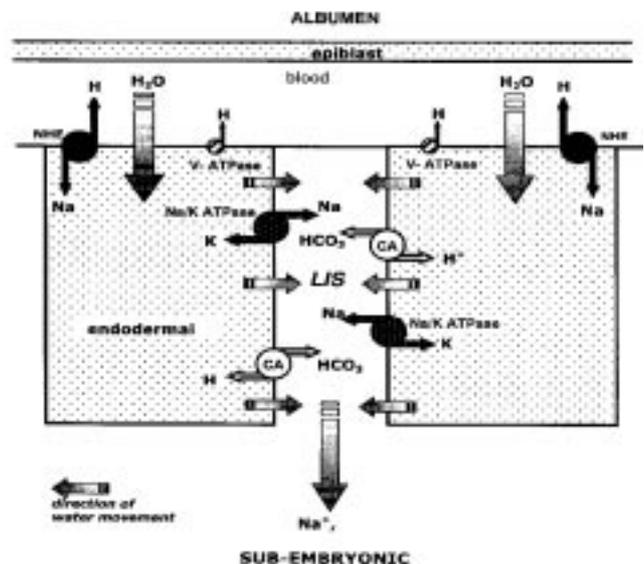


**Fig. 1** A transverse section of the blastoderm of the pheasant at Hamburger–Hamilton stage 18. The dark stain indicating the location of carbonic anhydrase activity (CA) is localised at the intercellular junctions of the endodermal cells. Note also the large vacuoles within the cells. The tissue was fixed in 2.5 % glutaraldehyde, embedded in the plastic resin JB4, and cut at  $20\mu$ . A modification of Ridderstrale's (1976) adaptation of Hansson's (1968) cobalt sulphide precipitation method was used to visualise carbonic anhydrase activity.

cells is the membrane-associated form, CA IV (Dodgson, 1991a).

A model of production of SEF (Latter and Baggott, 2002) is summarised in Figure 2 and proposes that hydration of carbon dioxide, catalysed by carbonic anhydrase, occurs in the endodermal cell of the blastoderm. This provides protons for the  $\text{Na}^+/\text{H}^+$  exchanger located on this cell's plasma membrane adjacent to the vascular endothelium. Thus in exchange for protons, the  $\text{Na}^+/\text{H}^+$  exchanger would furnish cytosolic sodium to the  $\text{Na}^+/\text{K}^+$  ATPase located on the basolateral membrane of the endodermal cell, so ensuring the transport of sodium into the SEF.

As exchangers for bicarbonate and chloride appear not to be present in the quail blastoderm (Babiker and Baggott, 1995; Latter and Baggott, 2000a, 2002), the fate of the bicarbonate ions produced by hydration of carbon dioxide remains unresolved. Latter and Baggott



**Fig. 2** A simplified model of the mechanism for sub-embryonic fluid secretion by the quail blastoderm (after Latter and Baggott, 2002). The stippled rectangles represent two endothelial cells enclosing a lateral intercellular space (LIS).  $\text{Na}^+/\text{K}^+$  ATPase and carbonic anhydrase (CA) are located on the endodermal cell lateral plasma membranes, and arrows show the proposed direction of ion movements:  $\text{HCO}_3^-$  and  $\text{Na}^+$  into the LIS;  $\text{K}^+$  and  $\text{H}^+$  into the cytosol. The sodium ion/hydrogen exchanger (NHE) located on the plasma membrane facing the blood vessels exchanges  $\text{H}^+$  for  $\text{Na}^+$ , the latter moving into the cytosol. There is a small outward movement of  $\text{H}^+$  due to V-ATPase on this membrane. Water is indicated as passing through the endodermal cytosol as large vacuoles are present in these cells.

(2002) suggested that the membrane-associated carbonic anhydrase of the endodermal cell possesses vectorial properties leading to the exit of bicarbonate ions into the SEF and the delivery of protons into the cytosol. They cite two pieces of evidence in support of this novel role for the enzyme. Firstly, *in ovo* the bicarbonate ion concentration of SEF increases between 60 and 72 h of incubation (Babiker and Baggott, 1991) and, secondly, transport of protons and bicarbonate ions in opposite directions has been reported for CA IV inserted into an artificial bilayer (Diaz *et al.*, 1982). Cytosolic carbonic anhydrase was not detected histochemically in endodermal cells (Latter and Baggott, 2002) making an intracellular generation of protons unlikely. This is in contrast to the role of carbonic anhydrase in kidney proximal tubule cells (Brown *et al.*, 1990; Dodgson, 1991b). Rather, the production of fluid and the transport of ions by the quail blastoderm resemble both these processes in the epithelial cells of the vertebrate gall bladder. These cells produce an isosmotic fluid utilising an apical  $\text{Na}^+/\text{H}^+$  exchanger (Reuss, 1989) and a membrane-associated carbonic anhydrase (Persson and Larson, 1986; Parkkila *et al.*, 1996). A proton-motive V-ATPase, an enzyme that is widely distributed in transporting epithelia (Wieczorek *et al.*, 1999), was also detected on the apical surface of the quail blastoderm (Latter and Baggott, 2000a, 2002), but was found to be of minor importance in SEF production.

Some aspects of ion transport and fluid production by the quail blastoderm remain unresolved. For example, Babiker and Baggott (1995) found that chloride-depleted media reduced substantially fluid production by the blastoderm in culture, yet neither furosemide, an inhibitor of  $\text{Na}^+/2\text{Cl}^-/\text{K}^+$  co-transport (O'Grady *et al.*, 1990), nor DIDS, an inhibitor of chloride/bicarbonate exchange, altered fluid production. For chloride transport across the blastoderm at least two alternatives remain to be investigated: the movement of chloride by paracellular route and/or a transcellular route (Zeuthen, 1992). In contrast to those blastoderms cultured on a sodium-deficient medium, those cultured on a chloride-deficient medium were unable to maintain higher concentrations of chloride, or sodium, in the SEF compared with the medium (Babiker and Baggott, 1995). This suggests that chloride acts as a counter ion for the sodium in the transport process and is additional evidence that it is sodium that is the actively transported ion.

It is striking that the blastoderm possesses the histoarchitecture necessary for the production of local osmotic gradients. The endodermal cells of the *area vasculosa* are separated by narrow, long lateral intercellular spaces (LIS, Figure 2) with tight junctions at their apical ends (Mobbs and MacMillan, 1979). Although in culture, blastoderms produced SEF with  $[\text{Na}]$  in excess of the  $[\text{Na}]$  of albumen on which they were cultured, there was no difference in the osmolality of SEF and albumen (Latter and Baggott, 2002), suggesting that fluid was generated by local osmotic gradients. The mechanism suggested for the quail blastoderm (Babiker and Baggott, 1995) would be similar to that reported for the mammalian gall bladder (Diamond, 1968). In this tissue isosmotic fluid transport is brought about by a standing osmotic gradient located in the LIS, with subsequent equilibration resulting in isosmotic fluid secretion (Tormey and Diamond, 1967). The action of sulphonamides and inhibitors of ion transport on SEF production was consistent with fluid generation by local osmotic gradients within the LIS (Babiker and Baggott, 1995; Latter and Baggott, 2002): when SEF production was reduced there was no detectable difference in osmolality of SEF and albumen.

These experimental observations contrast with measurements *in ovo*, where osmolality, as well as sodium concentration, of SEF has been reported to exceed that of the albumen (Howard, 1957; Babiker and Baggott, 1992). However, measurements of fluid osmolality *in ovo* and in culture are not necessarily strictly comparable. In culture, osmolality measurements are likely to represent those found at the blastoderm surfaces, whereas *in ovo* they may reflect values for fluid not necessarily adjacent to the blastoderm. Moreover, local osmotic gradients in LIS are able to produce a hyperosmotic fluid if the LIS are wide, short and the fluid transport rate is low, or if solute transport occurs over most of the LIS, rather than just at the apex (Diamond, 1979; Diamond and Bossert, 1967). So whilst, isosmotic fluid secretion *sensu stricto* may not occur in the blastoderm, the balance of evidence suggests that water flow from albumen to SEF is produced by local osmotic gradients within the LIS, rather than by a macroscopic osmotic gradient between SEF and albumen. For the quail blastoderm, Latter and Baggott (2002) suggested that the route for water flow through the endodermal cell is transcellular, as is the case for the gall-bladder epithelium (Persson and Larson, 1986). They based their conclusion upon

the presence of large intracellular vacuoles within the endodermal cells that they observed in their histochemical preparations, noting that the vacuoles had probably been fluid-filled before processing, as the tissues were embedded in a water-based medium.

In summary, it would appear that in early development a primary function of the endodermal cells of the blastoderm is the production of SEF, whereas formerly it was presumed that they were mainly concerned with the uptake of yolk (Mobbs and MacMillan, 1981). It is also evident that carbon dioxide plays an essential role in the production of this fluid because carbonic anhydrase plays a pivotal role in the generation of this fluid.

## 5. EFFECTS OF TURNING ON THE WATER AND ELECTROLYTE BALANCE OF THE AVIAN EMBRYO

### 5.1. Effect of egg turning on yolk buoyancy

In the domestic fowl the density of albumen increases as incubation progresses, although the density of SEF and yolk does not change (Meuer and Egbers, 1990). By contrast, in Japanese quail, the density of both SEF and yolk decreased as incubation progressed whereas the density of albumen increased (Babiker and Baggott, 1992). By 36 hours of incubation the density of SEF was  $1.004 \text{ kg L}^{-1}$ , which was less than yolk ( $1.0294 \text{ kg L}^{-1}$ ), and the yolk density itself was less than that of albumen ( $1.0356 \text{ kg L}^{-1}$ ). However, by 84 hours of incubation changes in the water content of albumen and SEF had increased the differences in density between these egg components. SEF density had decreased to  $0.9918 \text{ kg L}^{-1}$ , yolk density decreased to  $1.0196 \text{ kg L}^{-1}$ , whereas albumen density had increased to  $1.0708 \text{ kg L}^{-1}$ . Therefore, compared with eggs at 36 hours of incubation, the increased density of the albumen combined with the lower densities of yolk and SEF would lead to a greater buoyancy of the yolk sac in the albumen. Thus the blastoderm on top of the yolk sac would move closer to the shell (New, 1956; Romanoff, 1960) so facilitating respiratory gas exchange due to a thinner albumen layer above the embryo (Meuer and Baumann, 1987; Meuer and Egbers, 1990). Certainly, by 3 days of incubation in the domestic fowl the decrease in  $\text{Po}_2$  from shell membrane to blastoderm is very marked, with parts of the vascular system approaching a  $\text{Po}_2$  of zero, which contrasted markedly with much smaller gradient outside the *sinus terminalis* (Lomholt, 1984).

Although in Japanese quail the density of SEF of unturned eggs was no different from turned eggs (except at 96 h), unturned eggs had a smaller SEF volume, and by 84 hours of incubation a greater yolk density ( $1.0212 \text{ kg L}^{-1}$  in unturned *versus*  $1.0196 \text{ kg L}^{-1}$  in turned eggs). Moreover, the yolk sac of unturned eggs was immersed in albumen of a lower density ( $1.0652 \text{ kg L}^{-1}$  in unturned *versus*  $1.0708 \text{ kg L}^{-1}$  in turned eggs). The yolk sac of the unturned egg would, therefore, experience a lower buoyancy force, so would 'float' less readily in albumen, producing a thicker layer of albumen between yolk and the shell. It is significant that the effect of static incubation on densities of SEF, yolk and albumen is most marked after 72 hours of incubation when blastoderm growth is substantial, and when a failure to turn eggs of the fowl retards embryo growth and decreases hatchability (New, 1957; Deeming *et al.*, 1987; Deeming, 1989b).

Ar and Sidis (2002) suggest that failure to turn eggs may also lead to a localised shortage of water in the albumen overlying the blastoderm. Exacerbated by the loss of water vapour through the shell, the lack of water in the more concentrated albumen may be limiting ion transport and hence lower the rate of SEF formation.

### 5.2. Effect of egg turning on the mass of SEF and albumen

Sub-embryonic fluid is first measurable *in ovo* about 36 hours of incubation in the Japanese quail (Babiker and Baggott, 1992) or 48 hours in the domestic fowl (Romanoff and Romanoff, 1967). In the fowl egg Deeming (1989c) showed that SEF was significantly reduced by day 3 of incubation and thereafter, with the exception of day 4. Hence, at the end of the critical period of turning, 7 days in the fowl egg, there was around 10% less SEF in unturned egg (Deeming *et al.*, 1987). The SEF from unturned eggs had a lower solid content and a higher percentage water content (Table 1). This pattern was largely repeated for eggs of the Japanese quail, domestic turkey (*Meleagris gallopavo*) and domestic duck (*Anas platyrhynchos*) at an equivalent percentage of the whole incubation period (Table 1).

In the quail Babiker and Baggott (1992) measured the mass of SEF from 36 to 192 hours of incubation finding a peak at 108 hours of incubation. In this species the SEF mass for unturned eggs was lower at

**Table 1.** Effects of failure to turn eggs during incubation up to ~33% of the incubation period on mass, solid content and % water content of sub-embryonic fluid in eggs of the Japanese quail (Babiker, 1991; Babiker and Baggott, 1992), domestic fowl (Deeming *et al.*, 1987), domestic turkey and domestic duck (Deeming, 1989d)

	SEF mass (g)			SEF Solids mass (g)			SEF % water content		
	Turned	Unturned	Significance	Turned	Unturned	Significance	Turned	Unturned	Significance
Japanese quail (5 days)	3.22 ± 0.06	2.99 ± 0.03	*	0.0052 ± 0.0002	0.0042 ± 0.0002	*	94.8 ± 0.20	95.8 ± 0.20	NS
Domestic fowl (7 days)	15.47 ± 0.41	13.98 ± 0.48	**	1.48 ± 0.09	0.93 ± 0.05	***	90.50 ± 0.40	93.29 ± 0.34	***
Domestic turkey (8 days)	23.24 ± 0.45	21.63 ± 0.56	*	1.91 ± 0.13	0.89 ± 0.09	***	91.79 ± 0.53	95.93 ± 0.33	***
Domestic duck (9 days)	23.74 ± 0.66	18.24 ± 0.47	***	3.71 ± 0.13	2.54 ± 0.25	**	84.45 ± 0.80	86.24 ± 1.08	NS

Values are means ± SE. Significance determined by Student's two sample t tests (NS = not significant; \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ).

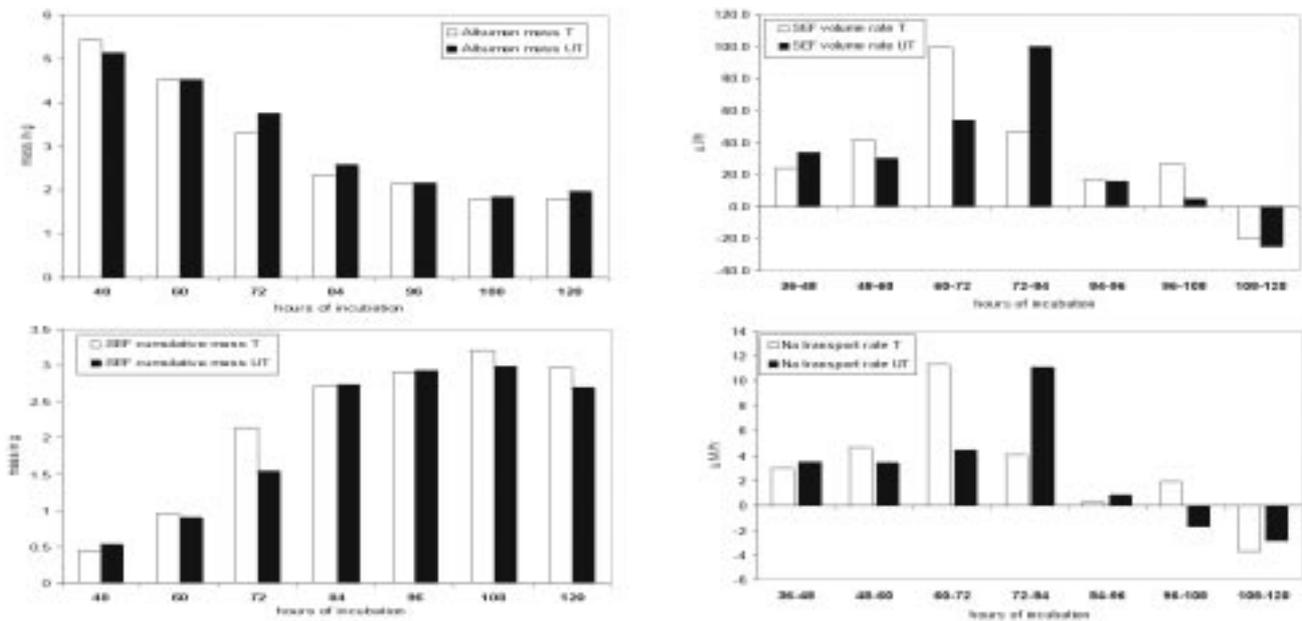
all times from 36 h to 120 h, but only significantly so at 72 hours, 108 hours and from 132–156 hours (Babiker and Baggott, 1992). However, it was the latter period when the fluid mass was decreasing and also the time when the dry weight of the fluid was substantially smaller in unturned eggs. In turned eggs the mass of SEF accumulated by 72 hours of incubation was greater than that in unturned eggs (Figure 3B) and the maximum rate of net SEF accumulation was greatest between 60–72 hours of incubation (Figure 3C). These differences between turned and unturned eggs in the accumulation of SEF were paralleled by decreases in the wet mass of albumen. At both 72 and 84 hours of incubation the wet mass of albumen of unturned eggs exceeded that of turned eggs (Figure 3A). Wittman and Kaltner (1988) also measured SEF volume in the Japanese quail, but only from 96 hours of incubation. Similarly, they found a peak in SEF for unturned eggs that was later than for turned eggs, although in their study it was very much later, at 120–168 hours of incubation, even though the decrease in albumen wet mass mainly occurred between 72 and 120 hours.

### 5.3. Effect of egg turning on the ionic composition of SEF, albumen and yolk

Deeming *et al.* (1987) were unable to show any significant effect of egg turning on the osmotic pressure, [Na] or potassium concentration ([K]) of domestic fowl eggs after 7 days of incubation. In the Japanese quail egg, the sodium concentration differed little over 60–72 hours of incubation (Figure 4A), this

was also the period during which there was the greatest net rate of accumulation of sodium in SEF (Figure 3D). Although after 84 hours of incubation the magnitude of the accumulated mass of SEF differed little between turned and unturned eggs (Figure 3B), prior to this time SEF accumulated more slowly in unturned eggs. For unturned eggs the peak rate of fluid accumulation occurred 12 hours later than in turned eggs, between 72–84 hours of incubation (Figure 3C). Likewise, the peak rate of net sodium movement into SEF for unturned eggs was recorded at this time (Figure 3D). After 108 hours of incubation the mass of SEF started to decrease (Babiker and Baggott, 1992) and there was a net loss of fluid mass and sodium from the SEF compartment (Figure 3D).

The composition of SEF and albumen from Japanese quail varied throughout this period, reflecting the substantial movements of ions that occur at this time. In turned eggs [Na] of the SEF increased between 36 and 48 hours of incubation (Babiker and Baggott, 1992; Figure 4A) and remained elevated until a decrease at 84 hours of incubation, despite a substantial increase in SEF mass. Albumen [Na] also showed no change over this period (Figure 4A). As albumen water content decreased substantially (by over 2 g) between 48–72 hours of incubation, there was a substantial movement of sodium out of this compartment at this time, as indicated in Figure 3C. This decrease in albumen mass was reflected in the increasing [K] in the albumen throughout the period 24–120 hours of incubation (Figure 4B). Chloride concentration ([Cl]) of the SEF exhibited changes similar to those of sodium, except that there was no



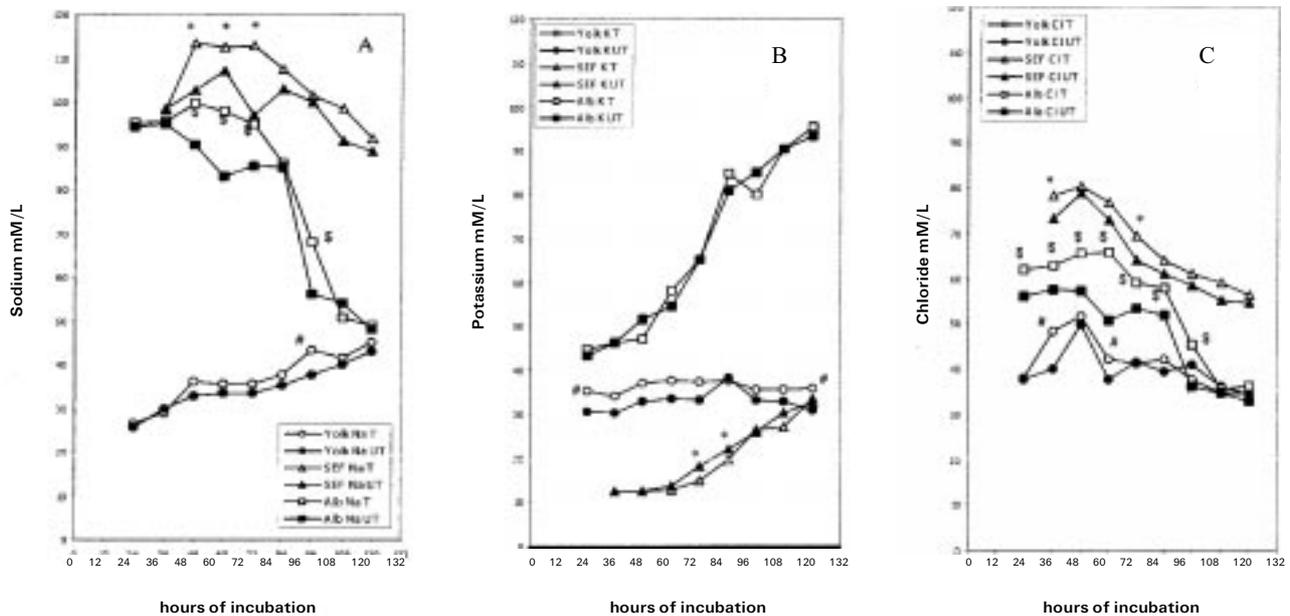
**Fig. 3** Changes in albumen mass (A), SEF cumulative mass (B), the rate of SEF volume produced (C), and the net rate of sodium transport into SEF (D) for incubation periods from 48 to 120 hours in the Japanese quail. Redrawn from Babiker and Baggott (1992) and Babiker (1991). T – turned; UT – unturned.

increase between 36 and 48 hours, as observed for [Na], and the subsequent decrease in concentration started at 72 hours of incubation. Albumen [Cl] changed little until 96 hours of incubation, again despite the substantial decrease in albumen mass (Figure 4C). It is clear, therefore, that sodium and chloride movements out of the albumen are sufficient so to not be reflected as an increase in their albumen concentrations; a situation that contrasts markedly with albumen [K].

It was between 48–72 hours of incubation when turning has the greatest influence on SEF and albumen composition in Japanese quail eggs. The [Na] of SEF of unturned eggs was lower than that of turned eggs for the whole of this period (Figure 4A); likewise [Na] of albumen was lower in unturned eggs. These changes in [Na] accurately reflect changes in the ion's activity as the activity coefficient for sodium throughout this period did not differ with period of incubation or turning treatment (range 0.777–0.781). In albumen, also, there was no detectable trend in the sodium activity coefficient with time or turning, although the value was lower (range 0.566–0.668; Babiker, 1991). The differences in [Cl] of SEF were less marked than for [Na]: turned eggs had a larger [Cl] than unturned eggs only at 36 and 72 hours of incubation, although the albumen concentration for this ion was lower for

unturned eggs throughout 24–84 hours of incubation. These differences in the albumen and SEF concentrations of sodium and chloride are consistent with the hypothesis that static incubation leads to a depletion of ions from the albumen. According to this hypothesis, as fewer ions are available for transport into the SEF in unturned eggs this fluid will have lower ion concentrations, which is observed.

Deeming *et al.* (1987) were the first to propose that static incubation leads to a depletion of sodium ions from 'unstirred layers' of albumen. Furthermore, they envisaged that the normal accumulation of SEF in turned eggs is due to the active transport of sodium into the yolk sac with water following osmotically. Hence, in unturned eggs, they suggested, a depletion of sodium from the layer of albumen adjacent to the blastoderm, due to a lack of 'stirring', would result in insufficient sodium for this process. Latter and Baggott (1996) investigated this proposal directly using eggs of the Japanese quail incubated from 24 to 72 hours. They tested the specific predictions that sodium would be depleted from the albumen adjacent to the yolk sac and that this would occur in eggs only where active sodium transport occurred, *i.e.* fertilised eggs. To test the first prediction, [Na] was measured in albumen adjacent to the inner shell membranes, as well as in albumen next to the yolk sac, for both sites at the



**Fig. 4** Changes in ion concentrations of albumen, SEF and yolk between 24 and 120 h in the Japanese quail. Redrawn from Babiker & Baggott (1992) and Babiker (1991). (A) Na – sodium; (B) K- potassium; and (C) Cl – Chloride; T – turned; UT – unturned; Yolk – yolk; SEF- sub-embryonic fluid; Alb – albumen. For SEF \*, for albumen \$, and for yolk # indicate that means for turned and unturned treatments differ significantly ( $P < 0.05$ ) at the period of incubation indicated.

yolk equator of the egg. Thus, if  $[Na]$  was greater in the albumen adjacent to the inner shell membranes, the difference,  $\Delta[Na]$ , would be positive.  $\Delta[Na]$  was always positive in both turned and unturned eggs, but was always larger for unturned eggs for all periods of incubation ( $+2.4 - +5.7 \text{ mM L}^{-1}$  in unturned eggs compared with  $+0.3 - +1.3 \text{ mM L}^{-1}$  in turned eggs).

To test the second prediction, Latter and Baggott (1996) measured  $\Delta[Na]$  at the vegetal pole of the yolk, where there was no blastoderm, and in unfertilised eggs with no embryonic development. Again in both these experimental groups,  $\Delta[Na]$  was positive in value and always larger for the unturned eggs. They concluded that static incubation depleted sodium from the albumen layers adjacent to the yolk sac, but that the depletion was not dependent upon the active transport of sodium, as predicted by Deeming *et al.* (1987). Rather, they suggested that depletion of sodium from this albumen layer arose because  $[Na]$  of albumen is substantially greater than yolk, of the order of  $44 - 52 \text{ mM L}^{-1}$  in turned or unturned eggs. Moreover, they reported that in unincubated eggs  $\Delta[Na]$  was about  $+3 \text{ mM L}^{-1}$ , a value that did not differ from that observed after 24 hours of incubation (Latter, 2000). Thus, it is apparent that even before

incubation is initiated  $[Na]$  of the albumen adjacent to the yolk sac is less than that of the albumen adjacent to the shell membranes. When eggs are incubated this difference persists, whether the embryo develops or not, but the effect of turning is to diminish the magnitude of  $\Delta[Na]$ . It is clear, therefore, that if eggs are fertilised turning will ensure a supply of sodium to the blastoderm for active transport by dissipating the 'unstirred', sodium-depleted layers of albumen adjacent to the yolk that are present at lay. It is, presumably, the mechanical effects of turning that are responsible for this, although the changes in albumen viscosity over this period in eggs incubated at  $38^\circ\text{C}$  (Romanoff and Romanoff, 1967) would presumably be a contributory factor.

For potassium the value of  $\Delta[K]$  did not differ for turned, unturned, fertilised or unfertilised eggs of Japanese quail during 24 to 72 hours of incubation: mean values ranged from  $+0.1$  to  $+0.28 \text{ mM L}^{-1}$  (Latter and Baggott, 2000b; Latter, 2000). As also observed for sodium,  $\Delta[K]$  of unincubated eggs and eggs incubated for 24 hours was the same (Latter, 2000). Thus, it is apparent that, unlike sodium, there is no layer of albumen depleted of potassium adjacent to the yolk sac in unturned eggs. Moreover, the increasing concentration of albumen  $[K]$  as incubation

proceeds suggests that it is the reduction in albumen mass that is the main factor determining albumen [K]. If this is so, it is perhaps surprising that the albumen [K] of turned and unturned eggs does not differ, as by 84 hours of incubation the albumen mass of unturned eggs remains larger than that of turned eggs. However, it is likely that potassium moves passively into the yolk sac, as albumen [K] exceeds yolk and SEF [K]. If this is so, differences in water content of the albumen due to turning treatment would not produce differing albumen [K]: an increase in [K] produced by a decreased albumen mass, for example in turned eggs, would concentrate potassium, but at the same time increase the passive movement of this ion out of the albumen. We would predict, however, that where turned eggs have a greater SEF mass than unturned eggs, a lower [K] would be found. In fact, SEF [K] of turned eggs first increased at 72 hours of incubation, when SEF mass of turned eggs was larger than that of unturned eggs, and [K] of SEF was less than that of unturned eggs at both 72 and 84 hours of incubation (Figure 4B). Also at 7 days of incubation there was no significant difference in SEF [K] in turned and unturned fowl eggs (Deeming *et al.*, 1987).

There are no published values for [Cl] of albumen adjacent to and distant from the yolk sac. However, in Japanese quail static incubation is known to decrease both albumen and SEF [Cl] during the period when SEF accumulation is maximal (see above), and that yolk [Cl] is lower than that of albumen (Figure 4C). This evidence points to the likelihood that unstirred layers of albumen depleted of chloride may be found next to the yolk sac. In the Japanese quail the osmolality of SEF increased markedly between 36 and 48 hours of incubation, when [Na] of SEF increased (Babiker and Baggott, 1992). Thereafter osmolality increased slightly up to 120 hours of incubation, as was also the case for albumen osmolality. However, the osmolality of SEF or albumen of turned eggs did not differ from that of unturned eggs during this period. Likewise, in the domestic fowl the osmotic pressures of SEF from turned and unturned eggs were not different at 7 days of incubation (Deeming *et al.*, 1987).

It is clear that changes in ionic composition of SEF are not faithfully reflected in the ionic composition of the yolk, and, therefore, that SEF constitutes a distinct compartment within the yolk sac. Whilst from 24 to 120 hours of incubation in Japanese quail, yolk [Na] and [K] were usually higher in unturned eggs than in

turned eggs, only three of these comparisons were statistically significant (Figure 4A,B), and did not correspond to significant changes in SEF ion concentrations. However, at 36 hours of incubation [Cl] of yolk of turned eggs was substantially higher than in unturned eggs (Figure 3C), a time when SEF [Cl] was higher the turned eggs, perhaps reflecting for this ion only the influence of SEF [Cl] on the yolk concentration.

### 5.3. Effect of turning on organic components of SEF

An obvious effect of the lack of turning is the reduction in the solid content of the SEF (Table 1). Deeming (1989c) attributed this difference to a reduced degree of disruption at the SEF-yolk interface that normally encourages yolk components to enter the SEF.

Babiker and Baggott (1992) and Babiker (1991) investigated the glucose, total carbohydrate, protein and lipid contents of SEF between 48 and 192 hours of incubation in eggs of Japanese quail. In both turned and unturned eggs glucose and total carbohydrate concentrations increased from their levels at 48 hours to peak at 72 h of incubation and declined thereafter. However, both glucose and total carbohydrate concentrations declined sooner in turned eggs, so that by 96 hours of incubation there was significantly greater glucose concentration in the SEF of unturned eggs, even though SEF volumes were not different. Wittman and Kaltner (1988) measured SEF glucose in Japanese quail from its peak value in turned eggs and thereafter, from 96 to 192 hours of incubation. The changes in glucose content reflected the more rapid disappearance of SEF in turned eggs, with unturned eggs having higher glucose contents of SEF in the period of 120 to 192 hours. It would appear likely that the lower concentrations of glucose in turned eggs reflect a greater utilisation, given that turning promotes embryonic growth and expansion of the *area vasculosa* (Deeming, 1989b) and that glucose is a primary source of energy early in development (Kucera *et al.* 1984).

After 48 hours of incubation protein concentrations were highest, and similar, in both turned and unturned eggs, but as SEF volume increased so the concentration declined substantially. In turned eggs by 96 h of incubation, however, SEF protein concentrations exceeded those in unturned eggs and this trend continued until 192 hours of incubation when SEF volume was decreasing. Likewise, in the domestic

fowl Deeming *et al.* (1987) found that protein concentrations of SEF was greater in turned eggs at 7 days of incubation. In the domestic fowl, the protein content of the SEF was significantly reduced from days 6 to 11 of incubation but protein concentration was not correlated with embryonic mass and Deeming (1989c) concluded that SEF-protein content was not of critical importance in the effect of turning on embryonic development. In Japanese quail, there were no consistent changes in lipid concentrations, either with period of incubation or turning treatment. However, the lipid concentrations were markedly higher in unturned eggs at 48 hours of incubation.

In summary, the main effect of static incubation on the organic components of the SEF was the delay in the changes in glucose and protein concentrations evident after their peak concentrations. In unturned eggs, glucose in the SEF declined at a later time than in turned eggs, and the protein concentration of the SEF increased later than in turned eggs.

#### 5.4. Why does static incubation reduce sub-embryonic fluid mass?

The evidence presented above demonstrates that static incubation has two major effects upon the developing blastoderm when secreting sub-embryonic fluid. First, an absence of egg turning results in a lack of mixing of albumen such that  $[Na]$  of albumen adjacent to the blastoderm is lower than that in turned eggs. Second, that static incubation reduces the rate of expansion of the *area vasculosa* during the critical period of SEF production. It is now clear that process of sub-embryonic fluid secretion requires both a  $Na^+/K^+$ ATPase, for the active transport of sodium, as well as membrane-associated carbonic anhydrase activity. Both these enzymes are located in the endodermal cells of the blastoderm. Thus static incubation would be expected to diminish the amount of fluid secreted as a consequence of reduced supply of sodium to the transporting cells of the blastoderm, and by a reduction in the numbers of these cells, due to the smaller size of the *area vasculosa* during the critical period for fluid production.

## 6. AREAS FOR FUTURE INVESTIGATION

The model proposed for SEF proposes a key role for carbonic anhydrase and so carbon dioxide is an important component of the process. Within the

oviduct there is a high level of dissolved  $CO_2$  in the egg and this begins to diminish after the egg is laid because of diffusive loss through the pores. The storage of eggs in air decreases the total  $CO_2$  content and the lower level of dissolved  $CO_2$  affects the acidity (pH) of the albumen and yolk and the environment within the egg becomes very alkaline (around pH of well over 9.0) (Cotterill *et al.*, 1958; Mueller, 1958). However, albumen pH can be a poor indicator of albumen  $CO_2$  content as an albumen pH in excess of 9.3 encompasses a wide range of  $CO_2$  contents (Mueller, 1958). Thus, the  $P_{CO_2}$  of the albumen, will, therefore, depend upon the  $CO_2$  buffering characteristics of the albumen, as well as the  $P_{CO_2}$  of the environment. Certainly, during prolonged egg storage the  $P_{CO_2}$  of the egg will be very low and the changes in the chemical nature of the environment surrounding the embryo may significantly influence the metabolism of the embryonic cells and cause cell damage. In the older literature, the reported effects of storing eggs in a  $CO_2$ -enriched environment on egg viability have not been consistent. For example, turkey eggs stored in  $CO_2$  before incubation exhibited decreased embryo viability when incubated (Becker *et al.*, 1968), but fowl eggs stored for 14 days with  $CO_2$  showed an increase in viability, and a lower albumen pH (Walsh *et al.*, 1995). However, it is not clear whether the known ability of  $CO_2$  to retard the thinning of albumen during storage (Cotterill and Gardner, 1957) is an important contributory factor to the changes in egg viability. More recently, the effects of holding stored eggs in a high  $CO_2$  environment were investigated by Sharon and Ar (1988) who stored eggs for 36 days in air. Two days before incubation started the eggs were either: (1) exposed to 95%  $N_2$  y 5%  $CO_2$ ; (2) 100%  $N_2$ ; or (3) just left in air as a control. Hatchability (of eggs set) of the three groups was 55%, 42% and 32% respectively (fresh eggs hatched at 84%) with the embryos exposed to the  $N_2$  y  $CO_2$  mix had lower early embryonic mortality than those only exposed to nitrogen alone. The high levels of  $CO_2$  mean that this gas diffuses into the egg and, by dissolving in the albumen, lowers the pH of the environment (from 9.6 to 7.9–8.1) making it more suitable for early development (Sharon, 1990). The reasons why replenishing the  $P_{CO_2}$  of the egg after storing is beneficial are unclear but it may reflect a need for a level of  $CO_2$  within the egg to be used by the carbonic anhydrase during SEF production. The role of partial pressure of

carbon dioxide (CO<sub>2</sub>) within the egg on SEF production is certainly an area for further investigation.

Egg turning is not restricted to incubation and during prolonged storage of poultry eggs it is often used to extend the viability of the embryo. As we have shown above low albumen [Na] adjacent to the yolk sac can lead to reduced production of SEF, and it would appear that this low level is present at lay. It is possible that it may well be beneficial to turn eggs during storage in order to minimise problems associated with development of these differences in [Na] but further study is required to confirm this idea.

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