Effect of local changes to shell permeability on the gas exchange of the avian embryo

This thesis is presented for the completion of the degree of
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Table of contents

List of tables, plates and figures

Tables

Plates

Figures

Abstract

The chicken embryo's ability to match the perfusion of its chorioallantoic membrane (CAM) to regional differences in shell conductance was investigated. Shell conductance was reduced locally by waxing one half of each egg. The impermeable shell area under the wax was balanced by an increase in the ambient oxygen fraction from 22.7% on day 10 to 31.0% on day 20 (wax/oxygen treatment). A second range of experiments (wax/hole treatment) was conducted at normoxia, where holes were drilled into the wax-free side, while overall shell conductance remained unchanged. They yielded very similar results. The resulting oxygen gradient indicated the low lateral diffusion under the shell.

Early chorioallantoic expansion was not altered by wax treatment. From day 10 to 15, the density of arterioles and venules (≤ 25 μm) was reduced under wax, independently of treatment timing. The free side of experimental eggs did not differ from controls. Similarly, capillary density and volume were reduced under wax, but did not differ between the free side of experimental eggs and controls. Barrier thickness during this time declined, apparently accelerated in experimental eggs. Capillary haematocrit was reduced under wax, possibly indicating a lower perfusion of these microvessels. On day 15, total blood volume under wax was somewhat greater than under the free side, which did not differ from the control eggs. The timing of the shell treatment had no effect on blood volume. Haemoglobin concentration of experimental eggs was lower than in the control eggs on day 15.

Although there was no observation of adaptation under the free side to compensate for the waxed side, the wax/oxygen treated embryos showed normal growth and respiration until day 20. Wax/hole treated embryos reduced mass and respiration in comparison to the control from day 15 and 16, respectively.

It appears that chorioallantoic blood flow in the CAM can be locally down-regulated, but not increased. As early embryos do not appear to reduce gas exchange and growth, adaptation may be unnecessary at this stage. Later oxygen uptake may then suffer, when vascular proliferation is inactive. The embryo may be relying on the even distribution of its shell conductance and is unable to adapt to regional changes.

Declaration:

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Kerstin Wagner

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1. Introduction

1.1 Gas exchange of the bird embryo

Bird eggs contain all the energy, solids and water needed for the development of the embryo (Romanoff and Romanoff, 1949; Ar and Rahn, 1980). The only resources that are exchanged with the environment are heat energy and some gases (Needham, 1931). The most important gases in this respect are the respiratory gases oxygen and carbon dioxide, as well as water vapour that is lost to the environment (Needham, 1931; Romijn and Lokhorst, 1951; Wangensteen and Rahn, 1970/71). These gases have to pass through the shell and the underlying outer and inner shell membrane (OSM and ISM, respectively), which split at the blunt end of the egg to form the aircell. The shell membranes consist of fibrous material that is loosely (OSM) to tightly (ISM) interlaced (Romanoff and Romanoff, 1949; Simons, 1971). Gas movement through these layers has to follow the interstitial spaces that are initially filled with water and dry progressively during the first half of incubation (Kutchai and Steen, 1971; Seymour and Piiper, 1988). Since the gas exchange ultimately is between the environment and the blood, the gases have to pass through further layers constituted by the albumen and the tissue of the respiratory membrane. During early incubation this respiratory organ is formed by the area vasculosa, a highly vascularised part of the yolk sac membrane. Later on, the chorioallantoic membrane (CAM) replaces the area vasculosa as the major gas exchange organ of the avian embryo.

The movement of the gases is almost exclusively driven by diffusion (Wangensteen *et al.*, 1970/71; Paganelli, 1980; Metcalfe *et al.*, 1981; Rahn *et al.*, 1987). It relies both on the inherent characteristics of the gas species and the medium (air, tissue) through which the gas moves, as well as on the difference in partial pressure of the relevant gas between the two sides of the barrier. The rate of gas flow, the diffusion rate, is proportional to the permeability of the barrier and to the partial pressure difference of the gas between the egg and the environment. It is best described by Fick's equation of diffusion (Rahn *et al.*, 1987).

The measure for the permeability of the barrier is called the diffusive conductance, the inverse of the diffusive resistance. The different layers of the barrier each have their own resistance to gas movement. They are commonly grouped into an outer barrier, formed by the shell and the outer shell membrane, and an inner barrier formed by the inner shell membrane, its limiting film and the tissue of the respiratory membrane (Wangensteen, 1972; Piiper *et al.*, 1980; Wangensteen and Weibel, 1982).

The major contributor to the diffusive resistance of the outer barrier is the eggshell. The outer shell membrane contributes only little resistance to the gas flow after it has dried (Wangensteen *et al.*, 1970/71; Paganelli *et al.*, 1978; Piiper *et al.*, 1980; Wangensteen and Weibel, 1982). Similarly, the inner shell membrane has a low diffusion resistance after drying, except for its limiting film (Kayar *et al.*, 1981; Tranter *et al.*, 1983; Visschedijk *et al.*, 1988; Ar and Girard, 1989). The film, however, increasingly fractures after the first four days of incubation, so that its resistance to gas movement is greatly diminished in the second half of incubation (Tranter *et al.*, 1983). The tissue barrier may also have only a very low resistance after the maturation of the CAM (Wangensteen and Weibel, 1982), and the greatest part of the resistance of the inner barrier to oxygen diffusion has been attributed to the oxygen binding in the blood (Piiper *et al.*, 1980; Wangensteen and Weibel, 1982). In the second half of incubation, the eggshell may thus provide the greatest resistance of the mechanical barrier for the gas flow to and from the avian embryo (Ar *et al.*, 1974).

As the calcareous material of the shell is impermeable to any gas, shell conductance depends on the number and size of its pores, and is inversely proportional to the shell thickness (Ar and Rahn, 1985; Rahn *et al.*, 1987). The shell conductance is constant in most species (Drent, 1970; Wangensteen *et al.*, 1970/71; Ar *et al.*, 1974; Ackerman and Rahn, 1980; Tazawa, 1980a), but may increase in some species during incubation (Carey, 1979; Hanka *et al.*, 1979; Sotherland *et al.*, 1980; Rahn *et al.*, 1983). This increase may be a consequence of shell thinning throughout incubation (Sotherland *et al.*, 1980; Booth and Seymour, 1987; Seymour *et al.*, 1987), or may be caused by the erosion of the organic cuticle on the egg surface (Rahn *et al.*, 1983). In case of the latter, the increase appears to occur usually in the first few days of incubation, and shell conductance remains relatively constant through the second half of incubation (Carey, 1979, Rahn *et al.*, 1983).

It has been suggested that a limited oxygen availability may restrict growth of the embryo in late incubation (Metcalfe *et al.*, 1981; Metcalfe *et al.*, 1984; Paganelli and Rahn, 1984). Oxygen appears to be a more important regulator of embryonic growth than carbon dioxide (Tullett and Burton, 1987), and its supply through a constant shell conductance can only be increased by lowering internal oxygen partial pressure (P_{O_2}), which raises the difference in P_{O_2} between the inside and the outside of the shell. However, internal P_{O_2} can only be lowered to a certain extent in order to increase the diffusion rate (Rahn *et al.*, 1974; Tullett and Deeming, 1982). The existence of oxygen

limitation was confirmed by Metcalfe *et al.* (1981), Visschedijk (1980), Stock and Metcalfe (1987) and Xu and Mortola (1989), who showed that enriching oxygen in the environment enhances embryonic growth rates. However, other authors argue that oxygen supply is abundant (C. Vleck et al., 1979; D. Vleck et al., 1980; Williams and Swift, 1988) and excess oxygen might even be detrimental to development (Smart, 1991; Ar and Mover, 1994). Nevertheless, oxygen availability is widely accepted as the primarily limiting factor for embryonic growth. Its limitation cannot be overcome simply by enhancing the conductance of the eggshell, for example by increasing the pore number or size, as this would also increase water loss (Rahn, 1984).

A certain amount of water loss during incubation is necessary (in most species) to maintain body composition (Hoyt, 1979; Rahn, 1984), to allow the formation of the aircell and to dry the shell membranes in order to improve gas exchange (Seymour and Piiper, 1988). However, water loss exceeding its optimum reduces growth and hatchability (Drent, 1975; Simkiss, 1980; Davis *et al.*, 1988; Deeming, 1995). The internal atmosphere of the egg is always saturated with water vapour (Paganelli *et al.*, 1981). Water loss, therefore, depends on the incubation temperature and nest humidity, as well as the shell conductance. Birds have not yet been shown to manipulate ambient (nest) humidity successfully (Walsberg, 1980; Rahn, 1984; Andersen and Steen, 1986), yet some breed in the dry climate of deserts, high altitudes or polar regions (Rahn and Ar, 1974; Sotherland *et al.*, 1980; Rahn *et al.*, 1983; Arad *et al.*, 1988). In very dry environments, these birds adapt to low ambient humidity at the expense of oxygen availability by reducing shell conductance (Carey *et al.*, 1982; Arad *et al.*, 1988), indicating that under certain conditions adaptation to water loss is more important for growth than the maximisation of respiratory gas exchange. Yet even under milder conditions a compromise between respiratory gas exchange and water loss must be reached (Ar and Rahn, 1985; Rahn and Paganelli, 1990).

1.2 Diffusion/perfusion inequality

As the necessity to regulate water loss limits the conductance of the outer barrier, namely the shell, conductance of the inner barrier to oxygen has to be maximised. In particular, oxygen uptake into the blood has to be optimised. Not only has the diffusion distance through the inner barrier to be minimised by bringing the blood in closest contact with the gas space of the ISM, perfusion of the respiratory membrane has to be sufficiently large to allow oxygen uptake. However, the embryo should not waste

energy on excessive perfusion, and the blood flow should therefore match the amount of gas leaving or entering the egg. Ideally, the match of gas conductance and blood flow would not only be found on the whole system level (egg), but on every level of organisation (West, 1978). Yet there are local differences of eggshell conductance and blood flow in the underlying chorioallantois. Visschedijk (1968a) was the first to observe differences of the respiratory exchange ratio between the gas exchange of the aircell and the remainder of the egg ("allantoic part", Visschedijk, 1968a). While the shell thickness remains constant, pore density was observed to decline from the blunt to the pointed end of the chicken egg (Rokitka and Rahn, 1987), and surface-specific conductance of the shell was found to be greater at the poles than in the equatorial region (Seymour and Visschedijk, 1988). This increases P_{O_2} and decreases partial pressure of carbon dioxide (P_{CO_2}) in the aircell compared to the allantoic part of the egg (Sotherland *et al.*, 1984; Booth *et al.*, 1987; Seymour and Visschedijk, 1988). Accordingly, the respiratory exchange ratio (RE) of carbon dioxide produced to oxygen consumed is lower in the chorioallantoic than in the aircell region (Booth *et al.*, 1987; Paganelli *et al.*, 1988). This suggests that the diffusion/perfusion ratios of these regions are not equal (Rahn and Paganelli, 1985; Paganelli *et al.*, 1988; Seymour and Visschedijk, 1988).

The heterogeneous distribution of oxygen and carbon dioxide levels inside the egg are possible because lateral diffusion of gases between the different egg regions is small (Visschedijk *et al.*, 1988), yet this does not explain the origin of the diffusion/perfusion mismatch, *i.e.* why blood flow is not adjusted accordingly. An explanation may be found in the consideration of the control of blood vessel growth. On a global level, vessel growth is inversely related to ambient P_{O_2} (Metcalfe *et al.*, 1984): eggs incubated in an hyperoxic environment have reduced density of arterioles and venules, whereas eggs incubated under hypoxic conditions have increased vessel density (Strick *et al.*, 1991). Regulation on a local level would require the opposite – in order to match diffusion and perfusion, vessel density and blood flow would have to increase under high conductance areas of the shell and decrease under low conductance areas. It has yet to be shown if the avian embryo has the ability to adapt to the local variability of the shell conductance, what mechanisms are available to the embryo to prevent or counteract diffusion/perfusion inequality, and what the stimuli are that evoke the response.

1.3 Aims

This study therefore aims to verify and describe the existence of gradients of P_{O_2} under the shell as a consequence of low lateral diffusion. This is supported by the comparison of direct measurements of P_{O_2} under the chorioallantoic part and the aircell of the egg, as well as by the observation of the distribution of P_{O_2} under a shell widely altered in conductance. In the presence of such differences, the adaptive mechanisms that are available to the embryo to counter regional differences in shell conductance are studied. These could be expected to centre on blood flow, vessel density and barrier thickness in the chorioallantoic membrane, as this is the respiratory organ during the phase of strongest growth of the embryo and in the final phase of incubation. The capacity of these measures to compensate for the locally altered shell conductance is evaluated by observing embryonic growth and respiration.

The basic model for this study was the chicken embryo, as hen's eggs are easily available from breeders. They were particularly useful for this study because chickens are precocial. In precocial embryos, gas exchange reaches a maximum after about 75% to 80% of incubation (C. Vleck et al., 1979), which in chicken embryos is on day 16/17 of the 21 day incubation period (Romijn and Lokhorst, 1951). In the subsequent plateau phase, gas exchange is relative stable and embryo growth decelerates. This phase is of particular interest for the study of the effect of conductance manipulation, as shell conductance may limit oxygen availability. In contrast to altricial birds (Bucher and Barnhart, 1984), the chorioallantoic membrane attaches to the inner shell membrane and completely encloses the egg half-way through incubation (Ackerman and Rahn, 1980). This allows for sufficient time to alter regional shell conductance and to observe its effect on the development of chorioallantoic growth and vascularisation before and after attachment of the chorioallantoic membrane.

2. General Methods

2.1 Egg incubation

Crossbred, fertile chicken eggs were obtained from a local breeder (Globe Derby Poultry) prior to incubation. Upon delivery, eggs were cleaned with a damp cloth, dried, weighed to the nearest mg (Mettler AE 163 Dual Range Balance), measured for length and width (to 0.1 mm) and stored at 13°C (*cf.* Haque *et al.*, 1996) for up to a week until the experiment commenced. The measured initial mass was compared to the fresh mass (M) calculated from length (L) and width (B) of the eggs according to Romanoff and Romanoff (1949):

$$M = 0.5632 \times B^2 \times L \qquad (2.1)$$

No significant difference between the two values was observed, and the initial egg weight was therefore accepted as fresh egg weight.

Except for the first day of incubation during which shell gas conductance was measured (see below), up to 50 eggs were incubated in a free convection incubator (Bellsouth 100 Electronic Incubator, Bellsouth, Narre Warren, Vic) set to 39°C at the upper egg level as recommended by the incubator manufacturer. This resulted in an average egg temperature of approximately 38°C. Incubator humidity was approximately 50% to 60%. Average barometric pressure was 102.1 ± 0.4 kPa (means and SD). From day 4, eggs were rotated along their long axis every 3 h. This was maintained until day 12 of incubation, when the turner was switched off. Incubation was continued until the termination of the experiments.

2.2 Measuring the conductance of the eggshell

At the start of incubation, eggs were transferred from the cool room to a desiccator (a sealed container with a bed of silica gel) inside a constant temperature cabinet at incubation temperature (38°C). Water vapour conductance of the eggs was measured according to the method of Ar *et al.* (1974): after 8 h equilibration time, eggs were weighed quickly and returned to the container for approximately 24 h, after which they were weighed again to the nearest mg. The period of equilibration ensured that the temperature of the eggs and the box environment were as defined by the experiment and that any surplus water in the shell and its membranes had been removed.

All weight loss was considered to be water loss (Ar *et al.*, 1974). Water vapour conductance of the shell (G_{H_2O}, in mg H_2O d^{-1} kPa^{-1}) was calculated using Fick's equation of diffusion, assuming an ambient water vapour pressure (P_{H_2O}, in kPa) of 0 kPa (0%) in the silica box and the P_{H_2O} under the shell as 6.62 kPa (100% relative humidity at 38°C). The weight loss ($\dot{M}_{H_2O}$, in mg d^{-1}) was normalised for 24 h and the water vapour conductance determined as:

$$G_{H_2O} = \dot{M}_{H_2O} / \Delta P_{H_2O} \qquad (2.2)$$

From these values, the shell conductance for oxygen was calculated in later experiments according to Paganelli *et al.* (1978): G_{O_2} [ml O_2 d^{-1} kPa^{-1}] = 1.057 × G_{H_2O} [mg H_2O d^{-1} kPa^{-1}].

Eggs were allocated to groups so that similar group means and distribution of fresh egg mass, overall shell conductance and mass-specific shell conductance (= G_{H_2O} /mass, *cf* Tazawa *et al.*, 1983) were achieved.

2.3 Wax/oxygen treatment

To create eggs with regional differences in gas exchange ability, the eggshell was covered on one side (pole to pole) with a gas impermeable material, whereas the other was exposed to an environment with increased oxygen levels. Several different materials were tested to reduce the conductance of one hemisphere to zero. In the comparison of acrylic paint, varnish (water based), silicone and different waxes, a dental modelling wax (Kerr Set-up wax No 5 hard, Sybron Corporation, California) that did not melt at 38°C, proved most suitable. It reduced shell conductance to 2.5% of the initial value in eggs completely covered with it.

Oxygen fraction in the environment was regulated to an amount that would double the oxygen partial pressure (P_{O_2}) difference across the shell, thus potentially doubling oxygen uptake in the CAM under the wax-free half of the shell (cf. sections 5.3.1.2 and 5.4.1). Water loss rate was maintained at approximately the original level by incubation in a dry environment (see below), while the partial pressure of carbon dioxide (P_{CO_2}) was left at atmospheric level. These experiments concentrated therefore on the effect of oxygen availability.

Shell conductance was measured on the first day, after which the eggs were incubated until day 10 as described above. Thereafter they were kept according to

treatment group until termination of the experiment (days 12, 14, 15 and 20): the control group was left untreated and unturned in the incubator, where the humidity was regulated to match the water loss of the experimental eggs (fractional mass loss until day 20: control: 14.3% ± 1.8%, experiment: 12.4% ± 2.2%, difference not significant). The experimental eggs were covered to 50% with wax as described above. To increase ambient P_{O_2}, up to eleven treated eggs were placed on silica gel in a sealed chamber inside a constant temperature cabinet at 38°C, where they were likewise not turned. The chamber was provided with a controlled flow of dry atmospheric air (1.5 l min^{-1}; Mass-Trak Flow Controller), to which a controlled flow of oxygen was mixed (*cf.* appendix 7.1 and section 2.6). The oxygen fraction (measured at the out-flow with the 280 Combo portable Oxygen and Carbon Dioxide Analyser, David Bishop Instruments) was raised daily (tab. 2.1) to the extent needed to double the P_{O_2} difference between the inside and the outside of the shell as determined in prior measurements of P_{O_2} under normoxia in the gas space under the shell (fig. 2.1; section 3.2).

Incubation day	O_2 fraction
10	0.227
11	0.244
12	0.256
13	0.257
14	0.264
15	0.274
16	0.279
17	0.281
18	0.291
19	0.293
20	0.310

Tab. 2.1: Fraction of oxygen in the gas mixture for the experimental eggs in wax/oxygen treatment.

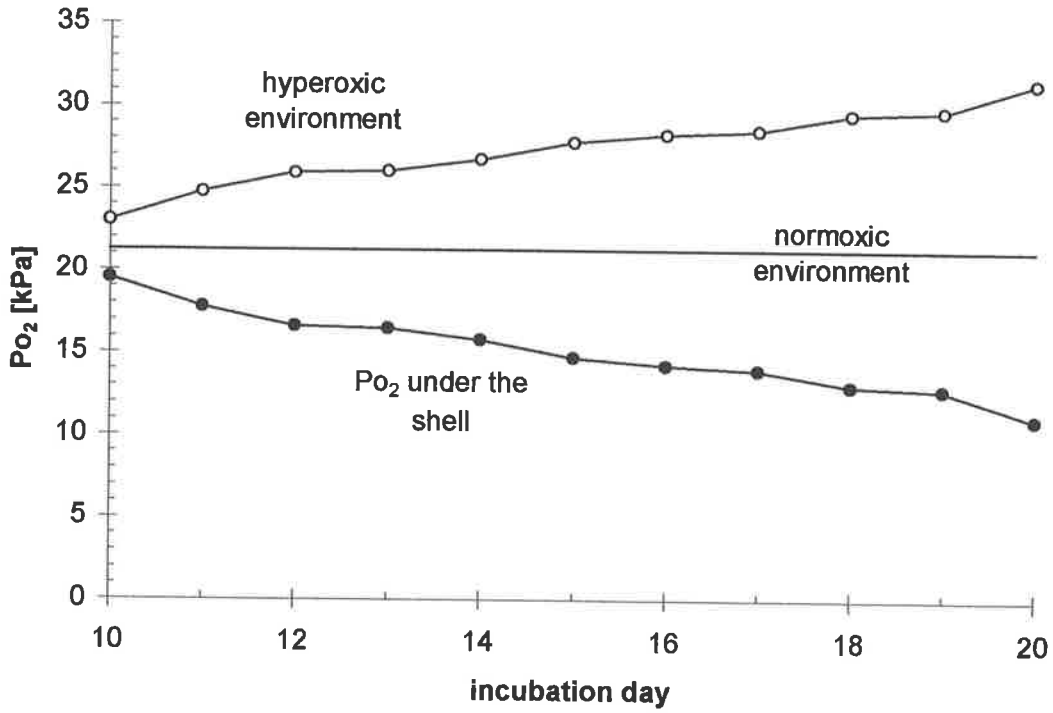


Fig. 2.1: Increase of oxygen partial pressure in the environment to match the decline of Po_2 under the shell.

2.4 Wax/hole shell treatment

2.4.1 Introducing artificial pores

In a different set of experiments, shell conductance was increased on one hemisphere (pole to pole) with artificial pores, to balance the loss of conductive surface area on the other side. Lateral movement of gases under the shell was then expected to smooth the transition and create a gradient.

Therefore one side of the egg was waxed as before, whereas shell conductance on the other side was increased by holes drilled through the eggshell. Since the total pore area of the chicken egg varies from 1.2 mm^2 (Wangensteen *et al.*, 1970/71) to 2 mm^2 (Wangensteen, 1972) (my own calculations for the eggs in these experiments showed an average around 1.42 mm^2 according to Ar *et al.* (1974), and 1.58 mm^2 according to Ar and Rahn, 1978), between 0.6 and 1 mm^2 of artificial pore area had to be created as compensation for the wax. Some experimentation was necessary to achieve a sufficient increase of shell conductance with the holes. Initially, two holes of 0.8 mm diameter, accounting for a surface area of approximately 1.01 mm^2, were drilled through the shell. However, they increased the total conductance only by 16% (13.98 mg d^{-1} kPa^{-1}, adjusted to 25°C) instead of the expected 50%. This may have been caused by the formation of a boundary layer. It has been calculated that the eggshell

pores have the largest possible diameter that does not create a boundary effect with a large resistance to gas movements (Rahn *et al.*, 1987). The artificial pores exceed this diameter greatly, so that diffusion through the pores is not proportional to the increase in their area. The impact of this is reduced under cooler conditions, and consequently the two holes increased the conductance almost twice as much in a measurement at 12°C (by 26.52 mg d^{-1} kPa^{-1}, adjusted to 25°C). Accordingly, the size of the drill bit was reduced to 0.6 mm, and four holes with a surface area of approximately 1.13 mm^2 increased the conductance sufficiently in some eggs, but only by about 40% in other (large, high conductance) eggs. The number of holes was therefore modified empirically to between four and seven holes of 0.6 mm diameter, depending on the initial conductance of the egg (see tab. 2.2). This compensated for the lost gas exchange surface under the wax in most cases.

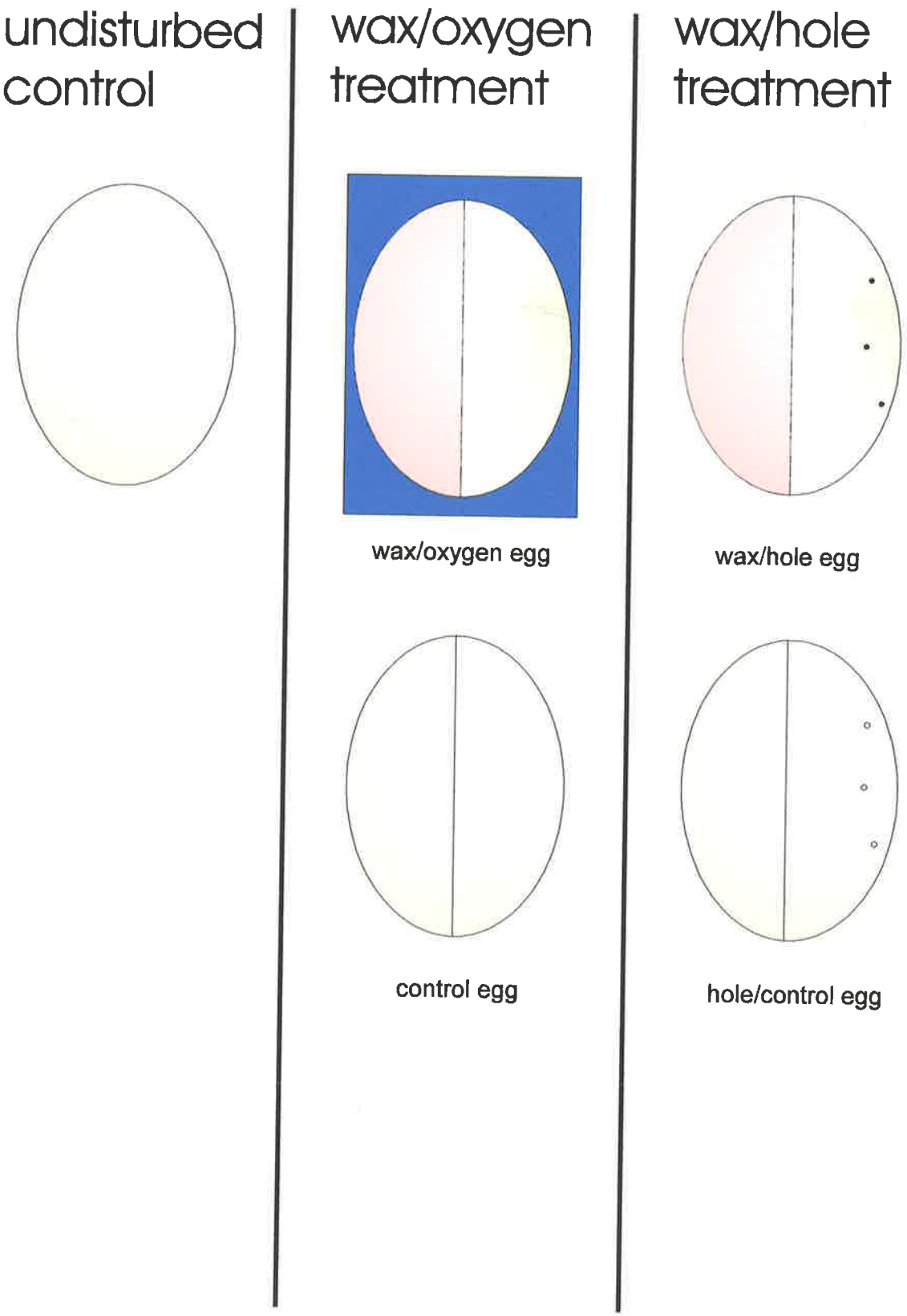
Initial shell conductance G_{H_2O} [mg d^{-1} kPa^{-1}]	Number of holes
< 80	4
$80 \leq G_{H_2O} < 95$	5
$95 \leq G_{H_2O} < 110$	6
$110 \leq G_{H_2O}$	7

Tab. 2.2: The number of holes (0.6 mm diameter) per egg drilled into shells in the wax/hole treatment depending on the initial shell conductance of the egg.

2.4.2 Experimental procedure

For the experiment of the effect of local differences in shell conductance (wax/hole treatment), a pencil line was drawn along the long axis of the egg. Molten wax was painted in a thin layer onto the lower side of the horizontally-held egg with a brush, thus trying to keep the heat (at that time about 50°C) away from the embryo. On the other half of the shell, the positions for the holes were marked in a circle with a pencil, with equal distance from the wax and each other (fig. 2A). The holes were drilled with a hand-held dental drill (12000 RPM, Renda, England) that had been fitted with a plastic sheath. This sheath prevented the drill-head from penetrating too far into the egg and wounding the membranes under the shell. The remaining shell debris was cleared from the artificial pores with sharp forceps and toothpicks.

Fig. 2A: Diagram of the different treatment groups and controls.



The procedural control eggs (hole/control) were handled in a similar way, i.e. the pencil line drawn and the egg handled as for waxing. The holes were drilled and then carefully filled with Araldite® (Selleys Chemical Company, Padstow NSW, Australia), a two-component epoxy adhesive. Between and after shell treatment, the eggs were placed into a box with silica gel and shell conductance was measured as described above. A second, undisturbed control was obtained from untreated eggs that were kept in the same incubators and desiccators. The treatments are illustrated in fig. 2A).

2.5 Experimental groups

Three different series of experiments were conducted, in which the effects of the wax/oxygen and the wax/hole treatment were observed during the second half of incubation, and the effect of the treatment timing on CAM adaptability was analysed (tab. 2.3).

Experiment	Treatment	day of treatment	day of termination
Series A	wax/oxygen	day 10	days 12, 14, 15 and 20
Series B	wax/hole	day 8	days 10, 12 and 14
	wax/hole	day 10	days 12, 14, 16, 18 and 20
Series C	wax/hole	before CAM attachment (days 1, 4, 6, 7 and 8)	day 15
	wax/hole	after CAM attachment (days 10 and 12)	day 15

Tab. 2.3: Overview of experimental groups according to shell treatment, day of experimental manipulation and day of termination

Series A: Wax/oxygen treatment - late incubation

After shell treatment on day 10 with wax, respirometry of individual eggs was done on days 12, 14, 16, 18 and 20 of incubation in the day 20 experiment, and just before termination in the day 12 and day 14 experiments. All eggs were placed into the

measuring chambers at the high P_{O_2} level determined for that day of incubation. This means that the control eggs experienced a high P_{O_2} environment for about 1 h on each of these days.

At the termination of the experiment, eggs on days 12, 14 and 15 were held for 15 to 20 s in liquid nitrogen which was found to be sufficient to freeze only the chorioallantoic tissue and blood, but not the inner compartments of the egg. After quickly cutting around the equator, the embryo and yolk sac were poured into a bowl, and the chorioallantoic arteries and vein were severed. Shell pieces with the CAM attached were removed for further measurements. Some were placed into a fixative (2.5% glutaraldehyde in phosphate buffer, appendix 7.2.1) for microscopic analysis (see chapter 4). For photometric analysis of the blood volume, the chorioallantois was separated from the shell pieces and stored in Drabkin's solution (Oser, 1965) (appendix 7.3.1). The shell pieces were stored in distilled water for later estimates of area (see section 4.2.3). The yolk sac was separated from the embryo and weighed to the nearest mg after external fluids had been drained. The embryo was separated from its membranes, blotted on paper tissue and weighed to the nearest mg. Both yolk sac and embryo were frozen in a freezer at -10°C and later freeze-dried. The position of the aircell was noted.

Embryos in the day 20 experiment were killed by breaking the neck with a haemostat, the yolk was extracted from the body cavity, and wet and dry mass for embryo and yolk were measured as described above.

Series B: Wax/hole treatment - long incubation

Some eggs were treated with wax and holes on day 8, and the development of the chorioallantoic membrane was compared with a procedural and an undisturbed control at the ages of 10, 12 and 14 days. In a second set of experiments designed to assess the impact of shell manipulation on embryonic growth during late development, eggshells were treated with wax and holes on day 10. The oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) were measured at days 12, 14, 16, 18 and 20 of development. On each of these days, a group of these eggs was opened along the equator immediately after measurement. The embryos were killed and separated from the membranes as above. In the day 20 group, the yolk was also extracted from the body cavity. Embryos and yolk were weighed to the nearest mg and freeze dried for determination of dry-mass.

Series C: Wax/hole treatment - timing of treatment

To compare the importance of stage of development at the time of experimental manipulation (i.e. at which age can the chorioallantoic membrane (CAM) still adapt to conductance changes), the wax/hole treatment was applied before (on days 1, 4, 6, 7, 8) and after (on days 10 and 12) CAM attachment to the inner shell membrane. As eggs were not turned in the first three days, wax/hole treated eggs on day 1 were positioned in the incubator so that the wax was on the side. This caused the embryo to be positioned in the middle between the high and the low gas exchange areas. The embryonic respiration ($\dot{V}O_2$, and $\dot{V}CO_2$) was measured on the day before and the day after shell treatment, as well as on days 10 and 13 if these days were not already covered by the treatment.

All experiments were terminated on day 15, when the development of the CAM and its vasculature is largely complete (Fitze-Gschwind, 1973; Strick *et al*, 1991; Burton and Palmer, 1992; Kurz *et al.*, 1995). For this, eggs were opened after a short (15 to 20 s) immersion in liquid nitrogen, and samples of the CAM, the embryo and yolk were taken as described for Series A.

2.6 Respirometry

2.6.1 Measurement

Oxygen consumption and carbon dioxide production of the embryos were measured using the 280 Combo portable Oxygen and Carbon Dioxide Analyser (David Bishop Instruments) in an open flow system operating on pressurised atmospheric air that had been dried (Drierite) and freed from carbon dioxide (Soda lime). A diagram of the setup can be seen in appendix 7.1. The oxygen analyser was calibrated against nitrogen and the dry, pressurised air. The carbon dioxide analyser was calibrated against the oxygen analyser with an ethanol flame. However, the calibration failed for very low flow rates, as its range was very narrow and not linear, resulting in an overestimation of the carbon dioxide production in young embryos.

The eggs were kept in sealed 185 ml polystyrene jars with removable lid inside a 38°C constant temperature cabinet that also housed the gas analyser for stability. Gas entered and left the egg chamber through PVC tubes in the lid. The flow rate was controlled with Model 810C Mass-Trak Flow Controllers (Sierra Instruments, Montery, USA) set for a flow rate of 70 ml min^{-1} (days 5 to 10) or 100 ml min^{-1} (after day 10). This replaced the gas volume inside the chamber every 4 - 5 min. After an initial

equilibration period of 15 min, oxygen and carbon dioxide fraction in the sample air were measured simultaneously every 10 s, alternating between three sample containers and a control channel every 5 min. The cycle was repeated three times. For each data point, eight measurements were averaged by the data collection software (acquisition program, Datacan V5.1, Sable Systems, Henderson, USA).

2.6.2 Analysis of respirometric measurements

The data were analysed using the analysis program of the Sable Datacan software. The stable phase of each 5 min signal (approximately 20 data points, minimum of 15) was averaged, and the three measurements per individual averaged again after the means had been corrected for a shift in the control value. Rates of oxygen consumption ($\dot{V}O_2$)and carbon dioxide production ($\dot{V}CO_2$) were calculated from these data using two iterative equations:

$$\dot{V}O_2 = [\dot{V}_i * (F_{i\text{-}O2}) - F_{e\text{-}O2})] / [1 - ((1 - RE) * F_{e\text{-}O2})] \quad (2.3)$$

$$\dot{V}CO_2 = [\dot{V}_i * F_{e\text{-}CO2}] / [1 + ((1/RE - 1) * F_{e\text{-}CO2})] \quad (2.4)$$

where $\dot{V}_i$ is the initial gas flow, $F_{i\text{-}O2}$ is the fraction of oxygen in the in-flowing air (0.2095), $F_{e\text{-}O2}$ the fraction of oxygen in the out-flowing air as measured, and $F_{e\text{-}CO2}$ is the fraction of carbon dioxide in the out-flowing air as measured. For a first estimate, the respiratory exchange ratio (RE) was assumed to be 0.8 and the equations were solved for $\dot{V}O_2$ and $\dot{V}CO_2$. From these values a new RE was calculated and the process repeated. The values stabilised by the third solution to these equations and the final RE was calculated from these.

3. Oxygen partial pressure under the eggshell

3.1 Introduction

Conductance of the chicken eggs to water vapour is constant throughout incubation (Drent, 1970; Wangensteen *et al.*, 1970/71; Ar *et al.*, 1974; Ackerman and Rahn, 1980). However, oxygen conductance increases in the first half of incubation as the shell membranes dry progressively (Kutchai and Steen, 1971; Lomholt, 1976), until day 11 of the 21 day incubation period, when practically all the interstitial spaces (65% of the volume) are filled with air (Seymour and Piiper, 1988). The drying of the shell membranes is important, as oxygen diffuses through air much faster than through liquid water. From day 12 onwards, the ratios of oxygen, carbon dioxide and water vapour conductance of the egg are similar to that of the diffusivity of these gases, indicating that the gases use the same pathways through the shell and membranes (Paganelli *et al.*, 1978). Once the shell membranes have dried, the diffusive conductance of the barrier of shell and shell membranes is fixed, but oxygen consumption of the embryo continues to increase as the embryo grows (Needham, 1931; Romijn and Lokhorst, 1951). This increase is partly supported by the maturation of the chorioallantoic membrane, the gas exchange organ of the bird embryo during the second half of incubation, and further by the continued increase of chorioallantoic blood flow (Tazawa and Mochizuki, 1976, 1977), haematocrit, haemoglobin concentration (Tazawa *et al.*, 1971b), and haemoglobin oxygen affinity (Reeves, 1984). As a consequence of increasing oxygen uptake, oxygen partial pressure (P_{O_2}) in the gas spaces of the egg continues to decrease (Romijn and Roos, 1938; Wangensteen and Rahn, 1970/71; Tazawa *et al.*, 1980).

The gas spaces of the egg are formed between the shell mamillae, in the shell membrane interstices and in the aircell. These different compartments are connected with the shell pores and each other, and for this reason it has previously been assumed that the same P_{O_2} can be found throughout the system (Wangensteen and Rahn, 1970/71; Rahn *et al.*, 1974; Paganelli *et al.*, 1978; Tazawa, 1980a; Piiper *et al.*, 1980). However, Visschedijk *et al.* (1988) calculated that the lateral diffusion coefficient of oxygen through the shell membranes is only 43% of the value through air. They calculated that the diffusive conductance in perpendicular direction over an area of 1 mm^2 (approximately the area serviced by one shell pore; Paganelli, 1980; Ar and Rahn, 1985; Rahn *et al.*, 1987) and over a diffusion distance of 0.0076 cm (thickness of the compound shell membrane) would be 173 times greater than in the lateral direction

through the same block of compound shell membrane over a diffusion distance of 0.1 cm and an area of 0.1×0.0076 cm^2.

The authors ignored the possible effects of the limiting film on the inside of the inner shell membrane (Simons, 1971; Wangensteen and Weibel, 1982; Tranter *et al.*, 1983) and assumed that the shell membranes are isotropic in regard to diffusion, i.e. gas permeability in perpendicular and lateral direction are the same. Ar and Girard (1989), taking the limiting film into account, refuted the assumption of isotropic diffusion characteristics of the membranes, and instead calculated that the Krogh's diffusion constant in the shell membranes is 100 times larger in lateral direction than in perpendicular direction. However, lateral diffusive conductance to or from a point source (abstraction of a pore) is independent of the area supplied, whereas perpendicular conductance increases with the surface area of the region under observation. Lateral conductance would therefore be only large enough to allow significant lateral gas movement over the service area of one pore. This is sufficient to achieve a "quasihomogenization of gas pressure…among adjacent pores" while the pores are closely spaced and free, but would not suffice if pore density is low, or if small areas of shell are blocked (Ar and Girard, 1989).

A homogeneous composition of the egg's gas spaces therefore depends partly on a similar shell conductance in all areas. While the shell thickness is constant, pore density declines from the blunt end to the pointed end of the egg and causes differences in local shell conductance (Rokitka and Rahn, 1987). A different result was obtained by Seymour and Visschedijk (1988), who found that surface-specific gas conductance was greater at the poles than at the equator, yet this still attests to local differences. Consequently, regional P_{O_2} may differ from the egg average in correlation with the local shell conductance, if the perfusion in the CAM does not match the local diffusive conductance. Some regulation of the chorioallantoic perfusion to match local conductance may be available to the embryo, as Seymour and Visschedijk (1988) calculated from their data a relative increase in perfusion under the aircell of 12%. However, this was insufficient to compensate for the difference in shell conductance, and the authors calculated a greater P_{O_2} in the aircell than in the reminder of the air space under the shell. This agrees with the experimental results of Booth *et al.* (1987) and Meir *et al.* (1999).

To obtain the results presented above, former researchers had to rely on calculations and intrusive or cumbersome methods. Conventional techniques used to

measure the gas composition of bird eggs generally require relative large volumes of gas (Wangensteen an Rahn, 1970/71; Rokitka and Rahn, 1987; Meir *et al.* 1999), perforation of the eggshell (Wangensteen and Rahn, 1970/71; Meir *et al.*, 1999) and large hypodermic needle hubs and attached syringes (Tazawa *et al.*, 1980), which make measurements difficult and can limit them to the aircell. To measure gas composition in other parts of the egg, some methods are intrusive, such as the artificial aircells employed by Sotherland *et al.* (1984) and Booth *et al.* (1987), or the removal of shell used by Meir *et al.* (1999). Non-intrusive, repetitive sampling of the gas spaces is necessary to confirm models of lateral diffusion under a partially blocked eggshell. Therefore I measured the development of P_{O_2} over different parts of the egg, using a modified technique of indirect sampling of the gas under the shell in a micro-compartment attached to the shell during the second half of incubation. The extent of lateral diffusion was further tested by subjecting the egg to shell treatment that either decreased (wax-cover) or increased (holes) regional shell conductance. Low lateral diffusion has implications on the regional gas composition in the egg and may necessitate the very local adaptation of the underlying vasculature and blood flow, which is studied in the following chapters. I tested the assumption that oxygen levels under the wax would decline in relation to the distance from the free shell area and that a gradient of P_{O_2} would form from areas of increased to areas of reduced shell conductance. Finally, direct measurements of P_{O_2} under areas of blocked shell were compared to the model of lateral diffusion by Ar and Girard (1989).

3.2 Material and Methods

3.2.1 Components of the gas analysis technique

The technique to measure P_{O_2} of the egg's gas spaces was based on the principle used by previous researchers (Tazawa *et al.*, 1980; Meir *et al.*, 1999), i.e. gas is sampled in an external compartment that equilibrates with the gas under the shell. Due to the present modifications, the gas volume in the compartment and the area covered by the glass were considerably smaller and therefore less influential on the measured values than in previous works. The smaller volume of the compartment reduced equilibrium time and therefore facilitated repeated measurements during the plateau phase. As the method was not intrusive, measurements could be taken without impact on the embryo.

The elements needed for the measurement of oxygen partial pressure involved a small gas compartment attached to the eggshell. The compartment was formed using approximately 1 cm long pieces of glass tubing with an internal diameter of 2.7 mm. The ends of the tube-pieces were first levelled and smoothened with a diamond grinder attached to a hand-held drill. One opening of each tube was partially reduced by heating the glass to form a lip, after which the other end was sealed to the shell, taking care to keep the area covered small. A drop of mercury was inserted inside the lip, sealing an airspace of approximately 0.05 ml volume from the environment.

Two sealing materials were tested, the dental wax (Kerr set-up wax No 5), that was used to block the shell pores in the waxed eggs, and the two-component epoxy adhesive Araldite®, that was used to attach the tube to the egg. To test the seal, a glass tube was glued to a glass slide with the sealant and the other opening sealed with mercury as described above. Using a syringe, the air was repeatedly sucked out and replaced with nitrogen, until the oxygen level inside the glass tube was reduced to less than 0.5 kPa.

The oxygen partial pressure inside the compartment was measured with a Clark type micro platinum-silver electrode (model 737, Diamond General, Ann Arbor, Michigan, USA), connected to the Sable systems Read Ox-4H O_2 analyzer (Sable Systems, Henderson, NV 89014, USA). The very fine tip of the electrode (ca. 100 μm) was inserted through the liquid mercury into the compartment (Fig. 3A.1). Measurements required a constant temperature and were therefore undertaken inside the controlled environment of a styrofoam box (50 × 30 × 30 cm) that was kept at 38°C with a custom made thermostat. The electrode was calibrated against nitrogen gas and dry atmospheric air at extremely low flow, by inserting the tip ca. 3 cm inside a PVC tube, inside the box. The box had two clear-plastic windows to observe the movement of the electrode that was inserted through a hole in one of the windows. A micromanipulator was used to position the electrode (Fig. 3A.2).

When the electrode was held in the compartment continuously, P_{O_2} gradually increased. After 23 min of continuous recording, the oxygen level in the compartment thus rose by 8 kPa. This was attributed to gas movement along the outside of the glass electrode, probably facilitated by adhering material, which apparently kept the mercury from sealing. In contrast, intermittent measurements, where the electrode was briefly inserted into the compartment after 20 and 40 min, yielded no changes to the P_{O_2} value. All further measurements were therefore done with the intermittent method, for which

the needle was inserted three times within 3 min and the values averaged as one measurement. Over a period of 85 min, the P_{O_2} level in the wax-attached compartment remained constant and it was therefore concluded that the dental wax was effectively sealing the glass for the purpose of this study. Similarly, over a period of 92 min there was no change to the P_{O_2} in the compartment sealed with the Araldite®. In the following, Araldite® was therefore used to attach and seal the glass tube to the eggs, while the wax was used to manipulate shell conductance.

3.2.2 Oxygen partial pressure in the gas spaces of the chicken egg during the second half of incubation

Oxygen partial pressure in the gas spaces under the shell was measured indirectly by measuring the gas composition in the sealed compartment that was in equilibrium with the egg gas. On day 9, eggs were placed for 4 h on silica gel to dry the shell in preparation for the sealant. Glass tubes were attached to the eggs with Araldite® as described above, after the egg's cuticle had been removed by gently sanding the egg at the position intended for the tube. The area covered by a glass tube and the thin ring of sealant around it was 19.6 mm^2, so that the two to three tubes attached to one egg represented less than 1% of the total egg surface. This was not considered to reduce total embryonic gas exchange significantly. The radius of the area covered by the glass tube and the sealant (approximately 2.5 mm) could be expected to lower the P_{O_2} measured in the centre of the area by about 0.4 kPa (Ar and Girard, 1989). As the internal diameter of the tube was 2.7 mm, a shell area of 4.2 mm^2, representing on average four pores (Ar and Rahn, 1985), was in contact with the gas in the glass compartment. Gas diffusion through these pores equilibrated the two gas spaces, which was completed after approximately 120 - 180 min (fig. 3.1), depending on the P_{O_2} value of the egg gas and the number of pores in the shell surface between the egg and glass tube.

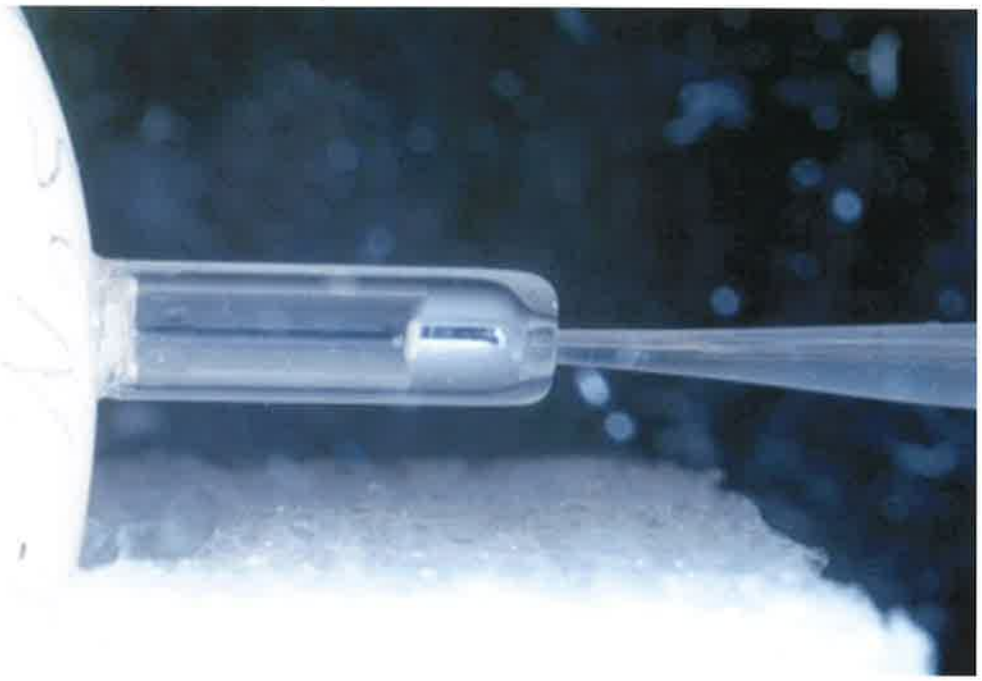


Fig 3A.1 Close-up image of the needle electrode inserted through the mercury seal into the glass tube compartment attached to a control egg.

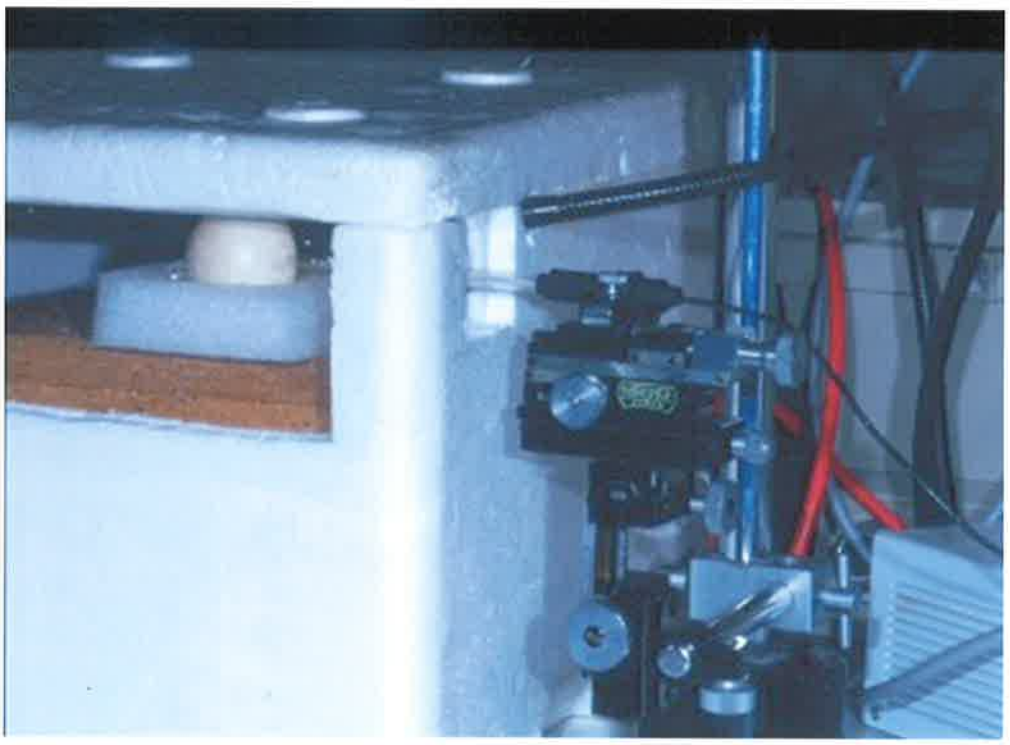


Fig 3A.2 The position of the electrode was controlled with a micromanipulator outside the styrofoam box and inserted through a window at the side. Egg and electrode inside the box were kept at 38 °C.

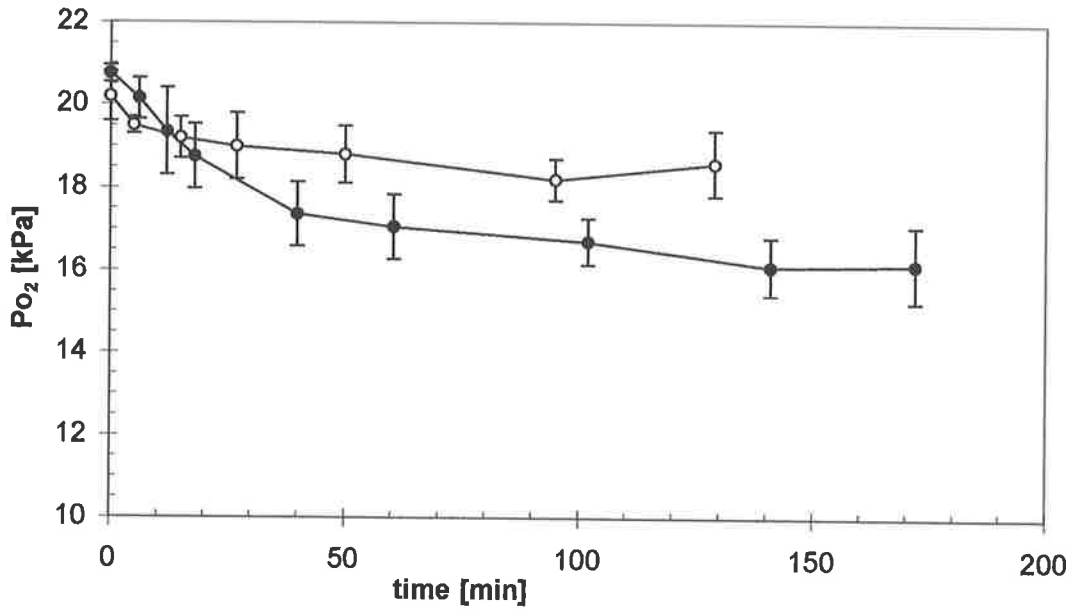


Fig. 3.1: Equilibration curves for Po_2 in glass compartments of two eggs on day 15. Three measurements averaged for each data point (+/- SD).

Changes in Po_2 during the second half of incubation were observed in eleven undisturbed control eggs (no shell treatment), and gas in the aircell was compared to the gas under the allantoic part of the shell. These eggs had one glass tube attached to the blunt end (*aircell*), and one to the equatorial region (*equator*) (fig. 3B.1). The oxygen consumption of these eggs was measured on days 12, 14, 16, 18, and 20 (cf section 2.6). From the known conductance of the eggs (cf section 2.2), it was possible to calculate the average partial pressure of oxygen under the shell and to compare it to the Po_2 measured in the aircell and the equatorial region of the egg.

Changes in Po_2 gradient under shell with locally altered gas conductance were observed in experimental eggs that were half covered with wax and had holes drilled through the opposing side of the shell as described in section 2.4. These experimental eggs had two or three glass tubes attached in various positions which were called *aircell* (blunt end of the egg), *hole* (inside the ring of holes), *½ hole* (between the holes and the wax border), *border* (at the border to the wax area), *½ wax* (halfway between the border and the centre of the wax) and *wax* (centre of the waxed area) (Fig. 3B.2); all of these positions except for *aircell* were on the equator of the egg. Due to the large modification of shell conductance, the measurements in these positions were considered independent for the purpose of analysis.

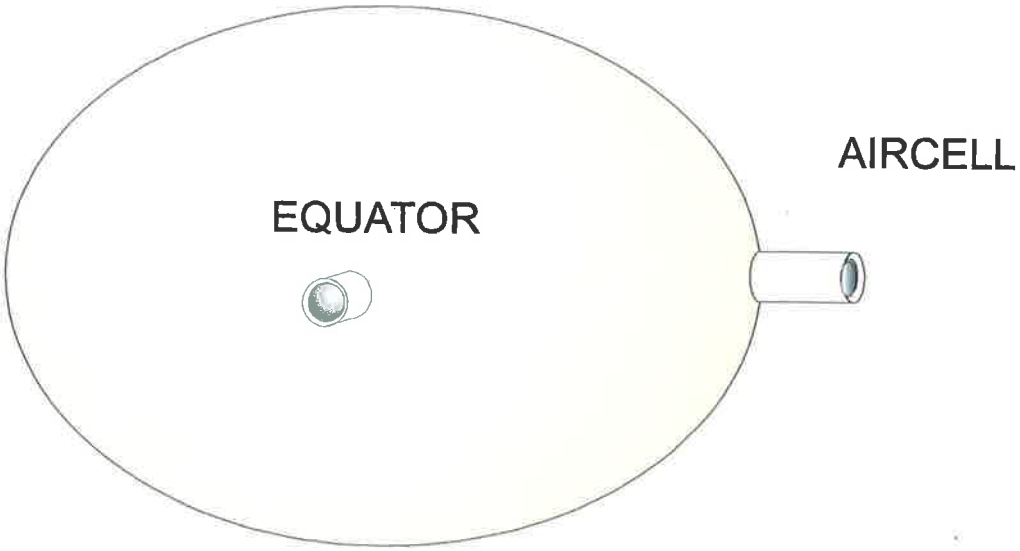


Fig. 3B.1 Positions of glass tubes for the measurement of oxygen partial pressure in undisturbed control eggs on the blunt end (above the aircell) and over the equator. Lateral view.

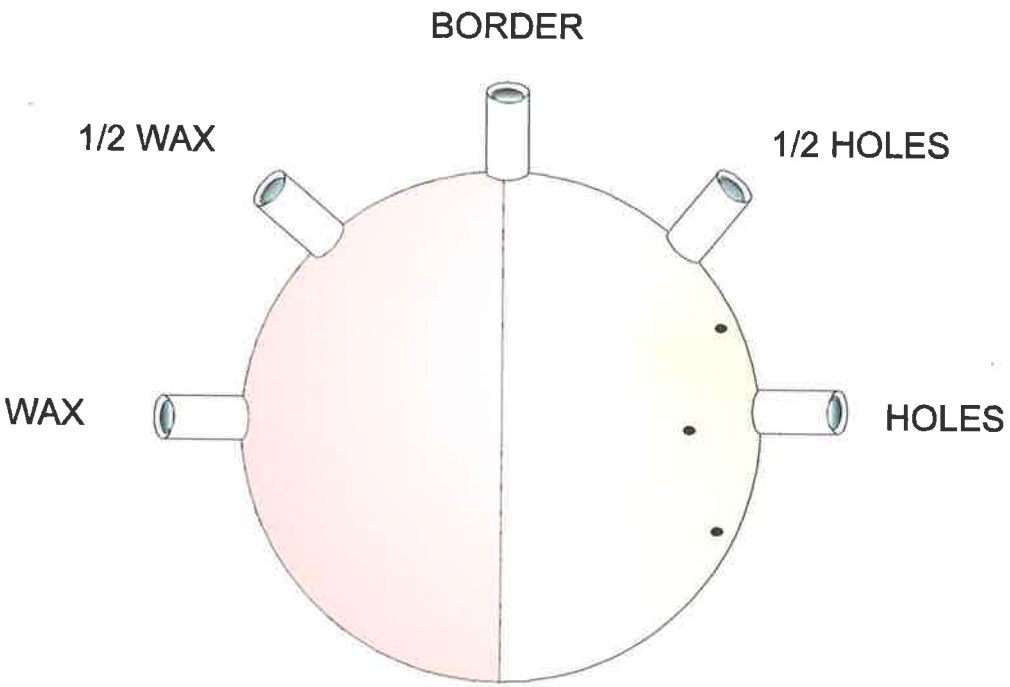


Fig. 3B.2 Positions of glass tubes for the measurement of oxygen partial pressure in eggs with wax/hole treatment. Only two to three of these positions were filled in any individual egg. Perspective from blunt end of egg.

After attachment of the glass tubes, eggs were turned manually twice daily until day 12. Oxygen partial pressure was measured daily from day 10 to day 19 of

incubation. Some measurements on day 20 were possible, but many embryos had commenced external pipping, and they were therefore excluded from measurement. In late incubation, water condensed on the inner side of the glass tube and on the mercury in some eggs. This affected the electrode to such an extent as to make measurements unreliable, and in these cases the mercury drop was removed between measurements. The mercury drop was replaced before the next measurement, and the air inside the tube was allowed to equilibrate for over 120 min.

3.2.3 A model for lateral diffusion

The change of P_{O_2} in the equatorial gas space under the shell was measured in correlation with an increasing area excluded from gas exchange with the environment by the application of wax. The P_{O_2} in the centre of the area was used to calculate Krogh's constant of diffusion for lateral oxygen movement in the compound shell membranes, which was then compared to the value calculated by Visschedijk *et al.* (1988). A model of lateral diffusion was developed for the effect of shell cover, and compared to the model by Ar and Girard (1989).

The P_{O_2} of eleven eggs was measured on day 19, when the chicken embryo is in the plateau phase of respiration (Romijn and Lokhorst, 1951; Rahn *et al.*, 1974). On day 18, the intended position for the tube on the egg was marked with a pencil, the shell sanded, a hole drilled with the dental drill as described in section 2.4 and the tube glued to the shell. The hole in the shell facilitated equilibration after only a few minutes. Five circles in 0.5 cm increments of the radius (0.5 to 2.5 cm) were drawn around the tube (fig. 3C). On day 19, these circles were successively covered with dental wax, and oxygen partial pressure was measured before and 90 min after each application of wax.

The lateral diffusion of oxygen under the shell was also investigated in a second experiment with wax circles of 0.5 cm and 1.0 cm radius that were observed during the second half of incubation (tab. 3.1). Shell treatment and tube attachment followed the outline above. Oxygen partial pressure was measured daily from day 10 to day 20. Where water condensed on the inside of tube and on the mercury, the mercury drops were taken out, and the glass tubes were kept open between measurements. This usually happened only during the last few days of incubation. Total water loss of the eggs was therefore not significantly increased. In the case of open glass tubes, measurements were started right after mercury was used to seal the compartment and repeated every minute until no further drop in P_{O_2} was noted.

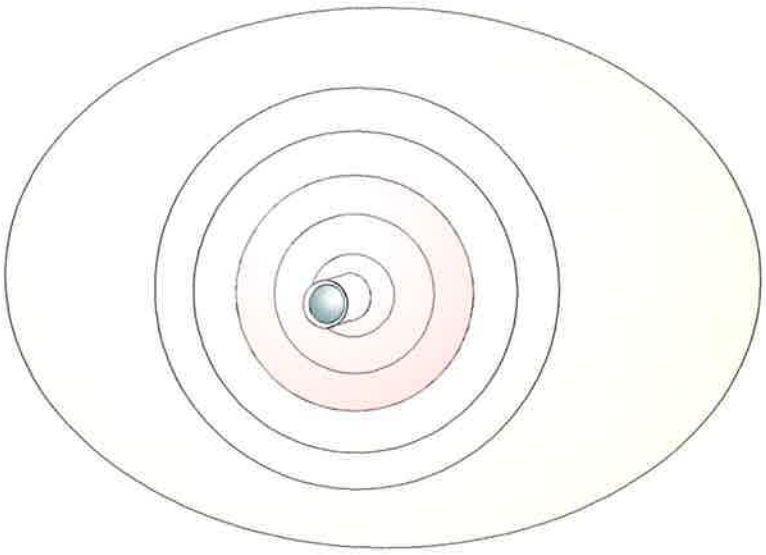


Fig. 3C Concentric circles in increments of 0.5 cm radius around a glass compartment. Three of the five circles (radius 0.5, 1.0 and 1.5 cm) around the glass tubes have already been covered with wax.

The rings marked on the eggs were covered with wax to varying degrees according to experimental group. Three groups of eggs were set up:

Group 1: nine eggs for control with two tubes on the equator (opposite to each other), and one over the blunt end. There was no wax around any of the tubes.

Group 2A: seven eggs for the test of lateral diffusion had two tubes on the equator (opposite): one without wax, the other with a wax ring of 0.5 cm radius.

Group 2B: six eggs for the test of lateral diffusion had two tubes on the equator (opposite): one with a wax ring of 0.5 cm radius, the other with a wax ring of 1 cm radius.

Experiment	group	treatment
Experiment 1	day 19	wax circles with increasing radius of 0.5 - 2.5 cm
Experiment 2	group 1	2 control tubes over equator
		1 control tube over aircell
	group 2A	2 tubes over equator: one control, one with wax circle (0.5 cm)
	group 2B	2 tubes over equator with wax circle of 0.5 cm and 1.0 cm radius

Tab. 3.1: Treatment groups in analysis of lateral diffusion.

3.3 Results

3.3.1 Oxygen partial pressure in the gas spaces of the chicken egg during the second half of incubation

3.3.1.1 Control eggs

Oxygen partial pressure over the aircell of the chicken egg decreased during the second half of incubation from 19.4 ± 0.3 kPa on day 10 to 13.3 ± 2.8 kPa on day 19. Values for the equatorial region of the eggs were slightly (0.4 ± 0.3 kPa), but not statistically significantly, lower than those over the aircell (MANOVA over differences against zero; fig. 3.2). From day 16 to 19, P_{O_2} values plateaued. On day 20, some embryos had commenced external pipping, and P_{O_2} levels in the remaining seven eggs averaged 11.5 ± 3.6 kPa. This decline probably resulted from increased activity of the embryos prior to hatching, and day 19 values were therefore assumed to be more representative of the P_{O_2} in the final phase of incubation. Neither day 19 nor day 20 oxygen partial pressure correlated with fresh egg mass, shell conductance or embryo mass (wet or dry) on day 20.

The sub-shell P_{O_2} calculated from the rate of oxygen consumption of the embryos ($\dot{M}O_2$, converted from $\dot{V}O_2$ for better comparison with literature) (cf. equation 2.2 in section 2.2, and section 2.6) was always higher than the values measured ($p < 0.05$, paired t-tests) (Fig. 3.3), with a mean difference of 1.5 kPa from the aircell measurements, excluding day 20 where the difference was 4.5 kPa. The measured

values from the aircell correlated with the $\dot{M}O_2$ and the calculated P_{O_2} only on day 14 ($r = -0.841$ and 0.863, respectively; $p < 0.01$). There was no correlation between the equatorial P_{O_2} and $\dot{M}O_2$ and the P_{O_2} obtained from the respiratory measurements on any of the days. The lack of a clear relationship between the measured and calculated P_{O_2} indicates the existence of differences in regional conductance and/or perfusion. The calculated P_{O_2} can only estimate the average over the egg surface.

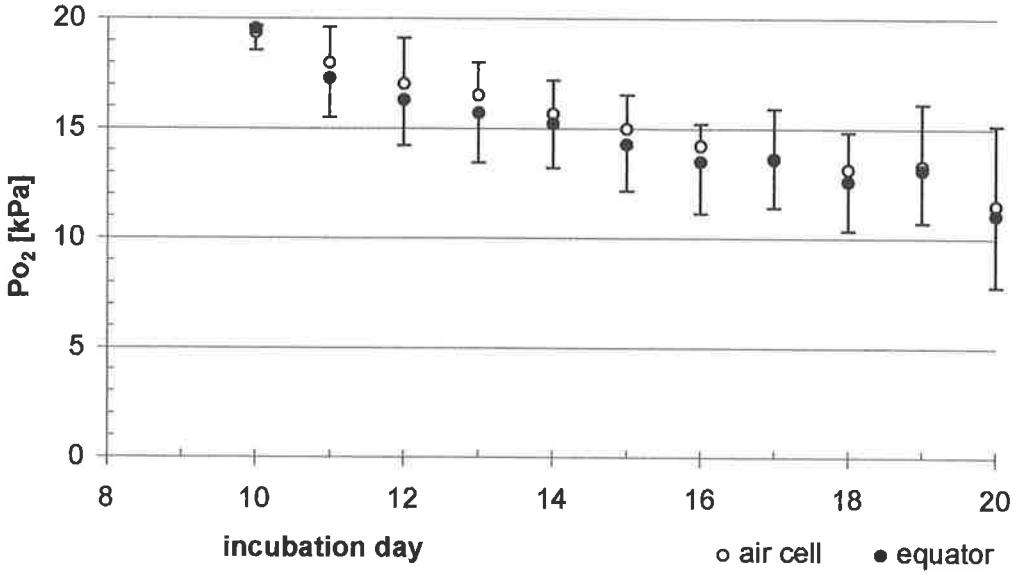


Fig. 3.2: Oxygen partial pressure [kPa] in the gas space under the shell. Comparison of the air cell and the equatorial region in undisturbed control eggs. Means and SD; n = 11; n = 7 on day 20.

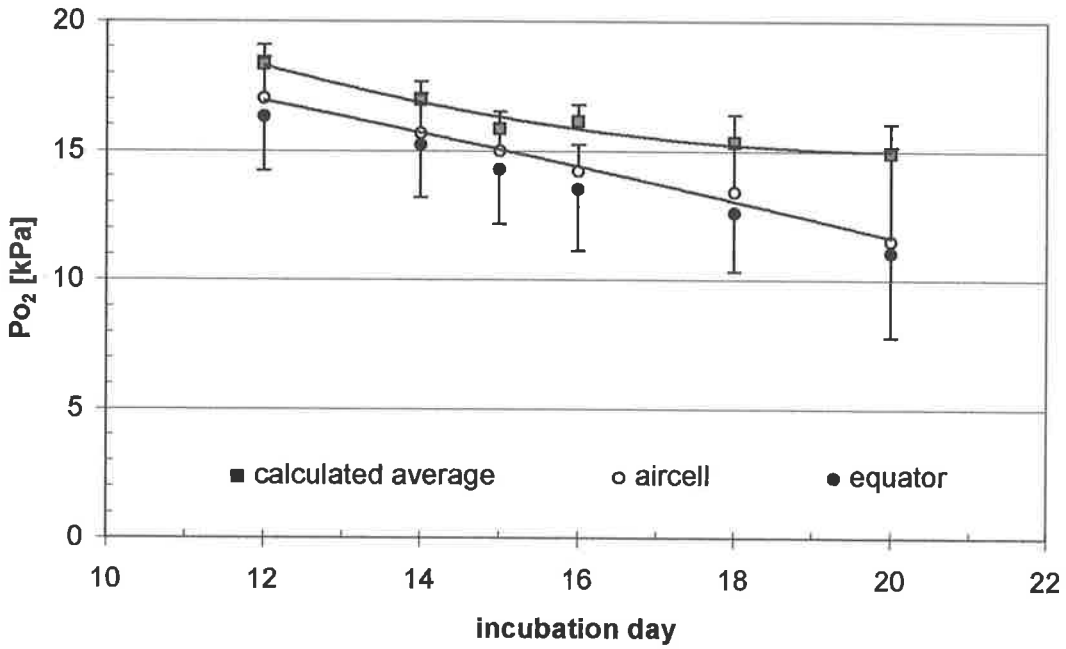


Fig. 3.3: Comparison of P_{O_2} calculated (from the oxygen consumption and shell conductance) and measured in air cell and equator. Means and SD, sample size = 10.

3.3.1.2 Wax/hole eggs

A gradient of oxygen partial pressure under the shell developed under the free side of the egg, from *holes* to *border*, whereas after a sharp drop along the borderline no differences were found between the two locations under the wax ($p < 0.0001$, $F_{4,11} = 16.3174$, MANOVA) (fig. 3.4). Under the waxed part of the eggshell, P_{O_2} was always reduced in comparison to the hole side, but although the difference increased until day 15 of incubation, there was no significant interaction between the groups and time ($F_{40,10} = 2.3704$, $p = 0.0789$, MANOVA). Partial pressure of oxygen under the waxed part of the shell reached a minimum around 3.8 kPa on day 18. Oxygen partial pressure of the aircell ($n = 4$) fell from 17.7 ± 1.9 kPa on day 10 to 9.6 ± 5.1 kPa on day 20, and lay consistently between values for *border* and *½ holes* (fig. 3.4).

The holes increased P_{O_2} under the shell to that above the control eggs until day 14, after which control values remained higher than that of the experimental hole side. Hole side P_{O_2} levels dropped significantly steeper from day 11 to day 19 (full sample size not available on days 10 and 20) than that of the control eggs ($p = 0.0023$, $F_{143,1} = 9.5931$, ANCOVA) (fig. 3.5).

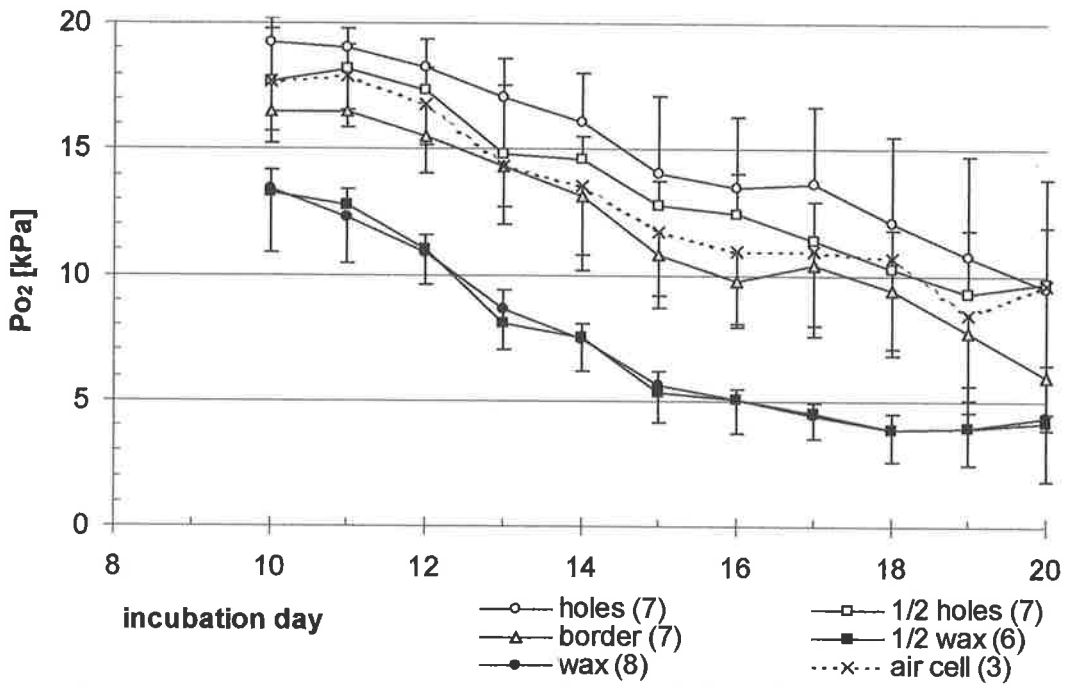


Fig. 3.4: Oxygen partial pressure under the egg shell in different positions in relation to the wax/hole treatment. Means and SD, sample size in brackets.

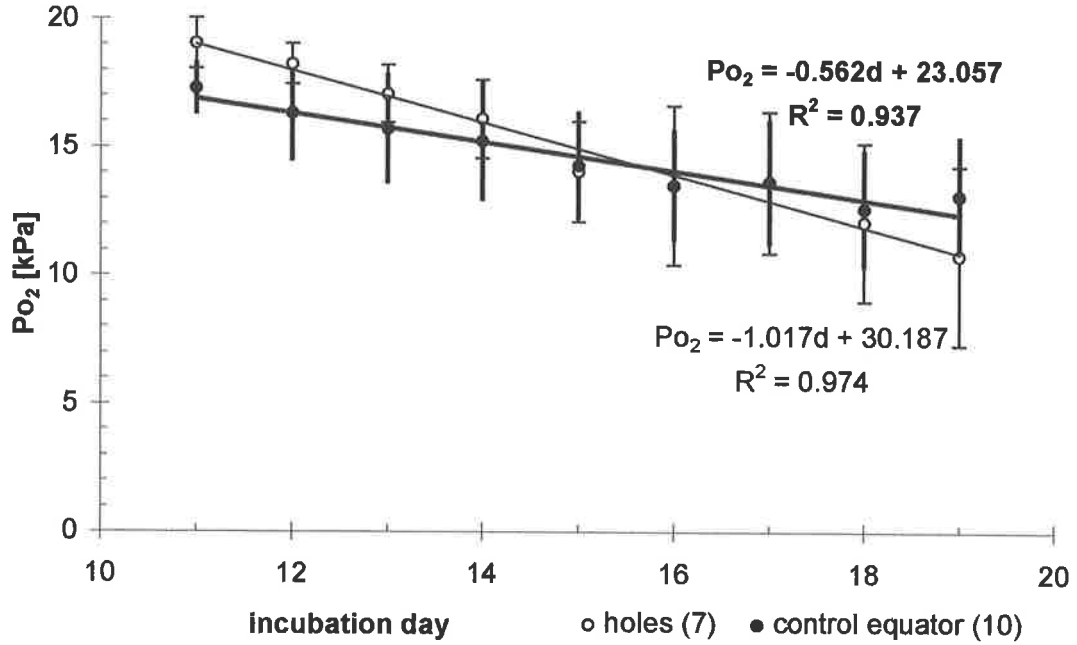


Fig. 3.5: Effect of the holes in the wax/hole treatment on the oxygen partial pressure under the shell in the equatorial region, comparison of position *holes* (wax/hole eggs) to the equatorial region of control eggs. Means and SD, sample size in brackets.

3.3.2 A model for lateral diffusion

3.3.2.1 Experiment 1

Oxygen partial pressure under the shell decreased with increasing radius of the area covered with wax. From an initial value of 13.2 ± 1.8 kPa under the glass tube only on day 19, Po_2 declined following an exponential curve over circle radius (r). The regression was calculated as

$$Po_2 = 15.396\, e^{-0.584r} \qquad (3.1)$$

so that Po_2 under the completely free shell (r = 0 cm) was extrapolated to 15.4 kPa (fig. 3.6).

The Po_2 measured at each radius was subtracted from this value (15.4 kPa) and the resulting difference in Po_2 (ΔPo_2) was compared directly to the corresponding values obtained from the model by Ar and Girard (1989) (fig. 3.7). Based on the measurements of Visschedijk *et al.* (1988), Ar and Girard described lateral oxygen

conductance ($G_{O_2\,lat}$) as the product of Krogh's constant of diffusion (K_{O_2}) through the shell membranes in lateral direction (3.2 mmol d^{-1} kPa^{-1} cm^{-1}) and the ratio of surface area to diffusion distance:

$$G_{O_2\,lat} = K_{O_2} \times 2\pi rh / r \qquad (3.2)$$

where r is the radius of the covered area and $2\pi rh$ is the surface area of a cylinder at the circumference of the area with the height (h) equal to the thickness of the shell membranes. The radius is also the diffusion distance in this model where the oxygen flow is to or from a central point source. As h is 0.0076 µm (Visschedijk *et al.*, 1988), $G_{O_2\,lat}$ equals 0.153 mmol d^{-1} kPa^{-1} for diffusion through a cylinder of any radius. Ar and Girard then calculated ΔP_{O_2} between the centre and the margin of the covered area from the oxygen consumption of the area covered (area-specific $\dot{M}_{O_2}$ multiplied by the area under cover). For the accurate comparison of ΔP_{O_2} according to the Ar and Girard model with the ΔP_{O_2} from experimental data, the area-specific mass flow of oxygen ($\dot{M}_{O_2}$) of the present study (0.31 mmol d^{-1} cm^{-2}) was used to calculate model data in fig. 3.7.

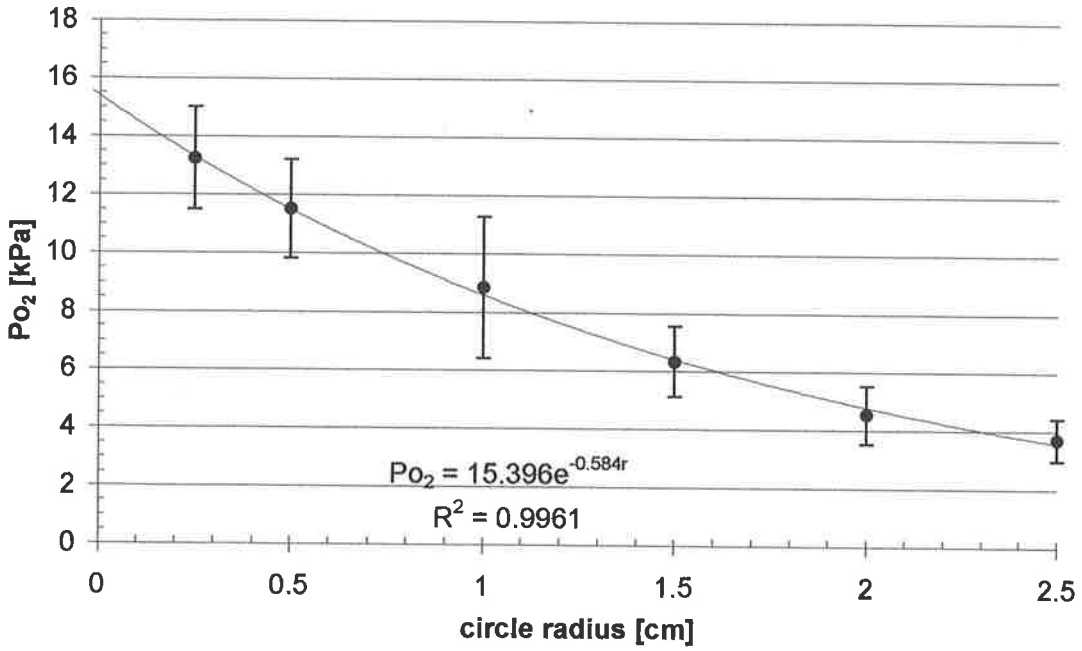


Fig. 3.6: Oxygen partial pressure on day 19 in the centre of shell covered with wax in circles of increasing radius (r) (n=11). Means and SD.

In contrast to the calculated exponential curve from the Ar and Girard model, the measurements yielded a polynomial function of ΔPo_2 that approached an asymptotic shape over the range of radius measured (fig. 3.7). The curves intersected at 1.07 cm radius. Below that radius, the measured reduction caused by the wax cover was increased in comparison to the model. Above that radius, measured Po_2 reduction was lower than expected by the model.

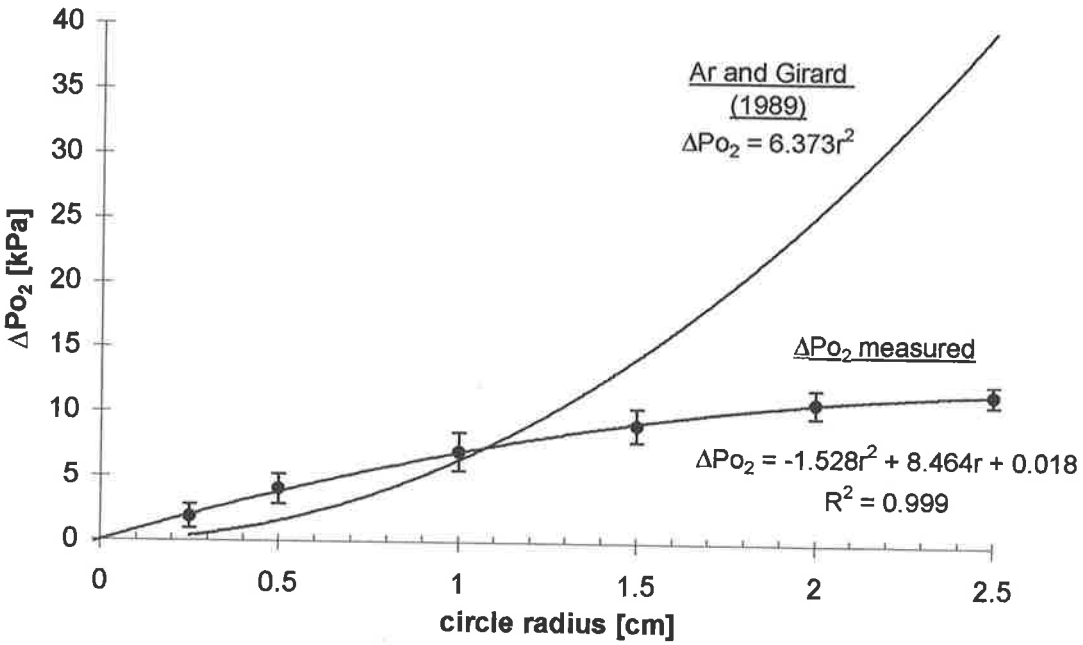


Fig. 3.7: The difference of Po_2 under the shell between the centre and the margin of wax circles of increasing radius (r) (n=11). Means and SD. Values are compared to calculated data from Ar and Girard (1989).

3.3.2.2 Experiment 2

In the eggs with wax circles of different sizes, observed throughout the second half of incubation (groups 1, 2A, 2B), Po_2 was increasingly reduced with increasing wax-cover. Group 2A eggs had consistently lower Po_2 values under the 0.5 cm wax circle than under the glass tube only (0.25 cm). The difference became significant on days 18 and 19 ($p < 0.05$, $n = 6$, paired t-test) and approached significance on days 10, 12, 14, and 17 ($p < 0.1$). Eggs in the group 2B had a lower Po_2 under the 1 cm wax ring than under the 0.5 cm wax ring. The difference was significant on days 12 to 18 ($p < 0.05$, $n = 5$, paired t-test). There was no difference in Po_2 under the 1 cm wax ring between groups 2A and 2B.

The average difference in Po_2 between 0.25 cm and 0.5 cm radius areas in group 2A and between the 0.5 cm and 1.0 cm radius areas in group 2B increased over time ($r = 0.895$ and $r = 0.897$, respectively; $n = 9$, $p < 0.005$). Under the 0.25 cm radius (group 2A), Po_2 declined from 19.7 ± 0.2 on day 10 to 13.7 ± 1.1 kPa on day 19 (no data available for day 20) (fig. 3.8). These values reached a plateau of about 14 kPa from day 15 and were not significantly different from the control eggs of group 1.

A comparison of the two opposing sides in the equator region of the same egg (group 1) showed differences between the sides. The average positive value of the difference remained quite constant through days 10 to 19 (1.1 ± 0.3 kPa, $n = 6$), but increased strongly on day 20 (3.1 ± 1.3 kPa). In five out of six eggs, the direction of the difference between the sides was not consistent, but rather one side or the other showed the greater value at different days.

Similar to the day 19 eggs in experiment 1, Po_2 under the free shell of the eggs in groups 2A and B was calculated from the regression curves of Po_2 under wax rings of increasing diameter (fig. 3.9). This extrapolation yielded a comparison for data obtained from direct measurements, in absence of the occluding effect of the glass tube. The values were greater than measured Po_2 under the glass tube (group 2A); the difference did not change over time and averaged 2.1 ± 0.8 kPa.

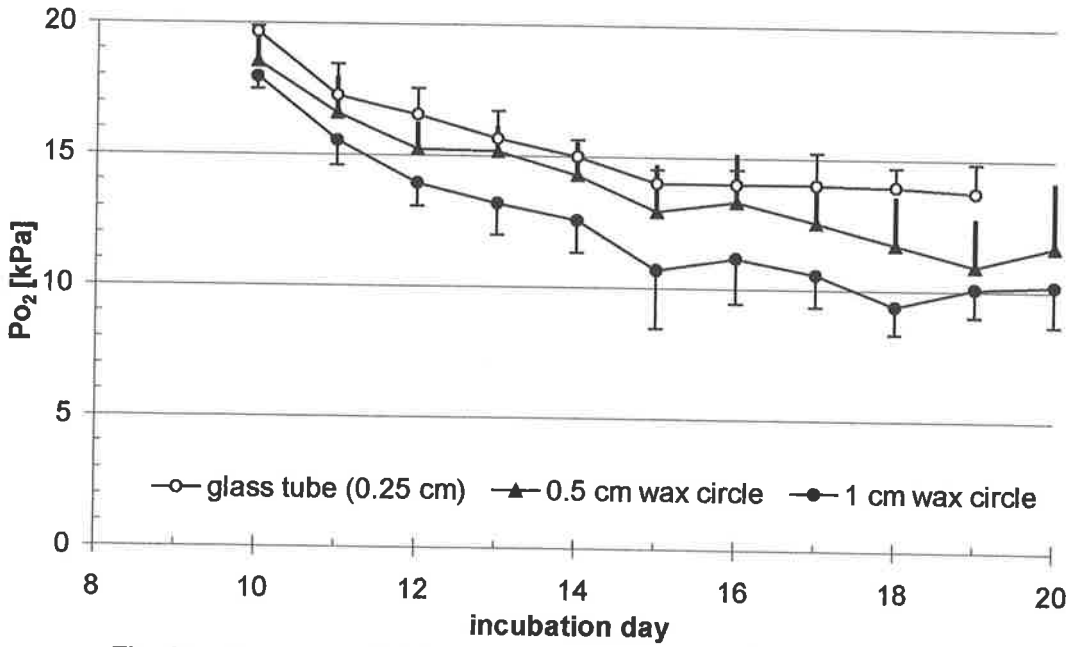


Fig. 3.8: Oxygen partial pressure [kPa] under eggshell with glass tube only (radius 0.25 cm, n=6), and in the centre of a wax circle with 0.5 cm (n=11) and 1.0 cm radius (n=5). Means and SD.

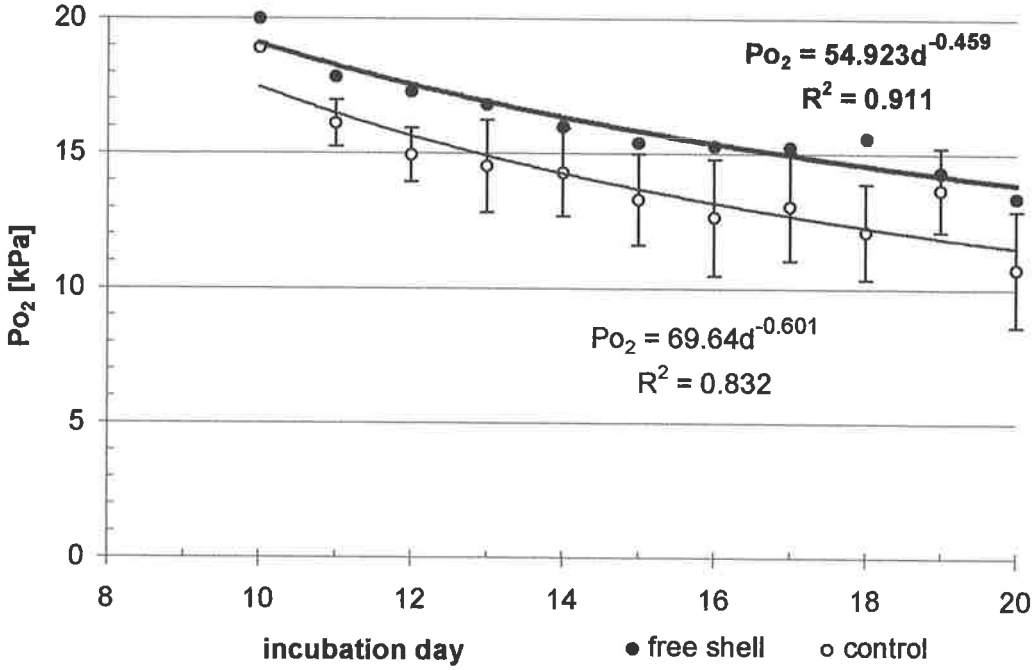


Fig. 3.9: Development of Po_2 under free shell as calculated from regression curves over wax circles, in comparison to measured values in control eggs. All equatorial positions.

3.4 Discussion

3.4.1 Oxygen partial pressure in the gas spaces of the chicken egg during the second half of incubation

The indirect technique to measure Po_2 under the eggshell was modified successfully in this study so that the equilibration time and the area under cover were reduced in comparison to the conventional method with large hypodermic needle hubs (Tazawa *et al.*, 1980). As expected from a constant shell conductance and increasing oxygen consumption of the embryo, Po_2 in the gas spaces under the eggshell declined in the second half of incubation and formed a plateau from day 16 to 19. This mirrors the change in oxygen consumption of chicken eggs (Romijn and Lokhorst, 1951; Rahn *et al.*, 1974; cf chapter 5). The range of data obtained for the Po_2 in the gas spaces under the shell (fig. 3.2) compares well to data from the literature (fig. 3.10). The glass-tube technique may be used to obtain values locally from other regions than the aircell, for example the equatorial region in the present study, without intrusive methods.

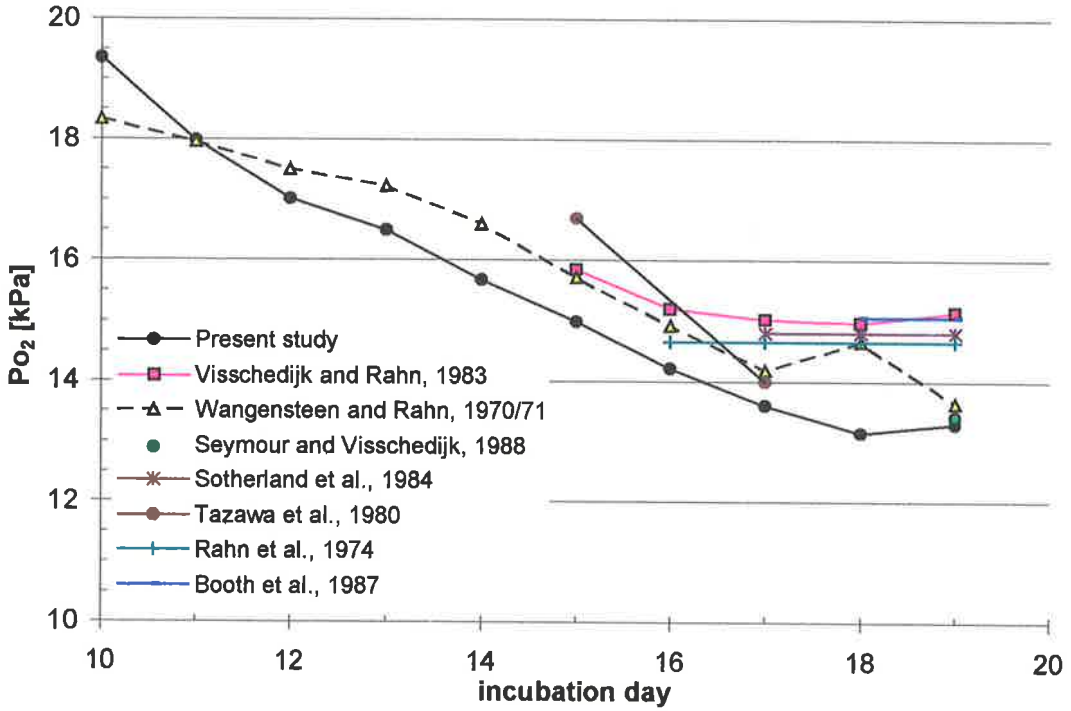


Fig. 3.10: Comparison of aircell Po_2 in the second half of incubation from the present study and published data.

Nevertheless, although the area covered by the glass tube was reduced compared to the conventional technique, there was a substantial drop in Po_2 under the tube in comparison to calculations for the free shell (2.2 kPa on day 19 in experiment 1, fig. 3.6; 2.1 ± 0.8 kPa from day 10 to 19 in experiment 2, section 3.3.2.2). This is most probably the consequence of limited lateral diffusion that allows the development of an oxygen gradient over the short distance of 2.5 mm radius from the outside to the centre of the area covered by the glass compartment. Similarly, the difference between the measurements of Po_2 and Po_2 values obtained from the shell conductance and oxygen consumption of the same eggs (fig. 3.3) may be explained by low lateral diffusion that affects the measured, but not the calculated values. It is therefore important to analyse lateral diffusion under the shell.

3.4.2 A model for lateral diffusion

Measuring Po_2 in the gas space above the equatorial region of the eggs with increasing shell cover yielded a different curve of the changes to Po_2 from that calculated in the model by Ar and Girard (1989) (fig. 3.7). The reasons for the differing curves are probably based on the assumptions and the purpose of the models. Ar and Girard

describe a model of lateral diffusion in the area serviced by a single pore, which can be abstracted as a point source for oxygen flow into the area. Of necessity the authors had to assume oxygen uptake occurred only at the margin of the disc (fig. 3D). As the area concerned was relatively small, the further assumption that embryonic oxygen consumption was independent of the extent of the shell cover was a reasonable estimate.

For the larger areas under cover in the present study, oxygen consumption of the embryo is clearly affected by the cover (chapter 5; cf Tazawa *et al.*, 1971a; Metcalfe *et al.*, 1981; McCutcheon *et al.*, 1982). This becomes clear when the *wax* and *½ wax* positions measured in section 3.3.1 (fig. 3.4) are compared. As the P_{O_2} measured in these two positions is identical, there can be no further oxygen uptake in the region between the positions. Accordingly, area-specific $\dot{M}_{O_2}$ would change along the diffusion path, as oxygen diffuses into the occluded area from the sides and is consumed throughout the area (fig. 3D). The oxygen diffusion rate into the area under cover is determined by the P_{O_2} in the immediate vicinity of the oxygen source, which in turn is determined by the local oxygen uptake as much as by the lateral conductance of the shell membranes. The diffusion rate is therefore not directly proportional to the difference in P_{O_2} between the centre of the disc and the margin.

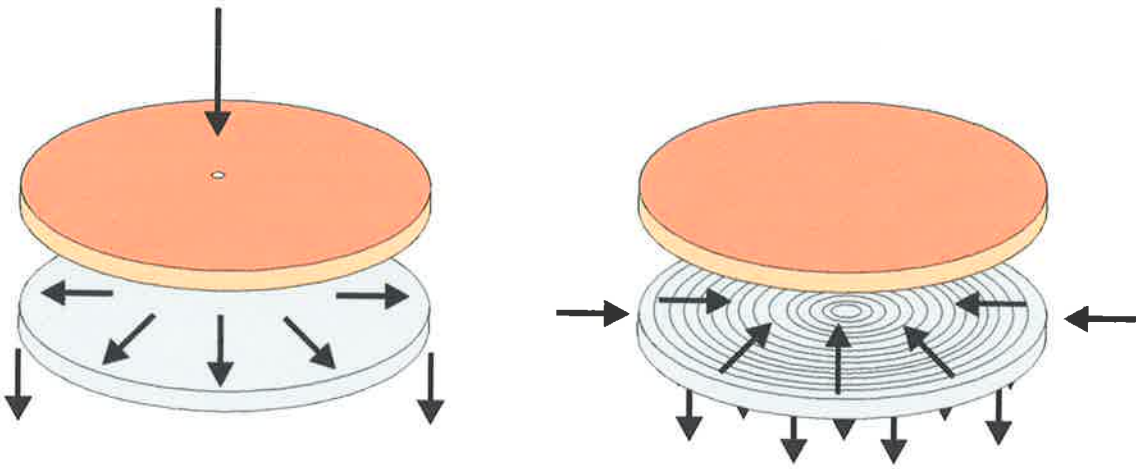


Fig. 3D Comparison of diffusion path under impermeable shell with pore in the model by Ar and Girard (1989) (left) and in the proposed model under a wax-cover (right). In the Ar and Girard model, oxygen diffuses outward from a single source in the centre to the margin of the area where it is consumed at a constant rate. In the proposed model, oxygen diffuses inward from the margin and is consumed everywhere along its diffusion path at a rate determined by local P_{O_2}.

Therefore, a model is proposed that uses the measured P_{O_2} difference between the centre of the disc and the margin to estimate Krogh's coefficient of lateral diffusion. On the basis that the area-specific $\dot{M}_{O_2}$ changes along the P_{O_2} gradient, it determines the Krogh's constant of lateral diffusion empirically so that the calculated ΔP_{O_2} matches the measured data.

The proposed model calculates P_{O_2} in the centre of a waxed area from the area-specific $\dot{M}_{O_2}$ on the basis of increments of radius (I), so that the area of radius R is divided into a series of concentric rings (annuli) with the width of the increment. The area of any annulus (A_i) is equal to the area of the circle formed by its outer radius r_i minus the area of the circle formed by its inner radius r_{i-1}:

$$A_i = \pi \times r_i^2 - \pi \times r_{i-1}^2 \tag{3.3}$$

The surface area (S_i) through which oxygen has to diffuse on its way from the margin to the centre of the area is formed by the average circumference of the annulus and the height of the cylinder h (thickness of the shell membranes):

$$S_i = \pi \times (r_i + r_{i-1}) \times h \tag{3.4}$$

Annulus-specific oxygen uptake ($\dot{M}_{O_{2\,i}}$) is equal to the area-specific oxygen uptake multiplied with the area of the annulus:

$$\dot{M}_{O_{2\,i}} = \dot{M}_{O_2} \times A_i \tag{3.5}$$

Total oxygen flow through an annulus ($\dot{M}_{O_{2\,lat}}$) includes oxygen uptake of that annulus and all the subsequent annuli:

$$\dot{M}_{O_{2\,lat}} = \Sigma_0^i \dot{M}_{O_{2\,i}} \tag{3.6}$$

Lateral conductance of oxygen flow through an annulus ($G_{O_{2\,i}}$) can be calculated analogous to equation 3.2:

$$G_{O_{2\,i}} = K_{O_2} \times S_i / I \tag{3.7}$$

The difference in P_{O_2} between the outer and inner radius of the annulus (ΔP_{O_2}) is then calculated from Fick's equation of diffusion:

$$\Delta P_{O_2} = \dot{M}_{O_2\ lat} / G_{O_2\ i} \tag{3.8}$$

This ΔP_{O_2} is subtracted from the P_{O_2} at the outer radius r_i ($P_{O_2\ i}$) to calculated $P_{O_2\ i-1}$ at the inner radius r_{i-1}:

$$P_{O_2\ i-1} = P_{O_2\ i} - \Delta P_{O_2} \tag{3.9}$$

For the first annulus, where $r_i = R$, $P_{O_2\ i}$ is equal to 15.4 kPa as obtained from equation 3.1.

The mathematical function that describes the gradient of P_{O_2} under the covered area is not known, because only the endpoints were measured, and $\dot{M}_{O_2}$ cannot be calculated directly. Accordingly, the model was solved iteratively. Starting with a constant $\dot{M}_{O_2}$ (control level calculated from uncovered eggs) throughout the area, the average P_{O_2} was calculated for each increment. Based on this average P_{O_2}, $\dot{M}_{O_2}$ was then calculated following a third degree polynomial equation that relates $\dot{M}_{O_2}$ to P_{O_2} (appendix 7.4). In a series of iterations (up to 20) the P_{O_2} in the centre of the covered area ($P_{O_2\ 0}$) was thus calculated, and then K_{O_2} was empirically modified so that $P_{O_2\ 0}$ would equal central P_{O_2} as measured (fig. 3.6). For r = 1 cm, where the curves of ΔP_{O_2} calculated from the two models intersected, K_{O_2} was equal to 1.2 mmol cm^{-1} d^{-1} kPa^{-1}. This is smaller than the 3.2 mmol cm^{-1} d^{-1} kPa^{-1} calculated by Ar and Girard (1989). As it is based on an extrapolation of P_{O_2} under the free shell and a certain relation between $\dot{M}_{O_2}$ and P_{O_2}, it might underestimate the lateral conductance of the shell membranes, and further experimental verification could prove useful. For more accurate calculations of K_{O_2} based on the measurements of P_{O_2}, knowledge of how the oxygen uptake into the blood depends on the P_{O_2} under the shell would be necessary.

As a consequence of low lateral diffusion, the P_{O_2} inside the area serviced by one pore (radius r = 0.05 cm) will not show a homogeneous P_{O_2}. Oxygen partial pressure midway between two adjacent pores (r = 0.05 cm) will be lowered by 0.44 kPa according to the regression equation of ΔP_{O_2} measured (fig. 3.7). As the greatest resistance to oxygen uptake has been calculated to derive from the oxygen binding in

the blood (Wangensteen and Wei bel, 1982), the small gradient in the area around a pore may not affect oxygen uptake significantly.

3.4.3 Formation of an oxygen gradient under wax/hole treated eggshell

The wax/hole treatment created a gradient of P_{O_2} under the eggshell that was not uniform around the egg. The abrupt decline in P_{O_2} from *border* to *½ wax*, and the homogeneously low values under wax confirmed a low lateral diffusion and showed that only the CAM underlaying the open side of the egg could have been involved in significant gas exchange for the embryo (fig. 3.4). Beyond the 1.7 cm average distance between the tube positions *border* and *½ wax* (the eighth part of the circumference of an egg), lateral diffusion was reduced to a non-measurable level. Similar low values were achieved in the lateral diffusion eggs (experiment 1) under wax rings with a radius of 2 cm, and therefore with a similar diffusion distance.

The oxygen levels under the waxed side showed substantial reductions and declined to a minimum of 3.8 kPa on day 18, which probably represented venous values, as similar P_{O_2} has been measured in venous blood of chicken embryos in late incubation (Piiper *et al.*, 1980: 3.2 kPa; Olszowka *et al.*, 1988: 3.3 kPa). However, the values failed to decline to lower venous P_{O_2} of 2.8 kPa as measured by Tazawa *et al.*, (1971b) and Tazawa (1973). That the P_{O_2} measured under the shell is equal to the venous P_{O_2} is supported by the observation that it does not decline further between the positions *½ wax* and *wax*. In this case the relatively high venous P_{O_2} could indicate that the embryos in the wax/hole treatment are not oxygen stressed, even late in incubation. An oxygen diffusion limitation in waxed eggs is, however, very likely (cf chapter 5). This should reduce venous P_{O_2} below control values as known from the literature (Tazawa *et al.*, 1983). Perhaps traditional measurements of blood P_{O_2} underestimate this variable, as the methods are necessarily intrusive. Oxygen consumption by the nucleated blood cells during and after sample taking, or the continued oxygen consumption of the embryonic and extra-embryonic tissue when the blood flow is obstructed by the catherisation, could possibly lower the P_{O_2} value measured below the venous level of the living embryo. In this case, blocking part of the respiratory surface area and measuring P_{O_2} under the shell in its centre may offer an alternative technique to measure venous P_{O_2} in late incubation, when the low lateral diffusion through the shell membranes does not allow enough gas movement to replace oxygen taken up by the embryo.

Further in agreement with a low lateral diffusion, the holes had no great effect on the P_{O_2} under the shell: only in the earlier phase of incubation (until day 14) were they successful in elevating the P_{O_2} under the shell to that above control values; thereafter the gas space under the holes was relatively hypoxic (fig. 3.5). A greater increase in $\dot{M}_{O_2}$ under the holes requires a greater diffusion rate across the shell and therefore a greater oxygen gradient across the shell. The increase in oxygen uptake can, however, only be achieved if blood P_{O_2} is also lowered or if the oxygen conductance across the CAM or oxygen binding in the blood is increased. Blood gas composition changes with shell conductance (Tazawa *et al.*, 1983; Tullett and Burton, 1985), and as the embryos in the experimental eggs had to rely solely on gas exchange through the wax-free side of the shell, blood P_{O_2} may have been reduced below the control values as a consequence of general hypoxia in wax/hole eggs. This may have been an adaptive process, as the embryo may have reacted to the altered shell conductance with a general decrease of venous P_{O_2} and the increase of haemoglobin oxygen affinity, which could be the result of accelerating the natural progression of these blood variables during the second half of incubation (Tazawa *et al.*, 1971b; Reeves, 1984). If the P_{O_2} measured under the shell did, however, represent venous levels, the data do not support a reduction of venous P_{O_2}. Alternatively, a lowering of blood P_{O_2} could have derived from increased blood flow, resulting in low transit times and incomplete equilibration of the blood, which in turn reduced P_{O_2} in the air space. As the haemoglobin oxygenation velocity declines with increasing saturation of the haemoglobin (Tazawa *et al.*, 1976b), and the oxygen dissociation curve of haemoglobin is not linear in relation to P_{O_2}, a reduction of transit time and blood P_{O_2} may reduce oxygen content proportionally less.

On the other hand, an increase of oxygen conductance in the inner barrier could involve a shorter diffusion distance across the tissue barrier of the chorioallantoic membrane, or a larger surface area of the microvasculature. As these variables do not change significantly in late incubation (Fitze-Gschwind, 1973, Ausprunk *et al.*, 1974) and the major resistance of the inner barrier has been calculated to lie in the rate of oxygen binding (Wangensteen and Weibel, 1982), this appears not very likely, and in agreement with these considerations, no decrease in barrier thickness was observed in eggs with wax/hole treatment (chapter 4). Additional oxygen binding in the blood would require increased oxygen capacity (haemoglobin content) or the acceleration of oxygenation and blood flow.

The oxygen uptake across all the shell on the hole side of the treated eggs may be assessed when P_{O_2} values are integrated from the positions *holes*, *½ holes* and *border* (tab. 3.2). Average P_{O_2} under the hole side was considerably lower than estimates for free shell in control eggs (calculated from oxygen consumption) after day 14 (tab. 3.2). This indicates that the holes were not sufficient to maintain oxygen diffusion across the shell at the normal level after day 14, so that the increase of the P_{O_2} gradient across the shell resulted from the increase of oxygen uptake on the hole side of the egg. By day 20, the P_{O_2} gradient across the shell of the hole side had almost doubled in comparison to the control values. This could indicate that the treated embryos were able to maintain total oxygen uptake at control levels and to compensate for the reduction of respiratory surface area under the wax. However, this increase of the P_{O_2} gradient across the shell was not uniform, as the P_{O_2} measurements for the different positions show (fig. 3.4). Consequently, blood oxygen loading was not uniform under the hole side. The embryos were therefore probably not able to maintain normal oxygen uptake under these conditions, which will be confirmed in chapter 5.

Day	$\dot{M}_{O_2}$ [mmol d^{-1}]	P_{O_2} [kPa] (control)	P_{O_2} [kPa] (integrated holes)	ΔP_{O_2} [kPa] (control)	ΔP_{O_2} [kPa] (integrated holes)	Change of ΔP_{O_2} for hole side
12	6.1 ± 0.9	18.5 ± 0.3	18.8 ± 1.2	1.4	1.1	-23%
14	14.2 ± 2.2	16.8 ± 0.3	16.3 ± 1.2	3.0	3.5	+16%
16	20.6 ± 2.9	16.0 ± 0.7	13.6 ± 1.7	3.8	6.3	+63%
18	23.5 ± 3.5	15.0 ± 1.1	12.3 ± 1.1	4.9	7.6	+55%
20	22.8 ± 5.3	14.9 ± 1.6	10.2 ± 2.0	5.0	9.7	+96%

Tab. 3.2: Comparison of the P_{O_2} averaged under the hole side in wax/hole treated eggs with the average P_{O_2} calculated from the oxygen consumption of untreated control eggs using Fick's equation of diffusion. A correction of 2.0 kPa was added to the measurements to account for low lateral conductance under the area covered by the glass tube. The oxygen gradient between the effective P_{O_2} of the air (at 100% water vapour saturation, cf Wangensteen and Rahn, 1970/71) and the sub-shell value was compared between the treatments.

Embryos are thought to tolerate only a certain minimum of P_{O_2} in the gas space: aircell P_{O_2} has been found to be very similar in many species towards the end of incubation, averaging 13.3 kPa (Tullett and Deeming, 1982; Rahn *et al.*, 1987). The maximum oxygen uptake in eggs with low shell conductance was assumed to be limited by this minimum (Tullett and Deeming, 1982). In this situation of an artificially changed shell conductance, only certain areas fell below this limiting value. However, as the integration of P_{O_2} over the hole side shows (tab. 3.2), average P_{O_2} under the wax-free shell fell below this minimum from day 18. As most of these embryos (12 out of 17) survived to day 20, this supports the view that embryos may be able to tolerated more hypoxic values in the gas spaces of the egg than thought (C. Vleck *et al.*, 1979; Carey *et al.*, 1982).

3.4.4 Conclusion

A modified technique was developed to measure P_{O_2} under the shell of bird eggs indirectly. As it required a smaller area of contact with the shell and a shorter equilibration period than more established techniques, it allowed the measurements of P_{O_2} in the centre of a shell area covered by wax in increasing diameter. This yielded an estimate of lateral oxygen conductance through the shell membranes of the chicken egg that was substantially lower than previous calculations and, if true, indicated the great effect the blockage of even a few pores has on underlying oxygen levels. Indirect measurements of P_{O_2} under the shell in the allantoic region of the egg therefore need to account for the effect of low lateral conductance in the shell membranes.

As a consequence of the low lateral conductance, holes drilled through the shell were sufficient to compensate for the loss of respiratory surface area elsewhere on the egg only until day 14 of incubation, during the period of chorioallantoic maturation. In late incubation, P_{O_2} under the holes was reduced in comparison to control eggs, and P_{O_2} under the wax reached venous level. Oxygen uptake in these eggs (wax/hole treatment) was probably suppressed in the last quarter of incubation.

4. Development of the chorioallantoic membrane

4.1 Introduction

The first and most important level where the bird embryo may address potential diffusion/perfusion heterogeneity caused by differential shell conductance is its respiratory organ, which, through most of incubation, is the chorioallantoic membrane (CAM). It is formed from the fifth day of incubation by fusion of the chorion and the allantois that grows as a small sac from the umbilical region of the embryo from day 3 (Fitze-Gschwind, 1973). The CAM reaches the inner shell membrane (ISM) on day 6 (Fitze-Gschwind, 1973) and attaches on day 10 (Seymour and Piiper, 1988). By day 12, its growth is complete (Ackerman and Rahn, 1980).

The directional growth of the CAM may be controlled by a variety of factors. Initially the CAM comes into contact with the ISM as it floats above the embryo that itself is positioned on the yolk sac, just under the upper part of the shell of the egg. There is some indication that the growth of the CAM is directed by oxygen availability. This evidence stems from shell-less and semi-shell-less culture techniques. In the shell-less culture, where embryo and yolk contents are kept in a petri dish (Auerbach *et al.*, 1974) or plastic-wrap tripod (Dunn and Boone, 1976), the CAM covers the open surface over the embryo and reaches only about $^2/_3$ of its size *in ovo* (Dunn and Boone, 1976). If the embryo is kept in a gas permeable plastic, the CAM may grow down the sides of the container (Dunn and Boone, 1978). In the semi-shell-less culture (Ono and Wakasugi, 1984), embryos and egg contents are kept in "surrogate" eggshells of larger species. This technique allows gas diffusion through the bottom of the container and provides calcium for the embryo. This promotes down-growth of the CAM and better albumen uptake (Dunn, 1991) and, as a consequence, the CAM develops normally (Rowlett and Simkiss, 1987). However, the shell-less and semi-shell-less cultures described above allow no egg turning, which is known to impede the completion of the chorioallantois (Tyrell *et al.*, 1954; Fitze-Gschwind, 1973; Tazawa, 1980b). The missing stimulus in this context may be the effect that gravity has on the CAM when the egg is turned, which may induce the CAM to cover all of the ISM, even under large sections of impermeable shell, in rotated eggs.

It is necessary to distinguish between the effect of gas exchange and the other factors that influence chorioallantoic growth. When confronted with an impermeable or low permeable shell section, directional growth of the CAM should change to avoid a

diffusion/perfusion mismatch on a large scale. Early research into the growth of the chorioallantoic membrane found a negative reaction of CAM growth to shell impermeability, but neglected turning of the eggs (Dareste, 1855).

On a finer scale, there are various mechanisms the embryo may use to adapt to local variances in shell conductance. Firstly, the extent of the vascularisation of the chorioallantoic membrane is subject to regulation. Embryos have been observed to adjust the density of pre- and post-capillary vessels in the CAM in response to an altered gas environment (Dusseau and Hutchins, 1988; Strick *et al.*, 1991). The second level of adjustment concerns the capillaries in the chorioallantoic membrane. Of the many blood vessels in the CAM, the capillaries are the most important for gas exchange. They form a system of interconnected vessels in the chorionic epithelium that is better described as a plexus than as a network (Burton and Palmer, 1989). From day 8 onwards, the capillaries move through the outer epithelium, until day 14, when only a very thin layer of attenuated cytoplasmatic processes remains between the blood and the gas space in the shell membranes (Fitze-Gschwind, 1973; Ausprunk *et al.* 1974). This minimises the diffusive distance for the exchange of respiratory gases and serves to optimise respiration during development (Fitze-Gschwind, 1973; Wangensteen and Weibel, 1982). It becomes crucial during late incubation when oxygen levels in the egg are low. Burton and Palmer (1992) showed that this process of capillary invagination may be accelerated or decelerated in hypoxic or hyperoxic environment, respectively.

While it is known that the density of the larger pre- and post-capillary vessels continues to increase until day 14 (Kurz *et al.*, 1995), proliferation of the capillary plexus is greatly reduced after day 10 of development (Ausprunk *et al.*, 1974; DeFouw *et al.*, 1989; Wilting and Christ, 1993, 1996). The proliferation of capillaries is growth-factor-dependant (Folkman, 1992). A large number of regulators (enhancers and inhibitors) of angiogenesis are known. Of all of them, the vascular endothelial growth factor (VEGF-A) appears to be the most important and best-studied agent, and the only one that affects endothelial cells exclusively (Wilting and Christ 1996). This factor is increasingly expressed during hypoxia in a variety of tissues, such as vascular smooth muscle cells (Brogi *et al.*, 1994; Stavri *et al.*, 1995; Gu and Adair, 1997), muscles (Minchenko *et al.*, 1994; Breen *et al.*, 1996), as well as in the kidney (Minchenko *et al.*, 1994; Tufromcreddie *et al.*, 1997; but see Kramer *et al.*, 1997) and in the brain (Minchenko *et al.*, 1994; Patt *et al.*, 1998), where it stimulates angiogenesis. However, information about respiratory tissues is rare. In chicken eggs, VEGF is known to

increase vascular density of the CAM when applied externally (Wilting and Christ, 1993; Kurz *et al.*, 1995). This effect is localised and could therefore present a mechanism for adaptive angiogenesis of capillaries and other small order vessels.

The blood volume in the microvasculature may also increase further during development by increasing the diameter rather then the density of the capillaries. An increase of capillary size was for example observed by Fisher *et al.* (1992) in capillaries of the rat cremaster muscle in response to hypoxia.

In the absence of vascular adjustment to variable shell conductance and in the presence of a diffusion/perfusion mismatch, the embryo may face hypoxic conditions. As a consequence, the oxygen binding capacity of the blood may be increased. Under normal conditions, blood haemoglobin concentration and haematocrit increase throughout incubation (Romanoff, 1967; Temple and Metcalfe, 1970; Tazawa *et al.*, 1971a, b; Tazawa, 1978, 1980a, 1984). As an adaptive reaction on the global level, these values are adjusted to changes of total shell conductance or ambient oxygen partial pressure (P_{O_2}) (Tazawa *et al.*, 1971a; Nakazawa and Tazawa, 1987, 1988; Tazawa *et al.*, 1988).

The aim of this study is to investigate whether the embryo is able to adapt the growth of its chorioallantoic membrane, and the chorioallantoic vascular system and perfusion and therefore achieve a match of diffusion and perfusion under locally variable shell conductance. To investigate if chorioallantoic growth is accelerated under the partly occluded eggshell, and if the direction of its growth is stimulated by oxygen or carbon dioxide levels as found under the shell, the size and position of the CAM were observed in partly waxed eggs before attachment on day 8, when the CAM covers 62%, and after attachment on day 10, when it covers 83% of the undisturbed egg (Ackerman and Rahn, 1980). Finer adjustments of vascular density, blood volume, haematocrit, haemoglobin concentration and microvasculature of the chorioallantoic membrane were compared between control eggs and those that had been treated with local changes in shell permeability.

Local reduction of shell conductance was achieved by waxing half of the eggshell, while on the other side (free side) oxygen availability was increased by incubation in a hyperoxic environment (wax/oxygen treatment) or by promoting shell conductance with holes drilled into the shell (wax/holes treatment). As specific local adaptation of the CAM to the variable shell conductance is only possible after attachment, the wax/oxygen treatment commenced on day 10. The wax/hole treatment

was performed at different days of development in order to investigate if there was a temporal "window of opportunity" for adaptation: shell treatment on day 4 was initiated before fusion of the chorion and the allantois, so that the embryo was exposed to the local differences in shell conductance throughout development of the CAM. Shell treatment on days 6, 7 and 8 fell into a time of very rapid CAM growth and vascularisation (Kurz *et al.*, 1995), before attachment to the inner shell membrane. Any reaction to the changes in shell conductance up to this time would encompass the whole organ. Finally, shell treatment on days 10 and 12 were undertaken after CAM attachment and after capillary proliferation declined in undisturbed eggs (Ausprunk *et al.*, 1974).

If the circulatory system in the CAM is regulated centrally by the embryo, levels of vessel density and blood volume would stay homogeneous throughout the CAM. The resulting diffusion/perfusion mismatch and functional hypoxia due to shunting of oxygenated blood from the wax-free area with oxygen-poor blood from the waxed area may stimulate accelerated chorioallantoic growth and development. In contrast, a strict local control of chorioallantoic development, independent of overall embryonic needs, may show increased growth and vascularisation under the wax, parallel to observations by Dusseau and Hutchins (1988) and Strick *et al.* (1991) in hypoxia, and decreased vascularity under the oxygen-rich wax-free side, resulting in an increased diffusion/perfusion mismatch. Only the integrated local control aimed at matching perfusion to diffusion would result in increased growth and vascularity under the wax-free side, to allow a greater local rate of oxygen uptake, while vessel density and blood volume under the wax should decline.

4.2 Material and Methods

All eggs were obtained from a local provider (Globe Derby Poultry), and measured and incubated as described in section 2.1. Shell conductance was measured on day 1 (cf section 2.2), and eggs were allocated to treatment groups so that average fresh mass, shell conductance and weight-specific shell conductance were matched between the groups.

Shell conductance was reduced locally on days 4, 6, 7, 8, 10 and 12 with wax, and this loss of gas exchange surface was compensated with holes as explained in section 2.4, or with a high oxygen environment as described in section 2.3.

4.2.1 Growth of the chorioallantoic membrane

In order to determine if growth of the chorioallantoic membrane is directed in response to a concentration gradient of respiratory gases, parts of the eggshell were waxed on day 6 of incubation. Before incubation, eggs had been marked with a grid drawn in pencil onto the shell, consisting of eight meridians at 45° angles (pole to pole, # 1 - 8) and seven longitudinal circles (parallel to the equator, "A" - "G") (fig. 4A.1). The waxed parts were either (a) the "blunt half" of the egg (from equator to blunt end), (b) the "pointed half" of the egg (from the equator to the pointed end) or (c) a "longitudinal half" from pole to pole (fig. 4A.2). The eggs were kept in the incubator under the conditions described in section 2.1, including the turner, with the exception of group (c). After waxing, the latter was kept in a temperature cabinet at a temperature and humidity equal to the incubator. These eggs were turned 180° over the ends twice daily by hand after waxing in order to maintain a position where a part of the border line of the wax was pointing upwards.

On days 8 and 10 of incubation eggs were candled, and the aircell and the position of the eye of the embryo were marked on the shell. The eggs were then held in liquid nitrogen for 20 s to fix the CAM in position and remove the wax, after which the eggs were quickly candled a second time and marking completed (where it had been prevented by the wax). Thereafter, the eggs were frozen solid in liquid nitrogen (1-2 min) and kept in a freezer overnight.

For analysis of the relative size of CAM cover, the shell was peeled away in small fields from the underlying frozen tissue and liquid. The outline of the underlying membranes (CAM and yolk sac) as seen on the frozen egg were transferred onto a standardised Sanson-Flamsteed projection (fig. 4B) which yields a true surface area (cf. Fitze-Gschwind, 1973). A quantitative analysis of the CAM cover was obtained by cutting the paper sheets and weighing sections. The CAM cover was established from the weight of the CAM section as percentage of the total for the relevant egg half, excluding the aircell area, and the waxed half was compared to the wax free half (hereafter termed "free half") of the egg. The weight of the aircell sections was used to establish the position of the aircell in relation to the wax cover in group (c).

The technique employed did not allow the comparison of CAM attachment under the free and the waxed side of the egg, as the shell would separate easily from the tissue that remained frozen to the contents of the egg.

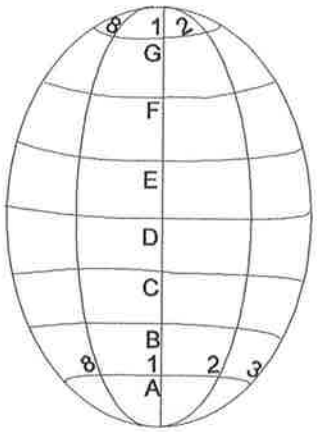


Fig. 4A.1 Gridlines on the eggshell: eight meridians at 45° angles (pole to pole, # 1 - 8) and seven longitudinal circles (parallel to the equator, "A" - "G").

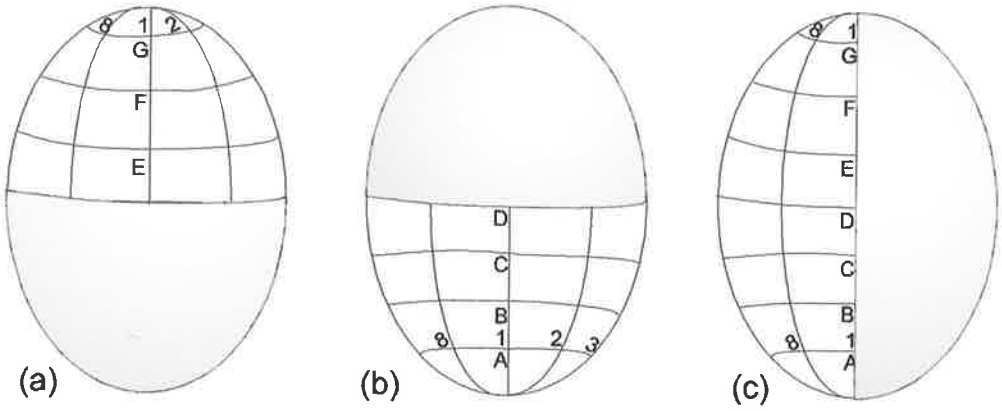


Fig. 4A.2 Treatment groups in CAM growth experiment: (a) "blunt half", (b) "pointed half" and (c) "longitudinal half" of the egg painted with wax.

In most eggs on day 8, the yolk sac membrane was clearly larger than the CAM (fig. 4C). The chorioallantoic membrane covered 29.1% - 56.7% of the aircell-free shell area. These values may be underestimated, because it was difficult to distinguish the CAM from the underlying yolk sac membrane, which had a similar vessel density and was fused to the CAM at the edge. However, since the yolk sac membrane lay directly under the shell in CAM-free areas and was as highly vascularised, it can be assumed that it functioned as a gas exchanger in these areas. Yolk sac and chorioallantoic membranes were therefore analysed as a unit in the day 8 experiments.

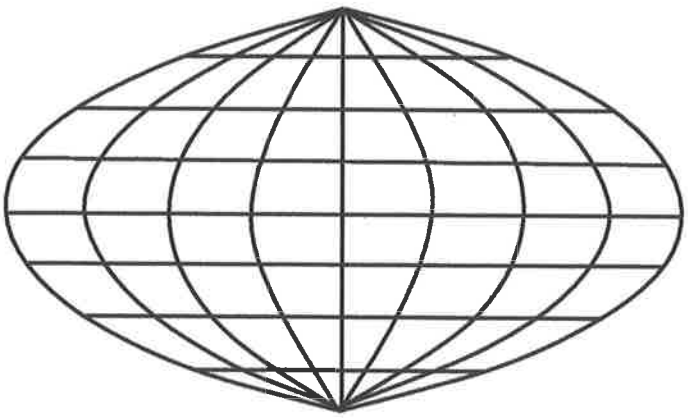


Fig. 4B Standardised Sanson-Flamsteed projection of grid on eggshell (about 70% of original size).

4.2.2 Density of pre- and post-capillary vessels

For an estimate of pre-capillary density, CAM pieces that had been stored in fixative (2.5% glutaraldehyde, appendix 7.2.1) were taken out, washed in phosphate buffer and distilled water (10 min each) and separated from the shell. Two pieces of approximately 3 - 4 cm^2 per egg were then placed on albumen-coated glass slides and allowed to dry overnight in the oven at 38°C. After drying, samples were stained in haematoxylin and eosin (see appendix 7.2.2) and viewed under a light microscope at 40x magnification. Of each section, five counts were undertaken in "vertical" and five in "horizontal" direction along the graticule in the eye-piece, so that the CAM of each individual egg was counted 20 times. The edges of the CAM sample were avoided. The intersections of vessels of less than 25 µm were easily recognised as one unit on the graticule and were counted. These included vessels of the first to third order of the centripetal ordering method (Fenton and Zweifach, 1981). A vessel was not counted twice unless it was branched between the two intersection points. As the blood had been fixed in position by freezing the egg in liquid nitrogen prior to the chemical fixation, erythrocytes made pre- and post-capillary vessels equally visible, and both were counted. By day 14, the size and number

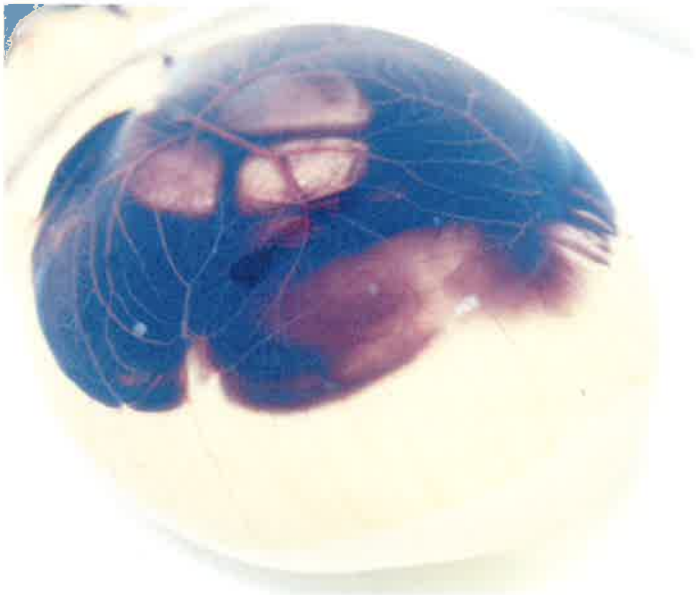


Fig. 4C Contents of a chicken egg on day 8. Ink injected into the allantoic compartment shows where the yolk sac membrane exceeds the CAM.

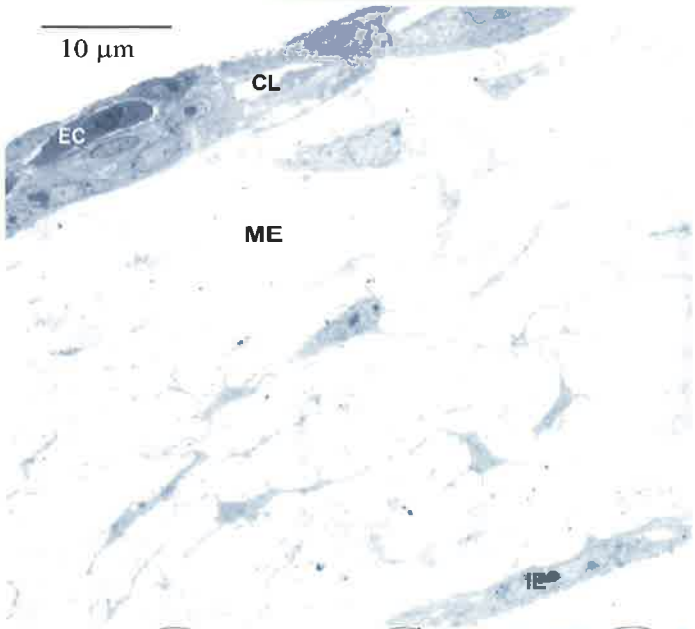


Fig. 4D.1 Cross-section of the CAM at 1250x. CL capillary lumen, EC erythrocyte, ME mesoderm, IE inner epithelium

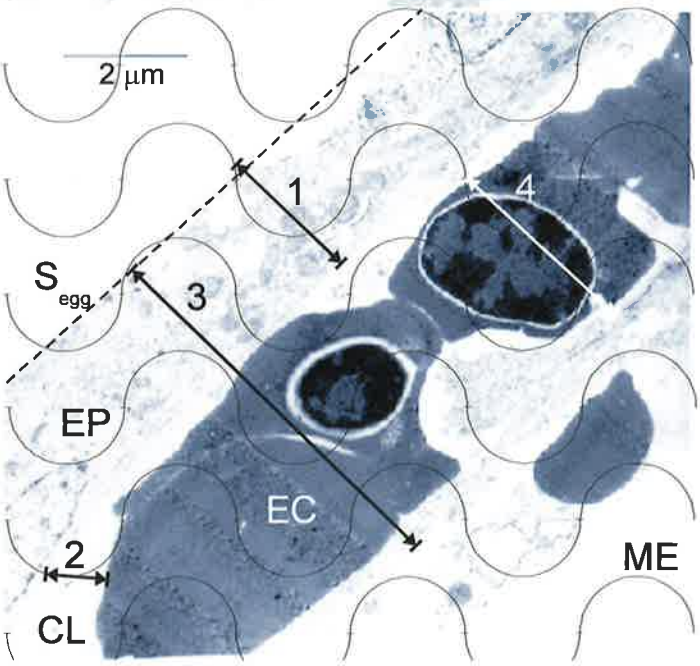


Fig. 4D.2 Isotropic grid overlayed on chorionic epithelium on day 10, 6200x. Segg reference surface, 1 distance surface/capillary, 2 distance capillary/erythrocyte, 3 thickness of chorionic epithelium, 4 diameter of capillary

of vessels of both types are comparable (DeFouw *et al.*, 1989). Calculation of the length density (L_a [vessels mm^{-1}]) of vessels followed Weibel (1979):

$$L_a = \pi/2 \times I_L , \qquad (4.1)$$

where I_L is the number of intersections per test line length.

4.2.3 Blood variables: haemoglobin, haematocrit and blood volume

4.2.3.1 Blood haemoglobin and haematocrit

Blood samples were collected from the allantoic vein or artery with a heparinised syringe at days 10, 12, 14, 15, and 16 for reference values of haemoglobin concentration. The calibration curves were calculated for the absorption of blood in concentrations of 5, 10 and 20 µl in 1.2 ml Drabkin's solution (Cyanomethaemoglobin method; Oser, 1965), measured at 540 nm with the Perkin-Elmer 124 Double Beam Spectrophotometer (Colena Instruments Division, Maywood, Illinois 60153, USA). An estimate of haemoglobin concentration was obtained by multiplying the absorption of the blood sample at a concentration of 1% in Drabkin's solution (10 µl blood in 1 ml Drabkin's) and at 540 nm with 14.8 (John Baldwin, pers. communication).

Haematocrit values were obtained for embryos on day 15. For this, approximately 60 µl blood in 80 µl micro-haematocrit capillary tubes (Pyrex Laboratory Glassware Corning, USA) was centrifuged for 5 min at 5400 G (Select a Fuge 24, Biodynamics). The haematocrit level was determined using a Hawksley micro-haematocrit reader (Hawksley, England).

4.2.3.2 Blood volume in the chorioallantoic membrane

The blood volume in different parts of the CAM was analysed photometrically. After quick-freezing the egg in liquid nitrogen and opening the eggs at the equator, CAM pieces (4.0 ± 0.9 cm^2 SD) were sampled, placed into 1.2 ml Drabkin's solution (appendix 7.3.1) and refrigerated until used (next day or the day after). The CAM piece was finely ground in the solution with a glass tissue grinder (Kontes Scientific glassware/instruments, New Jersey, USA), and the sample was then centrifuged for 3.5 min at 5400 G (Select a Fuge 24, Biodynamics). In cases of very high absorption,

samples were diluted with an equal amount of fresh Drabkin's solution. Absorption was measured at the wavelengths of 520, 540 and 560 nm against a control of Drabkin's solution. The data were analysed using a model by Lothian (1958) to correct for the other body fluids (e.g. lymph) within the tissue. It mathematically obtains the concentration of a known chromatophore within a "contaminated" solution of unknown absorption coefficient, assuming a linear absorption of the unknown substance(s) over the range of wavelengths selected. For the model, two wavelengths were selected that bracketed the target wavelength (540 nm) at equal distances ($\lambda_1 = 520$ and $\lambda_2 = 560$ nm), which showed the same absorption value in the pure solution (blood). The ratio of the maximum absorption to the bracket absorption of the pure solution, as well as the difference between the two bracket values of the contaminated solution, was used to calculated the concentration of the target solution within the mixed solution (see appendix 7.3.2).

The size of each CAM piece was considered to be the same as the piece of shell from which it had been separated. This was calculated by photocopying the shell piece on a commercial photocopier, cutting out the image and weighing it. The weight was directly proportional to the surface area involved and compared to three reference sections from the same paper sheet each time.

In the measurements of the blood volume in the whole CAM, the chorioallantoic surface area (S) was calculated from the fresh egg mass (W) according to Paganelli *et al.* (1974):

$$S = 4.835\ W^{0.662} \qquad (4.2)$$

After quick-freezing the egg in liquid nitrogen and opening the egg at the equator, both halves of the CAM were immediately transferred into a jar filled with 20 ml of Drabkin's solution, and under immersion cut into the smallest pieces possible with a pair of fine scissors. For absorption measurements samples were diluted by a factor of two and processed as above. The dilution factor of blood in Drabkin's solution was thus similar for all samples and within the linear absorption range.

4.2.4 Transmission electron microscopic analysis

4.2.4.1 Selection and processing of samples

All tissue samples for the transmission electron microscopy (TEM) were derived from the equatorial region of the eggs (from 30° N to 30° S). After freezing the intact egg in liquid nitrogen for 15 to 20 s, large sections (ca 4 cm^2) of the shell with the chorioallantoic membrane (CAM) attached were quickly cut and fixed in 2.5% glutaraldehyde. After 3-4 h the samples were rinsed in phosphate buffer and distilled water, and the CAM was separated from the shell. Thin (1 mm) strips were cut from the centre of the larger CAM sample and stored cold in fresh glutaraldehyde solution until used within 1-3 days.

For the TEM, final samples of about 1 mm x 2 mm were cut from these strips, post-fixed in osmium tetroxide, dehydrated in alcohol and embedded in TAAB resin according to the protocol in appendix 7.2.3.

When the samples were finally held in the resin blocks, the tissue samples rested flat on a bottom layer of resin that had been allowed to polymerise for 3 h before the remainder of the resin and the samples were added. Thus the perpendicular axis of the samples (in relation to the eggshell) was orientated vertically in the block. The surface plane of the samples did not move more than a few degrees out of the horizontal. Thick and thin sections were cut vertically through this plane using the Reichert Ultramicrotome OmU3. They were therefore orthogonal, non-random cross-sections of the CAM.

4.2.4.2 Imaging

The thick (1 µm) sections were stained with Methylene Blue and Fuchsin using the Adam Lockart method (see appendix 7.2.4.1) and viewed under the light microscope at 400x magnification in order to discern the suitability of the specimen. The thin (pale golden) sections of the tissue samples were stained with lead citrate and uranyl acetate (see appendix 7.2.4.2) and viewed under the Phillips CM100 transmission electron microscope at a high tension of 80 V. Images were captured along the full length of the sample at magnifications between 1100x and 1450x using a digital video camera installed in the vacuum chamber. This camera added to the magnification, so that the images were analysed at a magnification of approximately 10,000x (8,600x – 13,500x). The stored images were analysed with the Optimas 5.2 graphical analysis software.

4.2.4.3 Image analysis

Eight good-quality images of each sample were analysed and averaged. As the CAM cross-sections showed a high degree of anisotropy (fig. 4D.1), the images were overlaid with an isotropic test system L100 (Weibel, 1979, Vol. 1, p374) (fig. 4D.2). Measurements were taken from the intersection of the grid lines with the relevant tissue surface. The variables measured included:

D_{cap}: the shortest distance between surface and the inner capillary lining.

D_p : the shortest distance between the inner capillary lining and the erythrocytes.

I_{plane}: the number of intersections of grid lines with ideal surface (shortest line between the surface points of the chorionic epithelium at the image-margin: "plane").

I_{CAM} : the number of intersections of grid lines with surface of the chorionic epithelium.

I_{cap} : the number of intersections of grid lines with inner capillary lining.

- the point count of haematocrit (P_{hcrt}) and blood plasma (P_p)
- the orthogonal thickness of the chorionic epithelium and the capillary lumen from the intersection with the grid lines.

4.2.4.4 Calculations

The thickness of the air/blood barrier was calculated as an arithmetic and harmonic mean thickness of the tissue barrier (distance air surface/inner capillary wall) and plasma barrier (inner capillary wall/red blood cell) (Wangensteen and Weibel, 1982). Since the cross-sections of the tissue were orthogonal, the harmonic mean thickness was calculated directly from the inverse of the measurements. The thickness of the plasma barrier was calculated similarly as the harmonic mean thickness of the capillary wall/erythrocyte distance.

The haematocrit value was obtained as the ratio of blood cell count over total capillary lumen count:

$$Hcrt = P_{hcrt} / (P_{hcrt} + P_p) = P_{hcrt}/P_{cap} \qquad (4.3)$$

The actual surface area of the CAM (S_{CAM}) was calculated from the ratio of the number of intersections of the grid lines with the CAM surface (I_{CAM}) over the number of intersections with the ideal surface (plane) (I_{plane}), multiplied with the shell surface of

the egg (S_{egg}), which was calculated from the initial egg mass according to Paganelli *et al.* (1974, equation 4.2):

$$S_{CAM} = S_{egg} \times (I_{CAM}/I_{plane}) \quad (4.4)$$

Similarly, the capillary surface area (S_{cap}) was obtained from the ratio of the number of intersections of the grid lines with the inner surface of the capillaries (I_{cap}) over I_{plane}, multiplied with the shell surface:

$$S_{cap} = S_{egg} \times (I_{cap}/I_{plane}) \quad (4.5)$$

Only the half of the capillary surface that was directed towards the outside was measured in this context, as the side pointing towards the interior of the egg cannot be assumed to participate in gas exchange. The capillary volume (V_{cap}) per unit surface area was derived from the point count falling onto the capillary (P_{cap}), egg surface, the distance between the grid points (d) and the number of intersections of the grid lines with the plane (Weibel, 1979):

$$V_{cap} = S_{egg} \times \pi \times d/4 \times (P_{cap})/I_{plane} \quad (4.6)$$

Capillary diameter was calculated from the measured value by multiplying it with $4/\pi$ to obtain the value for a circular tube (Weibel, 1979). The thickness of the chorionic epithelium that contains the capillaries was used directly.

4.2.5 Statistical analysis

All values are given as means and standard deviation unless defined otherwise. Parametric and non-parametric tests were used appropriately, depending on tests for distribution normality. The two opposing sides of the same eggs were compared with a paired Student's t-test, when the distribution did not differ from normal. There was no correlation of vessel density, blood volume or barrier thickness between the two sides, arguing for independence of the values although they derived from the same individual. They were therefore compared to the control in an ANOVA where appropriate. Multiple

comparisons of group means with the undisturbed control used Dunnett's method (Dunnett, 1955).

4.3 Results

4.3.1 Growth of the chorioallantoic membrane

The wax cover on the shell did not impede growth of the chorioallantoic membrane. Although the vascularised area under the free half in group (b) (pointed end) was significantly greater than under the wax ($p = 0.0009$, $n = 5$, paired t-test) on day 8, this result was not repeated in any of the other groups on day 8 or day 10 (fig. 4.1a,b), and in group (c) on day 8 this was reversed, although not significantly.

Total CAM cover did not differ between same age groups. On day 8 it averaged 56.3% ± 4.2% of the egg surface (range 48.6% to 65.4%). On day 10, this had increased to 81.9% ± 7.8% (range 64.3% to 91.3%). By this day, only the chorioallantoic membrane functioned as gas exchange organ, and it covered the inner shell surface almost completely, with only small pockets of albumen remaining.

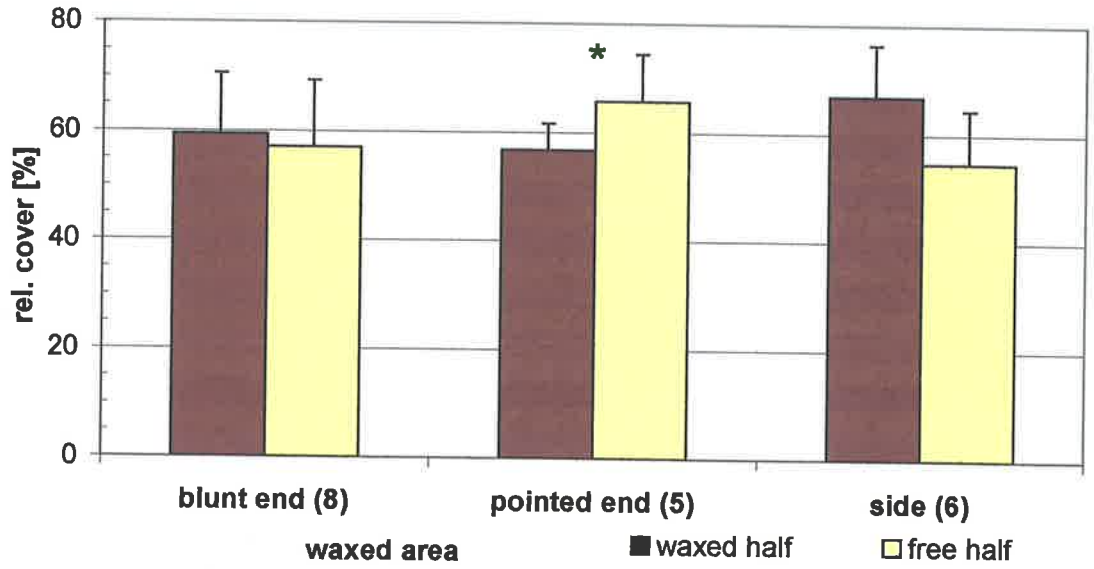


Fig. 4.1a: Relative cover of vascularised membrane under waxed and free shell, day 8. Means and SD, sample size in brackets. *indicates significant differences.

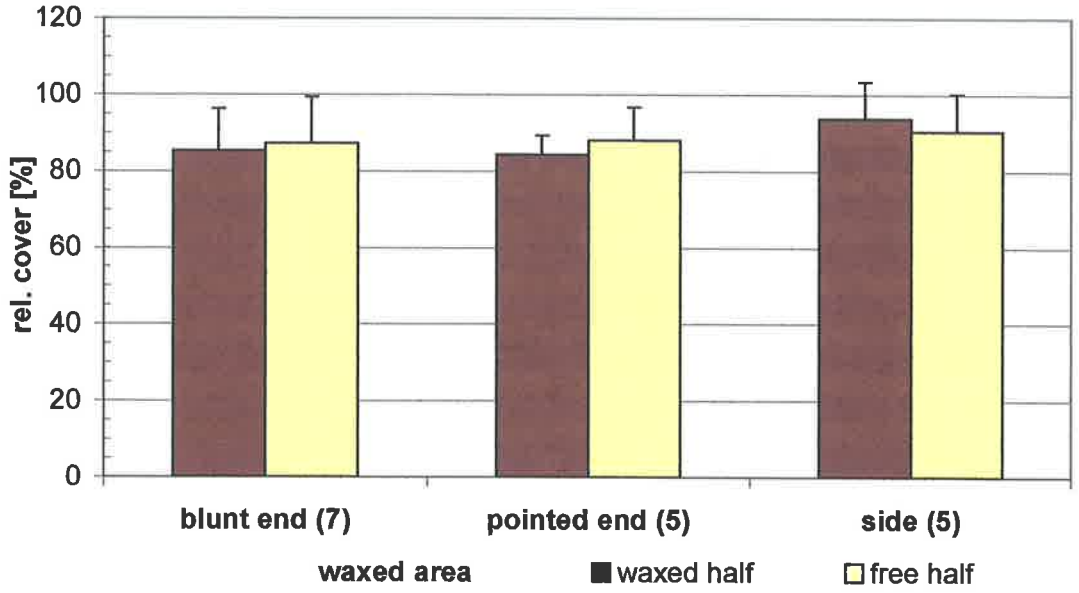


Fig. 4.1b: Relative cover of CAM under waxed and free shell, day 10. Means and SD, sample size in brackets.

Chorioallantoic surface area did not correlate with egg size (initial egg mass or egg surface area) or embryo mass (wet or dry) at any time in either individual groups or over all eggs. However, it correlated negatively with the relative size of the aircell (% surface area) as a measure of shell conductance in group (b) (significant on day 10, $r = -0.941$, $p < 0.01$, $n = 5$) and group (c) (significant on day 8, $r = -0.811$, $p = 0.05$, $n = 6$), but not in group (a). This may indicate that chorioallantoic growth is faster in eggs with lower shell conductance.

The position of the aircell was often slanted towards one side (not symmetrical around the egg's long axis), with a mean ratio of maximum to minimum distance from the pole of 1.8 ± 0.4 on day 8 and 2.4 ± 1.6 on day 10. However, this did not relate to the position of the wax in the "side" groups; the impermeable shell area of the waxed side was neither favoured nor avoided (paired t-test of aircell-shell area on waxed (2.8 ± 1.3 day 8; 3.7 ± 2.0 day 10) and free (3.6 ± 1.2 day 8; 3.7 ± 1.2 day 10) side).

In Brief:
The expansion of the chorioallantoic membrane was not influenced by the local reduction of shell conductance in partly waxed eggs.

4.3.2 Density of pre- and post-capillary vessels

During the incubation period from day 10 to day 15, the density of pre- and post-capillary (PPC) vessels increased in undisturbed eggs (fig. 4.2) ($p = 0.033$, $F_{3,45} = 3.1672$, ANOVA). This was significant from day 10 to 14, no further significant increase occurred to day 15 (Tukey HSD all comparisons). A similar result was obtained in the hole/control eggs, whereas wax/hole experimental eggs showed some indication that vessel density in the CAM appeared to be still increasing by day 15 (fig. 4.3). This was, however, not significant.

Wax/hole treatment at different ages

On day 15, after wax/hole treatment at different days, vessel density under the wax side of the egg were consistently smaller than under the hole side or in the control eggs (fig. 4.4), significantly after shell treatment on days 4 ($p = 0.020$), 7 ($p = 0.003$) and 12 ($p = 0.003$, t-test). Vessel density under the holes was often greater than in the hole/control group, but this was significant only after shell treatment on day 7 ($p = 0.006$, t-test).

The pattern was repeated in the combined groups of eggs that were manipulated before (days 4-8) and after (days 10-12) CAM attachment (fig. 4.5). Vessel density under the wax in both groups was significantly reduced in comparison to the hole side of the eggs and to the hole/control group after shell treatment on days 4-8 ($p < 0.0001$, $F_{2,59} = 12.7788$ days 4-8; $p = 0.0011$, $F_{2,43} = 8.0336$ days 10-12, ANOVA), but not to the hole/control after treatment on days 10 to 12. There was no significant difference between the hole side of experimental eggs and control eggs (Tukey HSD). Only the waxed (day 4-8) group and the hole (day 10-12) group deviated significantly from the undisturbed control (5.39 ± 1.10 mm^{-1}, $p < 0.05$, repeated means comparison, Dunnett's Method).

Vessel density did not change with age at treatment. The difference in vessel density between the wax and hole side of the [same] experimental eggs manipulated before CAM attachment (days 4-8; 0.85 ± 0.73) was not significantly increased compared to eggs with shell treatment after CAM attachment (days 10-12; 0.68 ± 0.83) (t-test). This would be expected as differences between wax and hole sides should emerge only after CAM attachment.

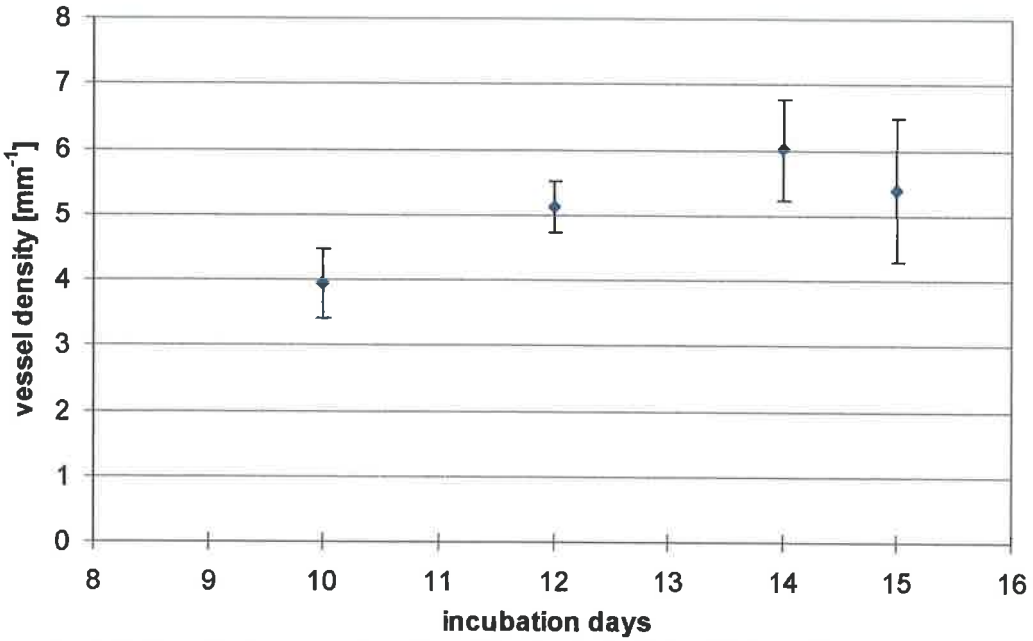


Fig. 4.2: Density of pre- and post-capillary vessels in the chicken CAM on day 10 (n = 4), day 12 (10), day 14 (3) and day 15 (32) of incubation. Means and SD.

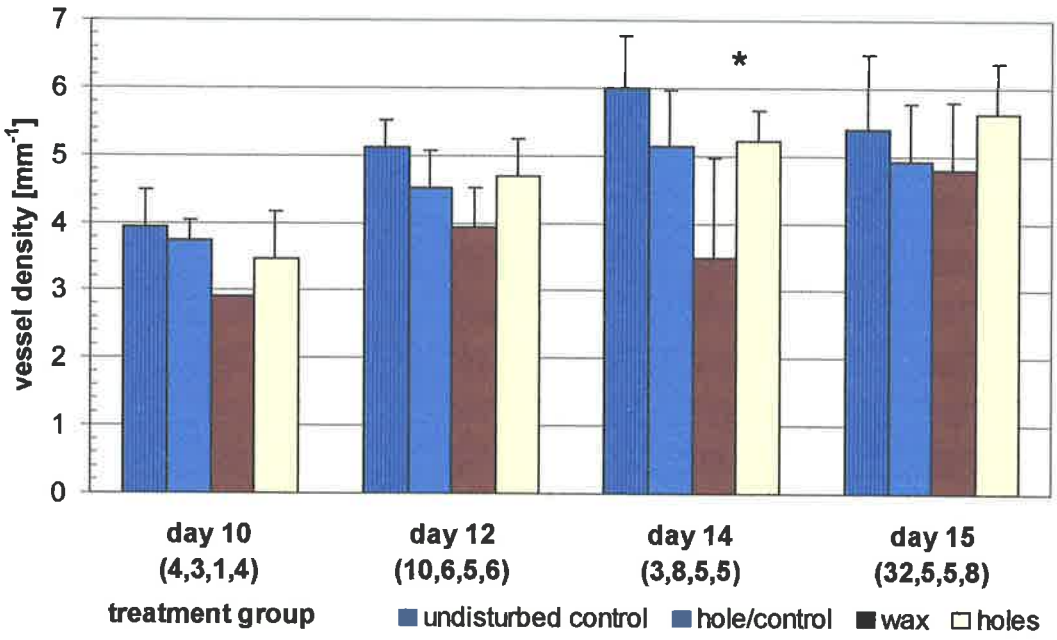


Fig. 4.3: Density of pre- and post-capillary vessels in CAM at different stages of incubation after wax/hole treatment on day 8. Means and SD, sample size in brackets. *indicates significant differences.

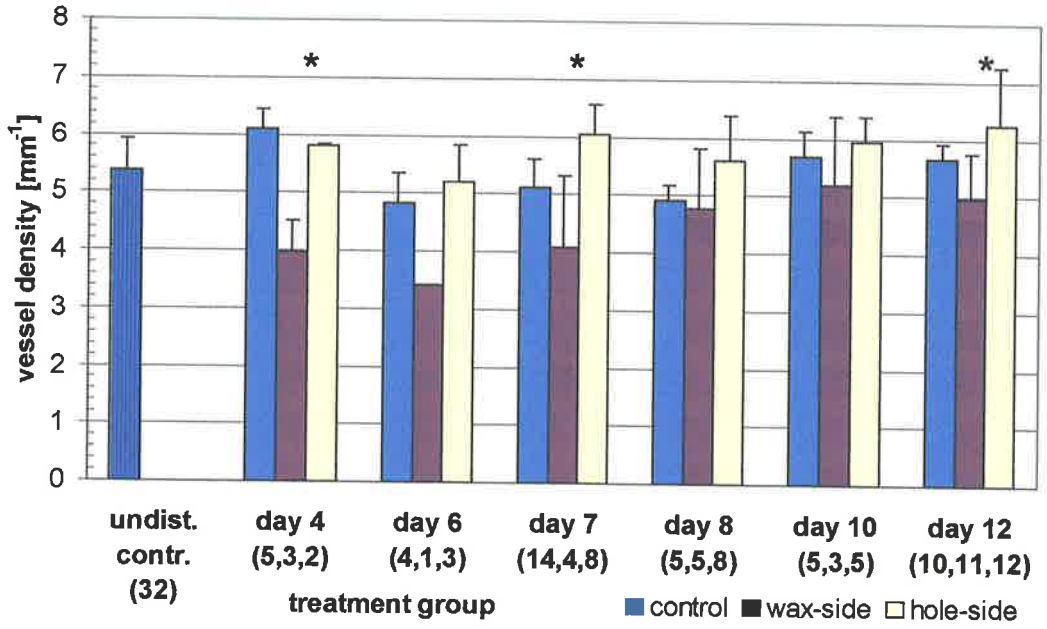


Fig. 4.4: Density of pre- and post-capillary vessels in CAM of 15 d old chicken eggs after wax/hole treatment at various days. Means and SD, sample size in brackets. *indicates significant differences.

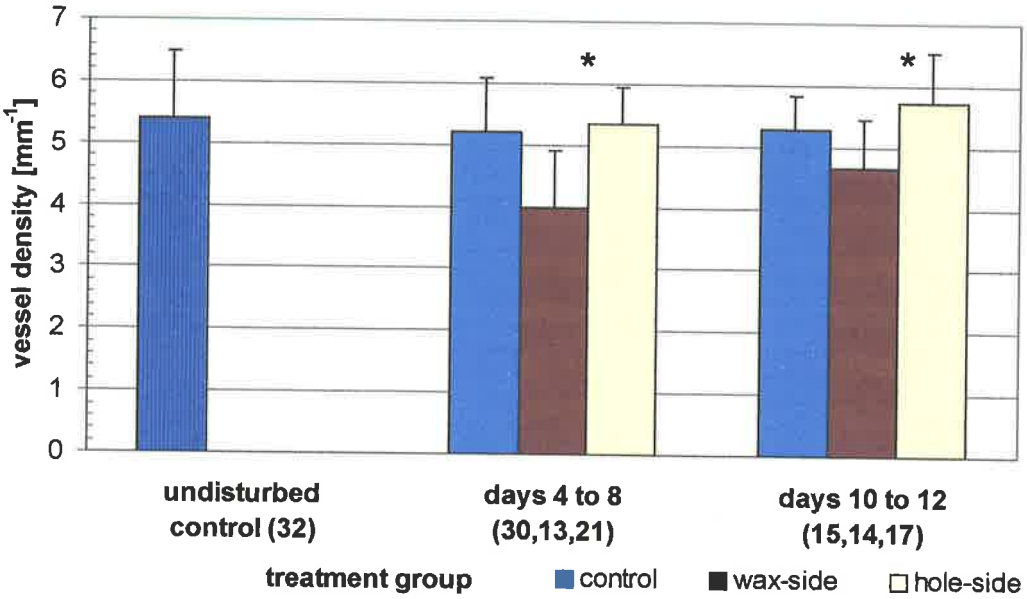


Fig. 4.5: Density of pre- and post-capillary vessels in chicken CAM on day 15 after wax/hole treatment before (days 4-8) and after (days 10-12) CAM attachment. Means and SD, sample size in brackets. *indicates significant differences.

Wax/oxygen treatment

The wax/oxygen treatment yielded very similar results (fig. 4.6). Overall vessel density increased from day 12 to day 14 in the control group ($p = 0.096$, t-test) and from day 12 to day 15 under the free side of the experimental eggs ($p = 0.0009$, $F_{2,22} = 9.7696$, ANOVA), but not under the waxed side. Pre- and post-capillary densities under the wax side were reduced at all times ($p = 0.059$ day 12, $p = 0.050$ day 14, $p = 0.006$ day 15, paired t-test) in comparison to the free side of the same eggs.

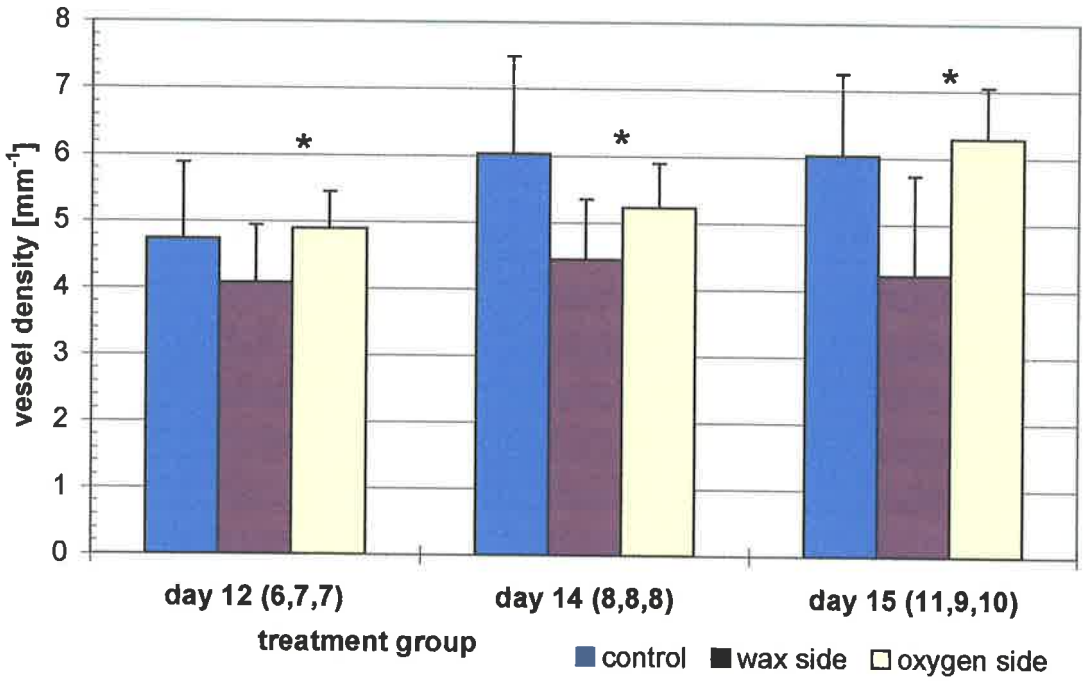


Fig. 4.6: Density of pre- and post-capillary vessels in CAM at various ages after wax/oxygen treatment from day 10. Means and SD, sample size in brackets. *indicates significant differences.

In Brief:

At any time on days 10 to 15, the density of pre- and post-capillary vessels was reduced under the wax in comparison to the free side in both wax/hole and wax/oxygen treatment. The density on the free side did not differ from that observed in control eggs. The timing of treatment had no effect on vessel density.

4.3.3 Blood measurements

4.3.3.1 Haemoglobin concentration and haematocrit

Haemoglobin concentration in the blood of undisturbed chicken embryos rose from day 10 (4.40 ± 0.63 g/100ml) to day 16 of incubation (6.55 ± 0.22 g/100ml) (fig. 4.7). It correlated positively with wet ($r = 0.840$, $p < 0.0001$, $n = 24$) and dry ($r = 0.819$, $p < 0.0001$) embryo mass, and with the age of the embryos ($r = 0.849$, $p < 0.0001$), but not with eggshell conductance ($r = -0.088$) or egg surface area ($r = 0.312$).

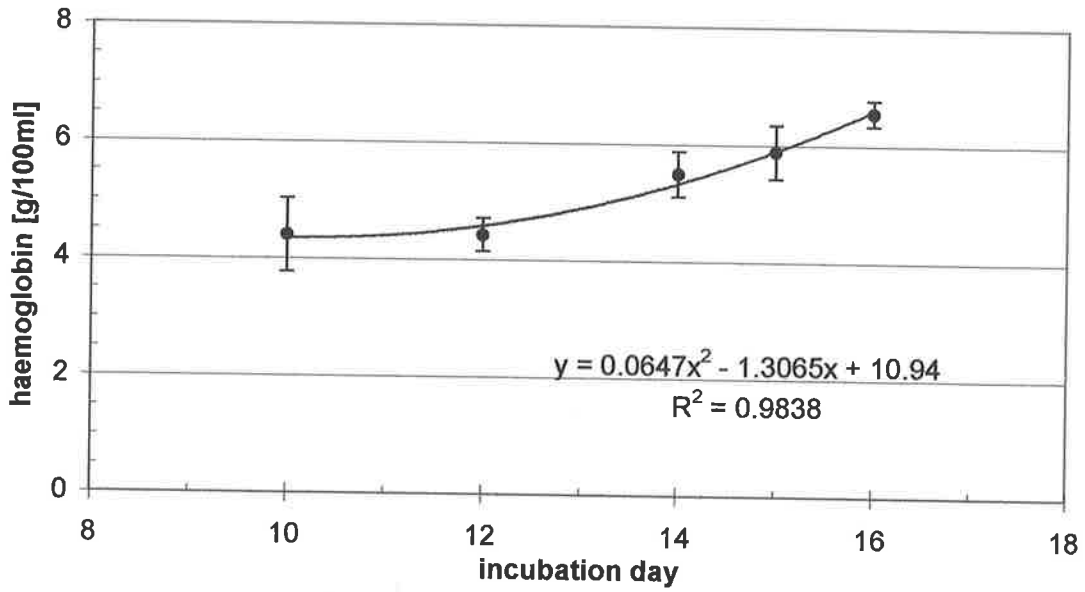


Fig. 4.7: Haemoglobin concentration in chicken blood from day 10 to 16 of incubation. Means and SD, sample size ranged from 3 to 8 per age group.

Haemoglobin concentration in wax/hole experimental eggs on day 15 (4.96 ± 0.92 g/100ml, $n = 8$) was significantly lower than in the hole/control (5.80 ± 0.44 g/100ml, $n = 11$) ($p = 0.016$, t-test). This corresponded to wet embryo mass, which was significantly lower in the experimental group (8.61 ± 1.69 g) than in the control group (10.05 ± 1.09 g) ($p = 0.038$, t-test). Haematocrit was slightly, but not significantly greater in the experimental eggs (28.5% ± 4.4%, $n = 4$) than in the control eggs (26.6% ± 1.8%, $n = 9$). It correlated significantly with haemoglobin concentration (control: $r = 0.921$, $p = 0.0004$; experiment: $r = 0.994$, $p = 0.0059$).

If mean corpuscular volume of the red blood cells was constant between treatments, the concentration of haemoglobin in the cells of the wax/hole eggs was

reduced by 13% in comparison to the control (obtained from the ratios of haemoglobin concentration over haematocrit).

In Brief:
The haemoglobin concentration, but not the haematocrit in the blood of wax/hole experimental embryos was reduced in comparison to the control group.

4.3.3.2 Blood volume in the chorioallantoic membrane

Undisturbed Control

The total blood volume of the whole CAM increased from day 10 (0.17 ± 0.07 ml) to day 16 (0.26 ± 0.06 ml) of incubation ($p = 0.0040$, $F_{3,41} = 5.1747$, $n_{d10} = 12$, $n_{d12} = 10$, $n_{d14} = 12$, $n_{d16} = 11$; ANOVA) (fig. 4.8). It did not correlate with the shell conductance or egg surface area within any age group.

On day 10, the CAM covers only about 83% of the shell, while from day 12 on it is complete (Ackerman and Rahn, 1980). This correction factor for day 10 was therefore included into the calculation for area-specific blood volume. This also increased significantly from day 10 (2.79 ± 1.18 µl cm^{-2}) to day 16 (3.56 ± 0.74 µl cm^{-2}) ($p = 0.0022$, $F_{3,41} = 5.7713$, ANOVA) (fig. 4.8). Mass-specific blood volume (in relation to dry embryo mass) decreased during this time ($p < 0.0001$, $F_{3,41} = 55.4138$, ANOVA) (fig. 4.9a). Since the water content of the embryo fell during this period from 93.1% ± 0.4% to 85.8% ± 1.8%, a slightly shallower, but similar curve was obtained for the wet-mass specific blood volume (fig. 4.9b).

There was a positive correlation between the total blood volume and the embryo mass over all samples ($r = 0.493$, wet mass, $p < 0.0001$; $r = 0.453$, dry mass, $p = 0.0014$; $n = 45$), as well as within the groups of day 10 ($r = 0.882$, $p < 0.0001$, $n = 12$), day 12 ($r = 0.563$, $p = 0.090$, $n = 10$) and day 16 ($r = 0.875$, $p = 0.0004$, $n = 11$) (dry embryo mass). These correlations persisted for the area-specific blood volume, where eggs were standardised for size (egg surface area).

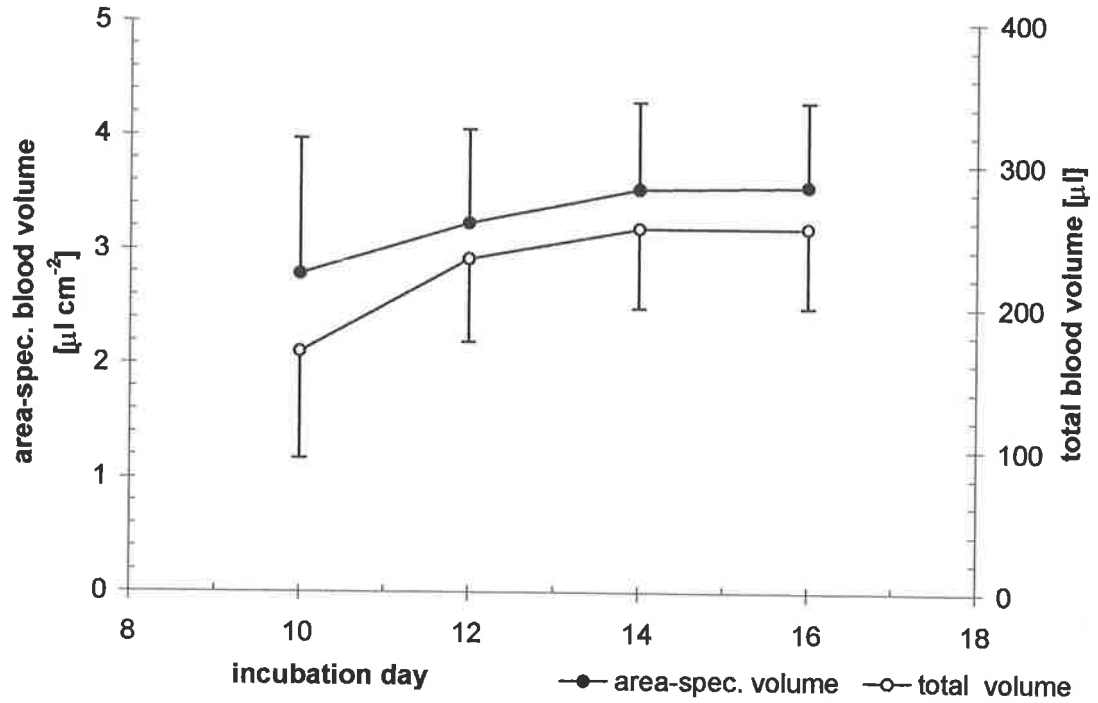


Fig. 4.8: Blood volume in CAM of undisturbed chicken eggs on days 10 to 16. Means and SD, 10 to 12 eggs per age group.

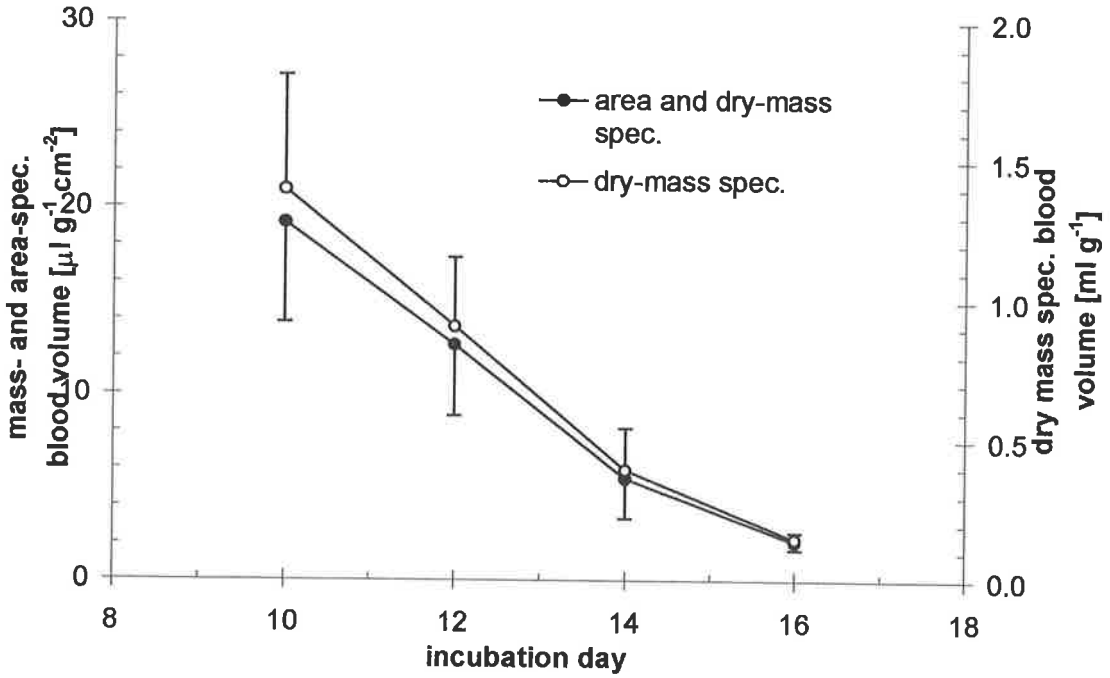


Fig. 4.9a: Dry-mass specific blood volume in CAM, absolute and area-specific, from days 10 to 16. Means and SD, 10 to 12 eggs per age group

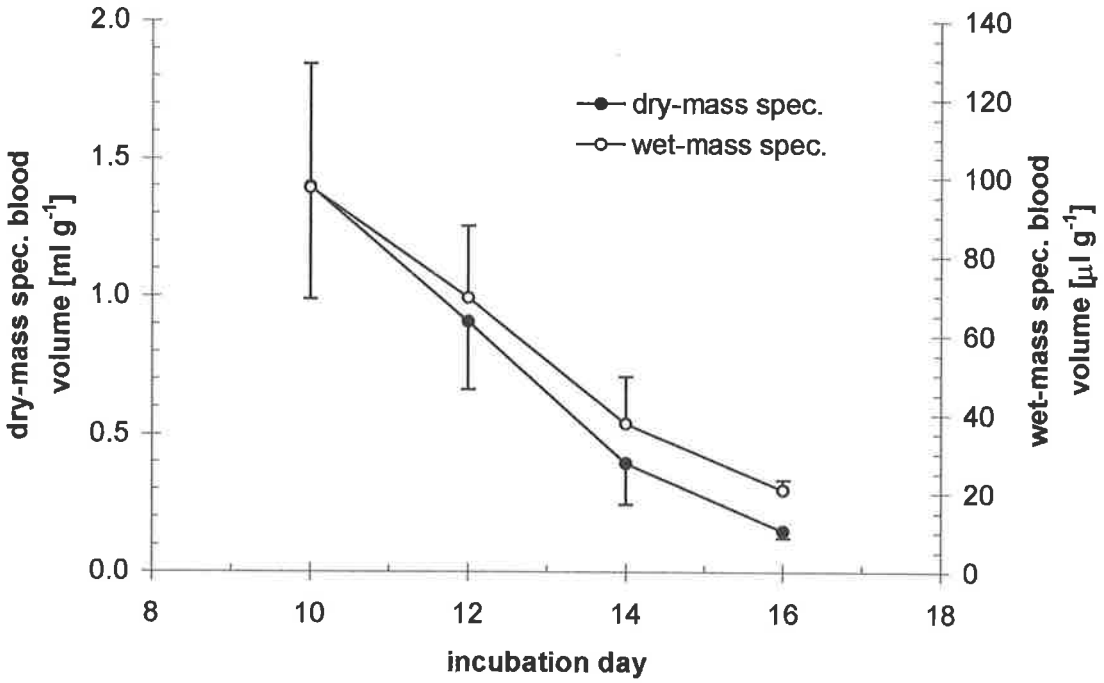


Fig. 4.9b: Comparison of dry and wet-mass-specific blood volume in CAM between days 10 and 16. Means and SD, 10 to 12 eggs per age group.

Wax/hole treatment

Over a similar time period, blood content of CAM pieces was established from day 10 to day 15 of incubation in undisturbed control eggs, hole/control eggs and both sides of experimental eggs (fig. 4.10). These measurements were corrected for the size of the pieces and therefore yielded not total, but area-specific blood volume. There was no increase in area-specific blood volume from day 10 to 14, but values on day 15 were significantly greater ($p < 0.001$ for the groups "hole/control", "wax" and "holes", ANOVA). Blood volume in the CAM under the waxed shell was greater than under the hole side, significantly on day 14 ($p = 0.027$, $n = 5$) and day 15 ($p = 0.023$, $n = 6, 8$). Values from the two sides of the same egg were not correlated. From day 12 to 15, the difference between the hole and the wax side of the same egg increased, although not significantly (ANOVA) (fig. 4.10).

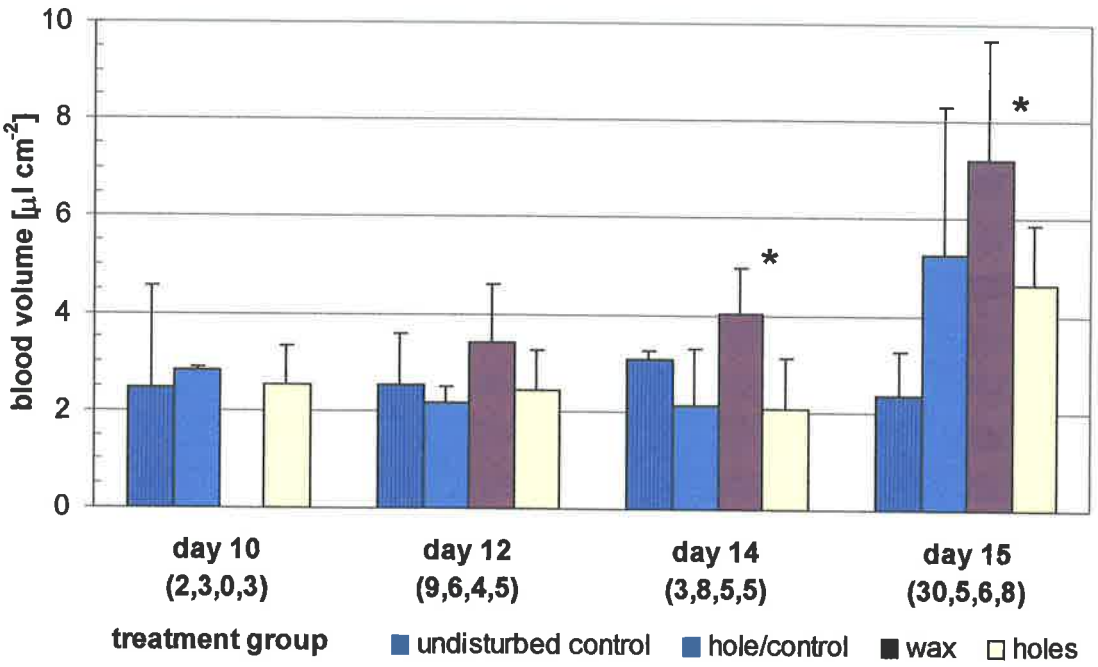


Fig. 4.10: Area-specific blood volume in CAM on days 10 to 15 after wax/hole treatment on day 8. Means and SD, sample size in brackets. *indicates significant differences.

Wax/hole treatment at different ages

In the experiments for the effect of treatment timing (wax/hole experiment), surface-specific blood volume on day 15 under the wax side of the shell was greater than under the hole side of the egg in all experiments but the day 6 treatment. This was significant on day 8 ($p = 0.025$, $n_{wax} = 6$, $n_{hole} = 8$, t-test, cf fig. 4.10) and day 12 ($p = 0.003$, $n_{wax} = 11$, $n_{hole} = 10$, t-test).

The combined groups manipulated before (days 4-8) and after (days 10-12) CAM attachment express this pattern clearly (fig. 4.11): the relative blood volume under the wax side was greater than under the hole side or in the control group (days 4-8: $p = 0.0050$, $F_{3,64} = 4.7054$; days 10-12: $p = 0.0003$, $F_{3,41} = 7.7846$, ANCOVA with the CAM piece size as co-variant). There was no significant difference between the hole/control eggs and the hole side of the experimental eggs at any time (Tukey HSD).

The area-specific blood volume in the CAM of all (day 4-8) groups and the waxed (day 10-12) group deviated significantly from the undisturbed control ($p < 0.05$, repeated means comparison, Dunnett's Method). The cause of these differences is not clear.

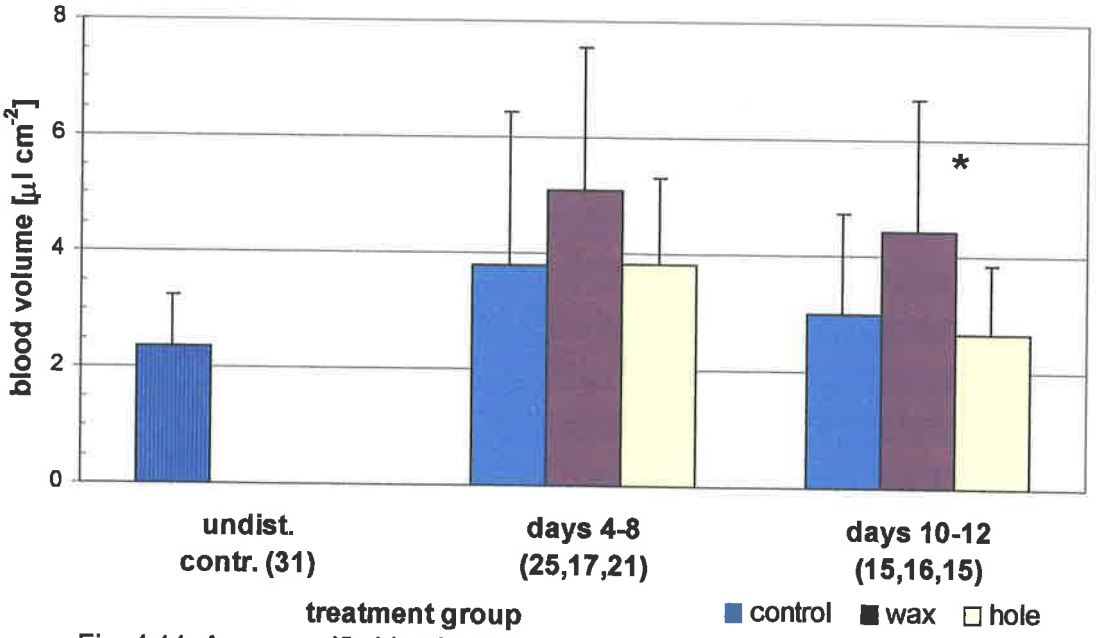


Fig. 4.11: Area-specific blood volume in CAM on day 15 after wax/hole treatment before (days 4-8) and after (days 10-12) CAM attachment. Means and SD, sample size in brackets. *indicates significant differences.

The difference in blood volume between the two sides of the [same] experimental eggs did not change significantly when manipulated before CAM attachment (0.0012 ± 0.0024 ml/cm^2) or after CAM attachment (0.0018 ± 0.0022 ml/cm^2). This would be expected, as differences between sides should appear only after CAM attachment.

Wax/oxygen treatment

In the wax/oxygen treatment, the surface-specific blood volume did not increase from day 12 to day 15 in any of the groups (Kruskal-Wallis test) (fig. 4.12). Blood volumes from the two opposing sides of the experimental eggs did not correlate significantly. On each day, the wax side of the experimental eggs had more blood in the CAM than the free side of the same egg in all groups, albeit not significantly (Wilcoxon Rank Test days 12 and 14, t-test day 15). Again there was no difference between the free side of experimental eggs and the control eggs.

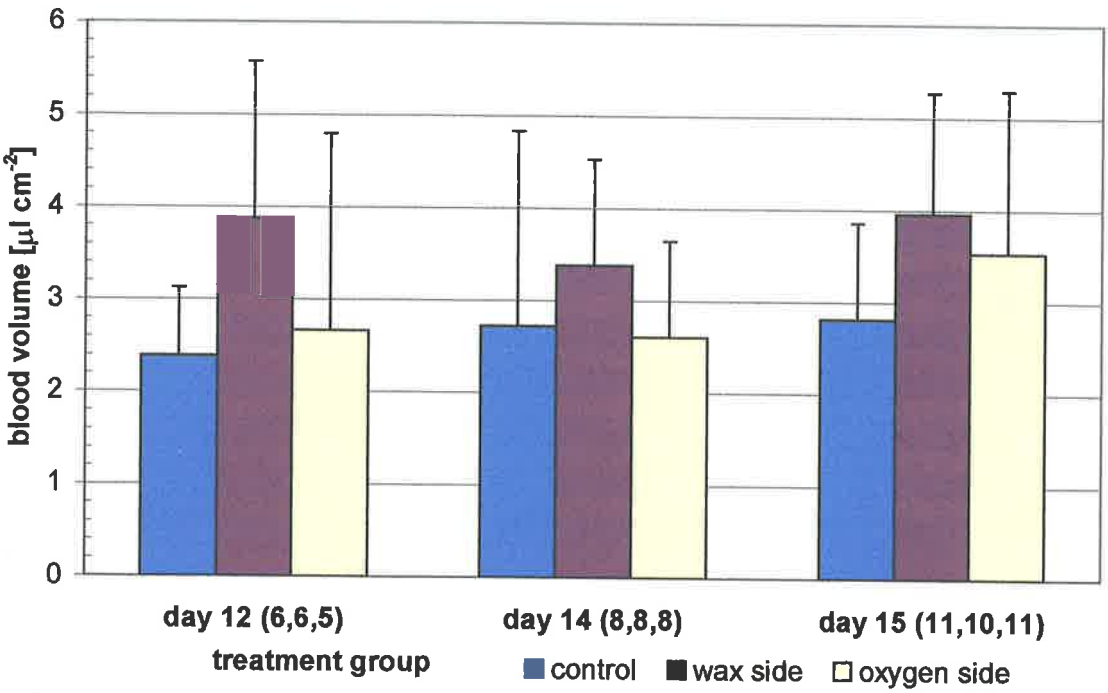


Fig. 4.12: Area-specific blood volume in CAM on days 12 to 15 after wax/oxygen treatment on day 10. Means and SD, sample size in brackets.

In Brief:

Total and area-specific blood volume in the CAM increased from day 10 to day 16 in undisturbed eggs. Total blood volume correlated positively with embryo mass. Area-specific blood volume was increased under the wax, but did not differ between the free side of experimental eggs and control eggs in wax/hole and wax/oxygen treatments. The timing of shell treatment had no effect on blood volume.

4.3.4 Capillary density

The CAM in the control eggs and under the free side of the experimental eggs in the wax/hole and wax/oxygen treatments showed an almost continuous layer of capillaries in the epithelial layer, covering between 76% and 91% of the reference surface ("plane") (fig. 4.13). Capillary surface area did not change over time (ANOVA). Most of the blood vessels appeared flattened and were filled almost completely with the nucleated red blood cells. Capillary surface area under the wax side of the experimental eggs ranged from 12% to 63% of the reference surface. It was reduced in comparison to the free side and the control group in all experiments (fig. 4.13), significantly in the wax/hole experiment on day 14 ($F_{2,13} = 4.117$, $p = 0.0412$), and in the wax/oxygen

experiment on day 12 ($F_{2,16} = 6.4725$, $p = 0.0087$) and day 14 ($F_{2,14} = 30.0234$, $p < 0.0001$, ANOVA). As the air surface of the CAM was only marginally greater than the reference surface (5%), the ratio of capillary to air surface was very similar, ranging from 70% to 87% in the control group and free side samples and from 11% to 50% under the wax.

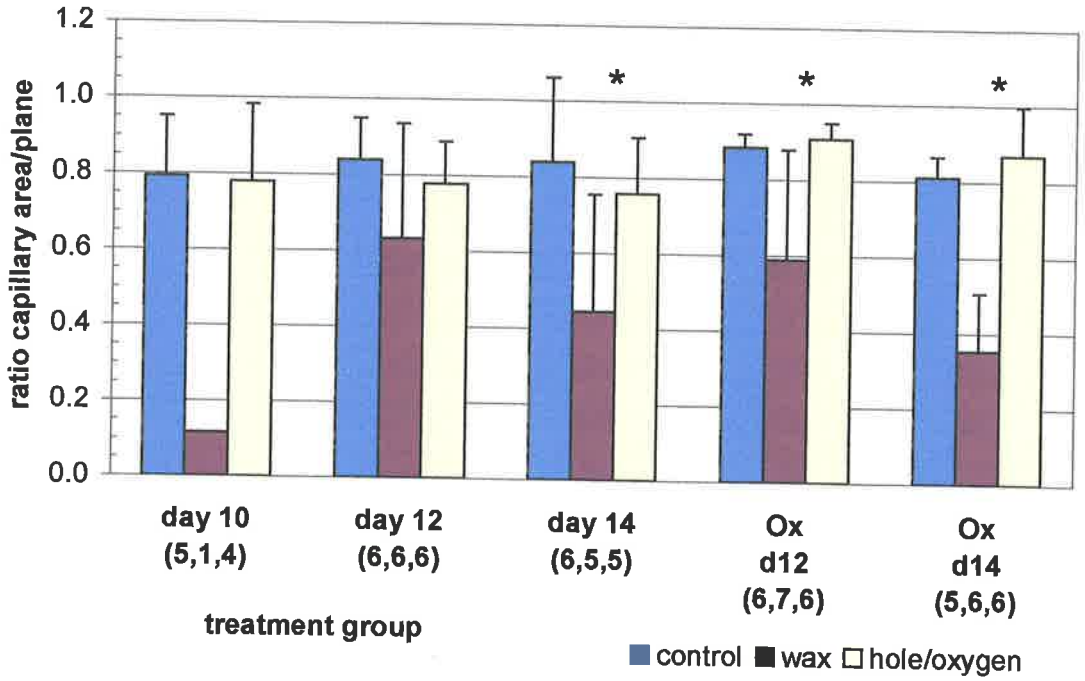


Fig. 4.13: Capillary surface area in relation to the reference surface (plane) in the chicken CAM from day 10 to 14. Means and SD, sample size in brackets. *indicates significant differences.

In Brief:

The capillary surface area did not change between days 10 and 14. It was reduced under the wax, but did not differ between free side of experimental eggs and control eggs in both wax/hole and wax/oxygen treatments.

4.3.5 Morphology of the epithelial layer of the chorioallantois

4.3.5.1 Tissue barrier

The capillaries in the CAM moved outwards in the chorionic epithelium between days 10 and 14. As a consequence, the harmonic mean thickness of the tissue barrier declined over time in the control groups of the wax/hole experiment from 2.5 ± 0.9 µm on day 10

(fig. 4E) to 0.6 ± 0.2 µm on day 14 (fig. 4F) ($p = 0.0026$, $F_{2,13} = 9.733$, ANOVA), and of the wax/oxygen treatment from 0.8 ± 0.2 µm on day 12 to 0.5 ± 0.1 µm on day 14 ($p = 0.0164$, t-test) (fig. 4.14a,b). Similarly, the thickness of the tissue barrier declined over time on wax and free sides of experimental eggs in the wax/hole treatment (fig 4.14a) ($F_{2,3} = 15.1966$, $p = 0.0617$), but not significantly in the wax/oxygen treatment, where day 10 was not measured (fig. 4.14b).

The epithelial layer of the CAM under the wax often appeared less clearly stratified and disorganised, with some leucocytes amongst the other cells (fig. 4G). Experimental eggs showed a greater thickness on the wax than on the free side (fig. 4.14a,b), but due to the high variation in the data this could not be verified statistically except perhaps for the wax/oxygen experiment on day 14 ($p = 0.0572$).

The arithmetic means of the tissue barrier thickness were in general greater than their respective harmonic mean, but showed the same pattern of decline over time. The close matches between the harmonic and arithmetic values in the control groups and under the free side in the experimental groups (wax/hole and wax/oxygen treatments) are an indication of a uniform distribution of the capillaries. The relatively greater arithmetic values under the wax indicate a non-uniform distribution of barrier thickness and are a consequence of the reduced capillary surface density in this tissue (fig. 4.14a,b).

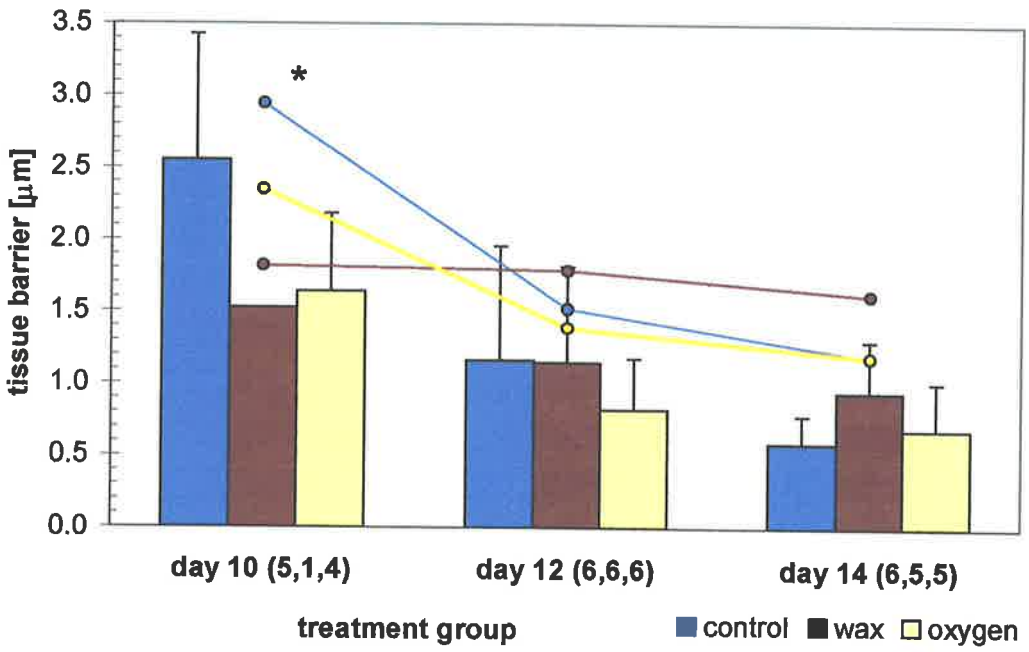


Fig. 4.14a: Harmonic mean thickness of tissue barrier in wax/hole experiment. Arithmetic means are shown for comparison as same-coloured lines. Means and SD, sample size in brackets. *indicates significant differences.

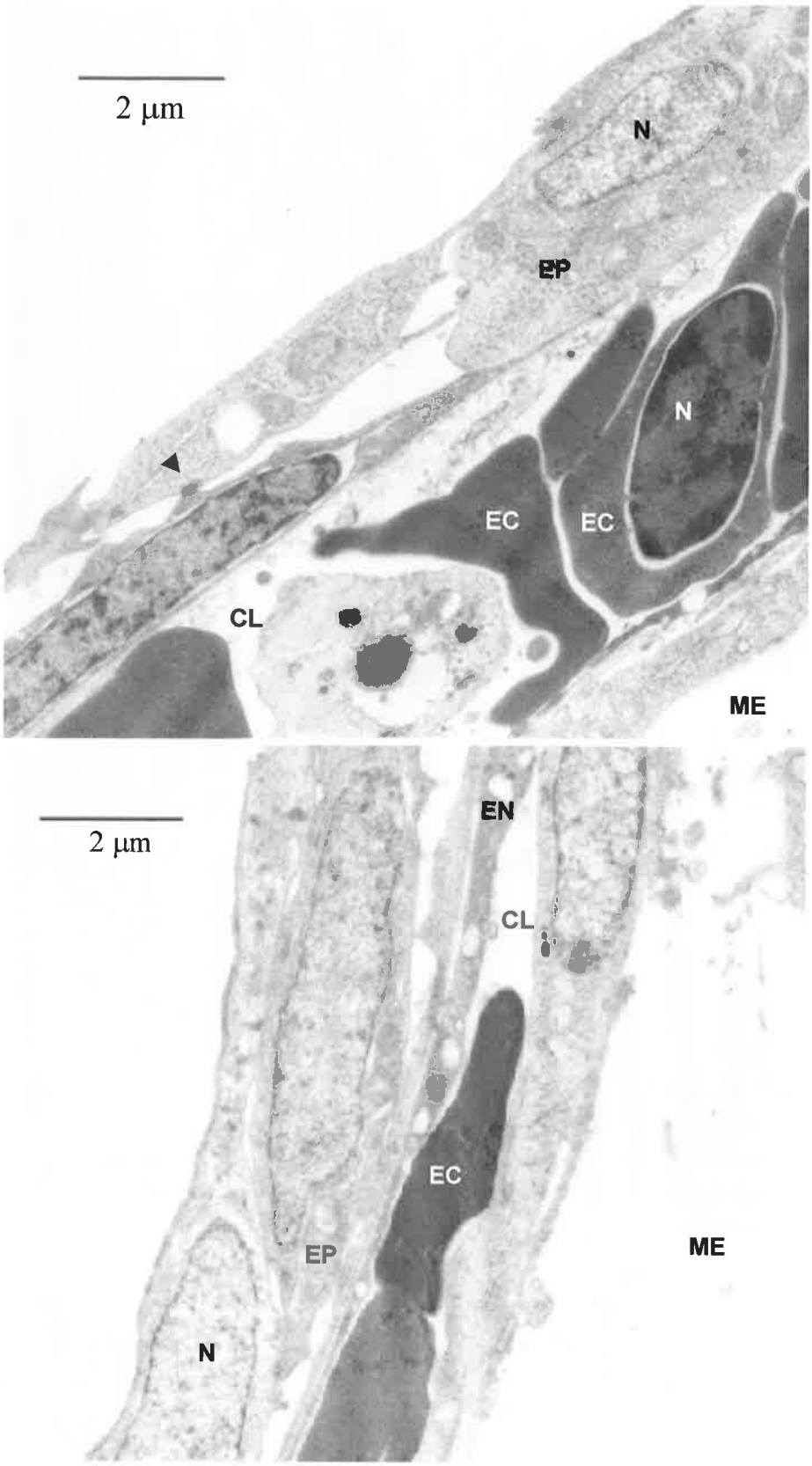


Fig. 4E Chorionic epithelium of the 10 d chicken at 6200x. Cell nuclei are interposed between the gas space (left) and the capillaries. EP epithelial cell, N nucleus, EC erythrocyte, CL capillary lumen, ME mesoderm, EN endothelial cell; arrow: tight junction.

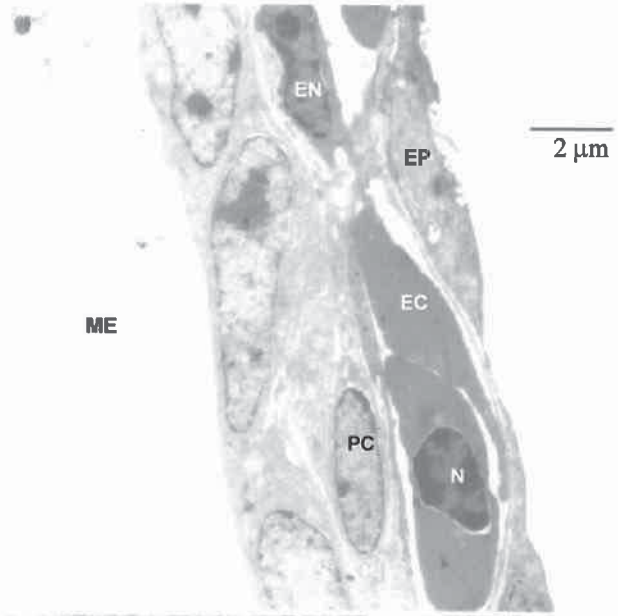


Fig. 4F.1 Chorionic epithelium of 14 d chicken embryo at 4400x. Capillaries have moved to close to the gas space (right). EP epithelial cell, EC erythrocyte, N nucleus, EN endothelial cell, PC pericyte, ME mesoderm.

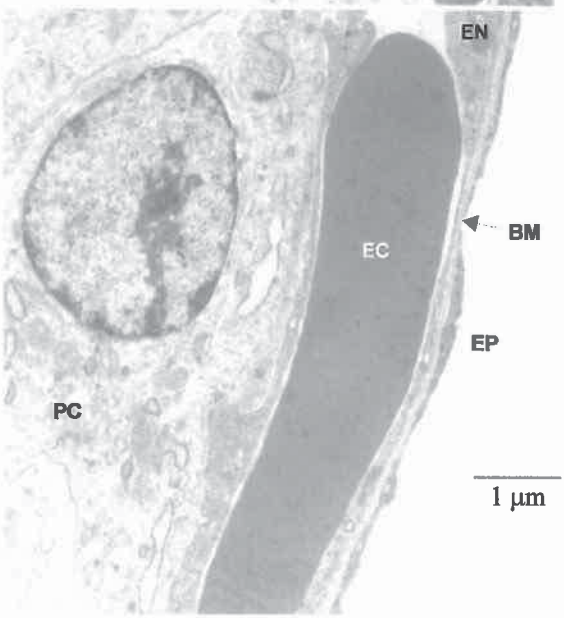


Fig. 4F.2 Surface of the chorionic epithelium of 14 d chicken embryo at 8700x. BM base membrane.

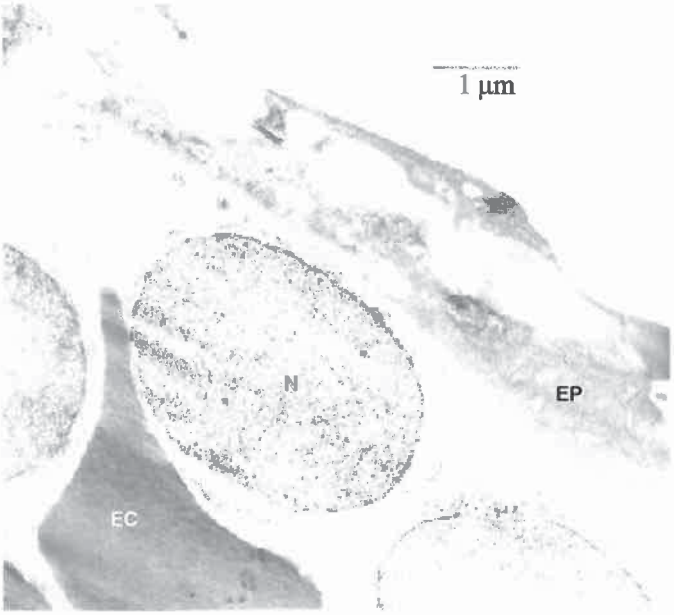


Fig. 4G Surface of the chorionic epithelium of 14 d chicken embryo under wax at 8700x. Unusually large vessel containing mainly leucocytes.

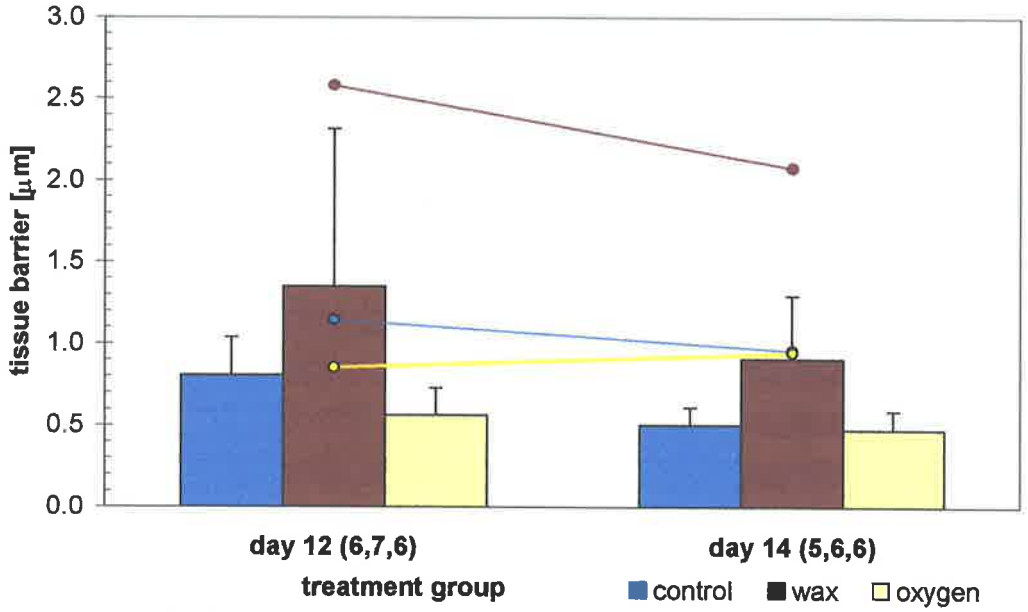


Fig. 4.14b: Harmonic mean thickness of tissue barrier in wax/oxygen treatment. Means and SD, sample size in brackets. *indicates significant differences. Arithmetic means are shown for comparison as same-coloured lines.

Experimental eggs (wax/hole and wax/oxygen) seemed to invert the capillary plexus of the free side sooner than the respective control eggs, as the harmonic mean thickness under the free side of the experimental eggs was smaller on days 10 and 12 than in the control groups. This was particularly apparent on day 10 in the wax/hole experiment ($p = 0.011$, t-test).

4.3.5.2 Plasma barrier

The harmonic mean thickness of the plasma barrier did not change over time and averaged 0.151 ± 0.046 µm in all controls. It was greater under the wax (0.483 ± 0.595 µm) than under the free (hole or oxygen) side (0.179 ± 0.100 µm, $p = 0.027$, $n = 21$, paired t-test). The thickness of the plasma barrier in the free sides of wax/hole and wax/oxygen experimental eggs did not differ from each other.

4.3.5.3 Capillary diameter

The diameter of the capillaries in the chorioallantoic epithelium did not change during the period from day 10 to 14 in the control eggs (averaging 3.5 ± 1.5 µm in the wax/hole and 3.2 ± 1.0 µm in the wax/oxygen experiment) or under the free side in the

experimental eggs (fig. 4.15), which latter did not differ significantly from the respective controls. Under the wax, capillary diameter was at first (day 12) larger than under the free side ($p = 0.0112$, $n = 6$ in wax/hole experiment; $p = 0.0756$, $n = 6$ in wax/oxygen experiment, paired t-test), but declined significantly ($p = 0.0644$, $n_{day\ 12} = 6$, $n_{day\ 14} = 5$ in wax/hole experiment; $p = 0.0108$, $n_{day\ 12} = 7$, $n_{day\ 14} = 5$ in wax/oxygen experiment, t-test) to control values on day 14. The capillaries appeared flattened and were frequently irregularly shaped where the endothelial cell bodies protruded into the capillary lumen. No truly circular section was observed at any time. Further, vessels were usually cut at an angle, and consequently the sections were ellipsoid to varying degrees, with a consequent deviation from the circular value. The diameter values presented here therefore overestimate the true vessel diameter.

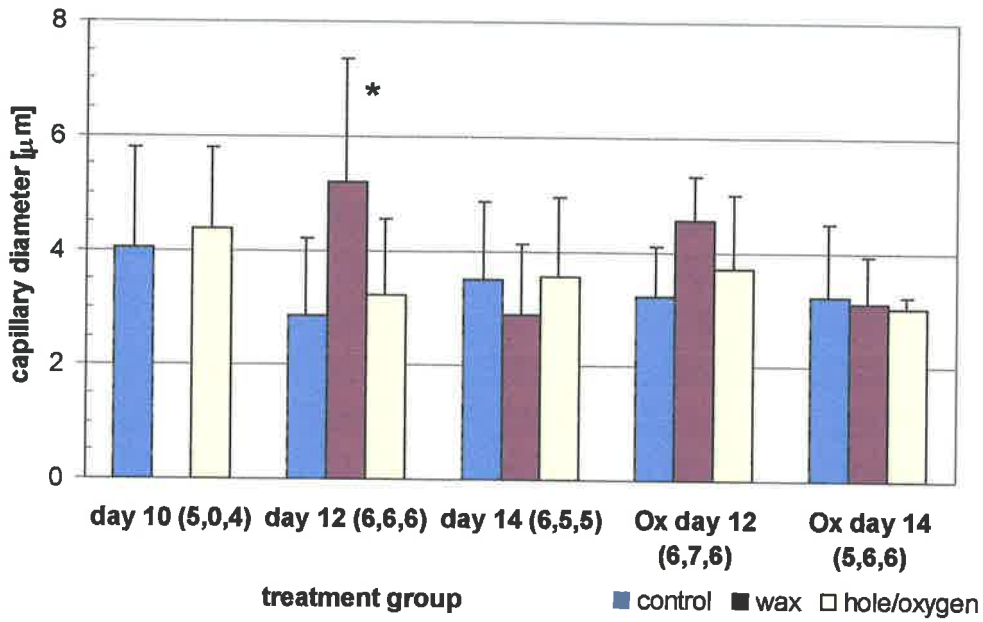


Fig. 4.15: Diameter of capillaries in chorioallantoic epithelium from day 10 to day 14 of incubation. Means and SD, sample size in brackets. *indicates significant differences.

A different estimate of mean capillary thickness was calculated as the ratio of capillary volume over capillary surface area. In this case the cross-sectional area was described as a flat rectangle, and the height of the rectangle ("diameter" of the capillaries) in the control averaged 2.0 ± 0.16 µm in the wax/hole and 1.9 ± 0.01 µm in the wax/oxygen experiments. Again, there was no change over time, and no difference between control and experimental groups except the indication of a reduced vessel

diameter under the wax in the wax/hole experiment on day 14 ($p = 0.085$, $n = 6$, paired t-test with hole side).

4.3.5.4 Thickness of the chorionic epithelium

In contrast to the diameter of the capillaries, the thickness of the chorionic epithelium increased in the control eggs of the wax/hole treatment from 5.7 ± 1.6 µm on day 12 to 8.9 ± 2.5 µm on day 14 ($p = 0.0342$, $F_{2,14} = 4.9363$, ANOVA) (fig. 4.16). Similarly, it increased in the control of the wax/oxygen treatment from 5.8 ± 1.0 µm on day 12 to 8.4 ± 1.3 µm on day 14 ($p = 0.0036$, $F_{1,19} = 15.2615$, ANOVA) (fig. 4.16). The free side of the experimental eggs yielded values similar to the control eggs. The thickness of the chorionic epithelium under the wax was greater than under the opposing side on day 12 ($p = 0.0194$, $n = 6$ in wax/hole; $p = 0.0279$, $n = 6$ in the wax/oxygen treatment, paired t-test), however it did not change towards day 14. By day 14 there was consequently no more difference between the waxed and free side.

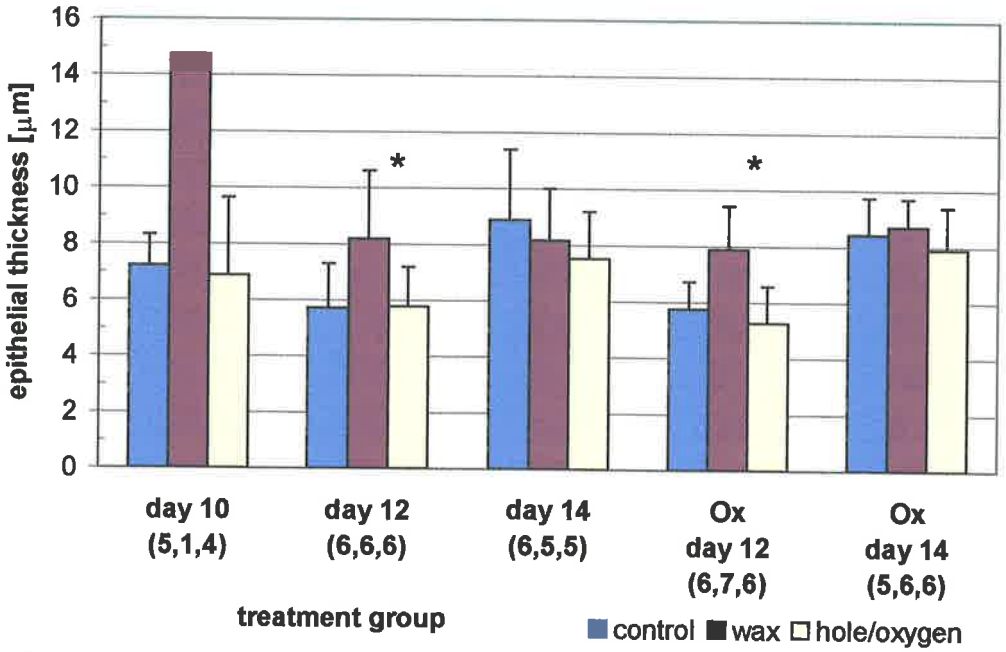


Fig. 4.16: Thickness of chorionic epithelium from day 10 to day 14 of incubation. Means and SD, sample size in brackets. *indicates significant differences.

In Brief:

The thickness of the tissue barrier declined between days 10 to 14. In wax/hole eggs this appeared to be somewhat accelerated. Tissue barrier thickness did not differ between wax and free side of experimental eggs. The thickness of the plasma barrier did not change over time. It was greater under the wax than under the free side of the experimental eggs in wax/hole and wax/oxygen treatment. The free sides did not differ from their respective controls. Capillary diameter and epithelial thickness were greater under the wax on day 12, but not on day 14 in both treatments.

4.3.6 Capillary blood volume

Capillary blood volume in the chorioallantoic membrane did not change over time in either control eggs or the free side of experimental eggs (fig 4.17). Capillary blood volume averaged 0.0124 ± 0.0062 ml and 0.0123 ± 0.0044 ml in the control eggs of the wax/hole and wax/oxygen experiments, respectively. Vessel volume under the waxed side seemed to decline from day 12 to day 14 in both experiments, but this was not statistically significant ($p = 0.129$ and $p = 0.074$, respectively, t test). However, blood volume under the wax was significantly less than under the free side on day 14 in both experiments ($p = 0.087$ wax/hole, $p = 0.0065$ wax/oxygen experiment, t-test). While leucocyte numbers in control eggs and under the holes were low, these cell types often appeared and sometimes dominated the capillaries under the wax (fig. 4G).

There was no correlation between capillary blood volume and shell conductance or embryo mass in any of the groups. This persisted when same-treatment groups of different ages were combined, which was justified due to the absence of change over time.

Capillary haematocrit varied greatly between individuals and did not change significantly over time in the control groups (ANOVA) (tab. 4.1). A difference between free and waxed sides of the same egg was significant only on day 14 in the wax/oxygen experimental group ($p = 0.011$, $n = 6$, paired t-test), where it was significantly reduced from day 12 in the wax/oxygen ($p = 0.015$, t-test). A similar decline in the wax/hole experiment was not significant. Total red blood cell volume as derived from the morphometric measurements is shown in fig. 4.18. It did not change significantly over time in the control eggs. Cell volume under the waxed side was lower than under the

free side on day 14 in both wax/hole ($p = 0.018$, $n = 5$, paired t-test) and wax/oxygen experiments ($p = 0.016$, $n = 6$).

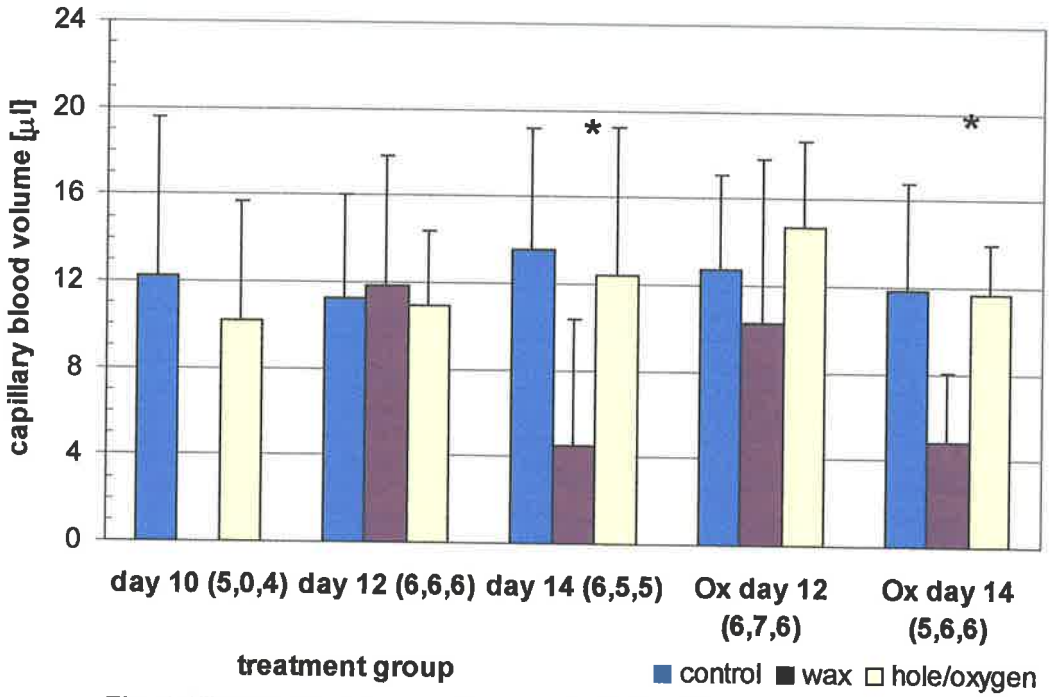


Fig. 4.17: Volume of the capillary plexus in the chicken CAM from day 10 to 14 as measured on the TEM micrographs. Means and SD, sample size in brackets. *indicates significant differences.

	Control	Wax side	Free side	Comparison wax/free side paired t-test
Wax/hole day 10	73.7 ± 8.1	No data	74.3 ± 10.6	n.a.
Wax/hole day 12	61.9 ± 14.7	67.1 ± 15.2	64.9 ± 11.2	n.s.
Wax/hole day 14	68.2 ± 4.9	50.3 ± 17.0	64.5 ± 17.6	n.s.
Wax/oxygen day 12	74.0 ± 4.7	69.2 ± 12.6	64.3 ± 12.4	n.s.
Wax/oxygen day 14	64.6 ± 13.8	46.9 ± 13.5	63.8 ± 14.8	p = 0.011

Tab. 4.1: Haematocrit values as percent of capillary volume in the CAM of day 10 to day 14 chicken eggs (mean and SD).

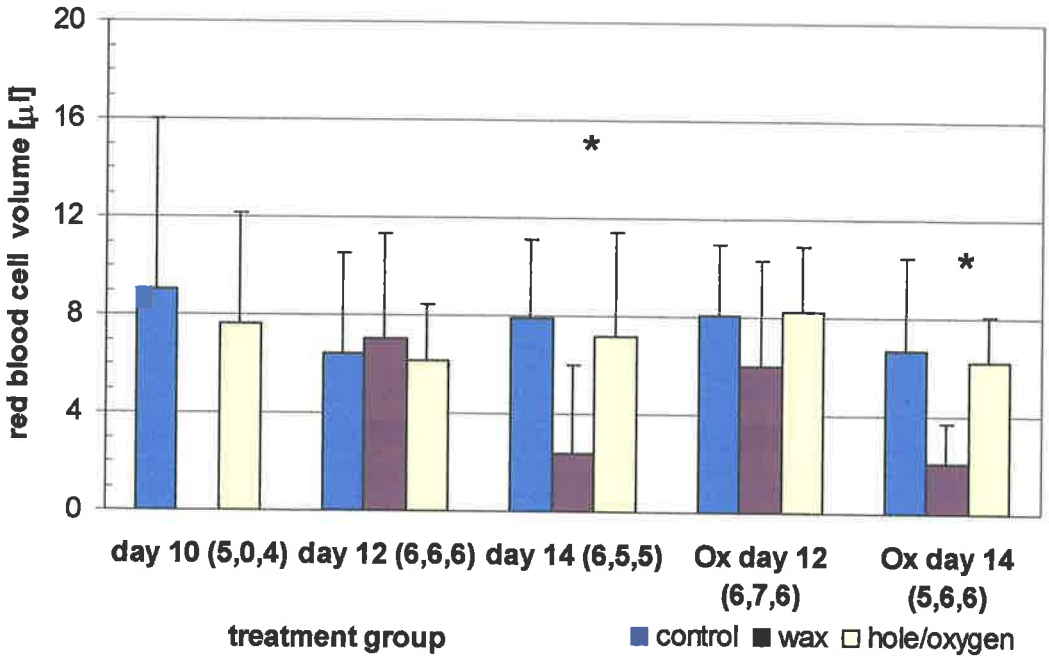


Fig. 4.18: Total red blood cell volume in the capillary plexus of the chicken CAM from day 10 to 14 as measured on the TEM micrographs. Means and SD, sample size in brackets. *indicates significant differences.

In Brief:

Capillary volume and haematocrit did not change between days 10 to 14. They were reduced under the wax, while the free side of the experimental eggs did not differ from the control group in wax/hole and wax/oxygen treatment.

4.4 Discussion

4.4.1 CAM growth and attachment

Shell permeability did not appear to influence chorioallantoic growth, as in all but one group the chorioallantoic membrane showed no decrease in area coverage under the wax (fig. 4.1). This is in contrast to observations by Baudrimont and St.-Ange (1847, as

obtained from Romanoff (1960), p 1115) and Dareste (1855), where the CAM grew towards the free shell areas, but agrees with Düsing's (1884) observation that the CAM does not grow preferentially towards better aerated areas (air tropism), instead it grows towards the nearest shell and spreads from there symmetrically in a mechanical process.

The relative surface cover of the CAM (56% of the egg surface on day 8 and 82% on day 10) agreed well with estimates by Ackerman and Rahn (1980) at these stages (62% and 83%, respectively). Although the area under the aircell was left out from the analysis in the present study, this probably had little impact on the results: on day 8 the percent coverage of the CAM over the aircell appeared similar to that over the rest of the egg. By day 10 the CAM usually covered the inner shell membrane over the aircell completely, so that total CAM may have covered an equivalent of 83.3% of the egg.

This agreement between the reports may indicate that the chorioallantoic growth rate is already at its maximum in undisturbed eggs, so that an accelerated growth in hypoxic eggs is not possible. Alternatively, the lack of acceleration in the spreading of the CAM may indicate that the small embryo may not experience oxygen limitation during this phase of incubation (cf chapter 5). The lack of oxygen limitation at this stage is supported by a comparison of the chorioallantoic growth in this study with the report by Burton and Palmer (1992), where a hypoxic environment reduced spreading of the CAM, while hyperoxic conditions had no effect. This study strongly suggests that chorioallantoic growth is not up- or down-regulated in response to oxygen availability, but rather reacts to metabolic suppression under hypoxia, possibly as a consequence of a general retardation of embryonic development.

In contrast to the growth of the CAM, its attachment under the wax appeared to be inhibited initially by the impermeable eggshell. Of the four (of six) surviving experimental eggs in the day 10 wax/hole experiment, the CAM was attached to the hole side, but only in one egg to the wax side of the shell. In contrast, all five (of six) surviving control eggs had the CAM attached all around. This may have been caused by an impeded absorption of the remaining thin albumen layer (cf Tazawa, 1980b) under the impermeable shell, possibly caused by the extremely low oxygen supply to the CAM or the lack of evaporation under the wax. Later in incubation, on day 15, it was often more difficult to separate CAM samples from the inner shell membrane after fixation on the wax side than on the hole side. The mechanism behind this difficulty remains unknown.

4.4.2 Vessel density and blood volume

4.4.2.1 Blood vessel density

The vascularisation of the CAM was influenced by shell permeability. The density of pre- and post-capillary blood vessels (PPC) under the wax side of experimental eggs was reduced from days 10 to 14 by 16.1% - 33.1% (wax/hole treatment) and 16.5% - 32.8% (wax/oxygen treatment), respectively, in comparison to the hole side. Similarly, PPC density was reduced on day 15 in all groups irrespective of the timing of shell manipulation by 12.3% to 34.4%. Further, capillary surface area under the waxed shell was lower than under the free shell by 19% - 59%. This indicates that angiogenesis was reacting negatively to the strongly hypoxic environment at any given time, in contrast to the study by Dusseau and Hutchins (1988) and Strick *et al.* (1991), where hypoxic conditions caused an overall increase in vascular density and a "corkscrew" growth of the vessels, and to Burton and Palmer (1992), where the capillary surface area was unchanged by hypoxia or hyperoxia. The corkscrew growth patterns were not discernible here. The level of hypoxia employed is possibly responsible for these differences, as well as the local vs global treatment. Whereas in low oxygen conditions an increased vascularity may compensate for the reduced Po_2, there is no use at all for blood vessels where no exchange is possible under the wax because of limited lateral diffusion in the shell membranes. The reduction in vessel density under the wax may thus constitute an adaptive response of the circulatory system. On the other hand, the oxygen limitation may have suppressed vascular proliferation. It also must be kept in mind that no other exchange of gases (e.g. carbon dioxide and water vapour) was permitted under the wax. Although oxygen is by far the most likely candidate to affect embryonic development (Tullett and Burton, 1987), other factors may well have had an effect.

In a similar work, Corona and Warburton (2000) found no change to chorioallantoic vessel density under wax in chicken eggs, which they attributed to a high degree of lateral diffusion under the shell. This would imply that gas exchange under the occluded area continued almost unchanged. However, as the lateral diffusion was rather low, causing extreme hypoxia under the waxed shell (cf chapter 3), respiratory gas exchange under the wax was severely limited, to which the reduction of vascular density under the occluded shell as observed in the present study corresponds better. The small remaining oxygen supply in the waxed region, provided by the lateral

diffusion, or an inflexible element of vascular development promoted blood vessel growth even under very hypoxic conditions. The lack of an increase in the difference between wax and hole side with earlier shell manipulation (compared to shell treatment on day 12) attests to this.

While the CAM was able or forced to reduce PPC and capillary density in response to the strong local hypoxia, the system was either not able, or not sufficiently challenged, to increase vessel density under the free side of the egg to compensate for the loss of respiratory surface area. This contrasts the observed decrease of the PPC density under hyperoxic conditions (45% and 70% oxygen, Strick *et al.*, 1991), but agrees with the unchanged level of capillary density observed by Burton and Palmer (1992, 40% oxygen). The lack of reaction to hyperoxia is possibly a consequence of the relatively low level of hyperoxia applied in this study (23% oxygen on day 10 to 26% oxygen on day 14). However, under the influence of the wax-cover, central regulation may have suppressed the expected decrease in vascularity under the free side. Correspondingly, vascular density under the free side did not differ from those obtained from control eggs.

Of the control eggs, the surface area of the capillary plexus in relation to the eggshell (range 76% to 91%) compared well to the results of 70% – 80% during late incubation in Tazawa (1978). As the relative surface area of the capillary plexus is thus very high under natural conditions, an increase may not have been feasible. Although Wangensteen and Weibel (1982) reported higher values of about 90% of the air surface at day 16, an increase in the capillary density after day 14 appears unlikely, as the capillary surface density has been found to reach its maximum at day 10 (Burton and Palmer, 1989) and reduce proliferation thereafter (Ausprunk *et al.*, 1974; DeFouw *et al.*, 1989; Wilting and Christ, 1993, 1996).

In contrast, arterioles and venules are known to maintain growth well after day 10 (Kurz *et al.*, 1995). The increase of the pre- and post-capillary density observed in this study from day 10 (3.95 ± 0.53 mm^{-1}) to day 15 (5.39 ± 1.1 mm^{-1}) (fig. 4.2) compares well with the results obtained by Strick *et al.* (1991) over this period (approximately 3.5 to 5.1 mm^{-1}). As described by Kurz *et al.* (1995), the length-density of these vessels peaked on day 14 (6.01 ± 0.77 mm^{-1}) with a value that was almost double the density described in that study of pre-capillaries (3.54 ± 0.23 mm^{-1}), with matches to the observation that the number of post-capillary vessels equals that of pre-capillary vessels at that stage (DeFouw *et al.*, 1989). This proliferation of PPC is not at

its maximum, as it can be increased under hypoxic conditions (Strick *et al.*, 1991). The absence of a similar increase in the experimental eggs of this study argues for the inability of the chorioallantoic membrane to increase vessel density on a local level in response to a whole system hypoxia, possibly because the immediate stimulus "hypoxia" is not present in that area, or the lack of an hypoxic challenge to the embryo. If the embryo is challenged, as indicated by the beginning retardation of embryonic growth at day 15 (see chapter 5), this argues for a independent local control of vascular growth. It is thus in agreement with the observation by Kurz *et al.* (1995) that endothelial proliferation is regulated by suppression on a local level.

4.4.2.2 Haemoglobin concentration and haematocrit

The wax/hole treatment caused a reduction of haemoglobin concentration by 14% on day 15. The reduction probably represents a delay of the normal increase of haemoglobin concentration during development (Tazawa *et al.*, 1971b). This was probably related to the difference in embryonic mass between the two groups, as haemoglobin concentration is correlated with embryo mass (Tazawa *et al.*, 1971b). No positive effect of the shell treatment on the haemoglobin concentration was therefore observed, which confirmed the observation by Baumann *et al.* (1983) and Baumann (1984) that the young (day 9) hypoxic embryo is unable to increase its total haemoglobin or blood volume and the changes to the oxygen affinity of the blood represent the principal adaptive mechanism.

The reduction of the haemoglobin concentration in smaller experimental embryos may have affected the measurements of blood volume that used the same age-specific blood standards for experimental and control eggs. As the earliest reduction in embryo mass of experimental eggs was observed in some groups on day 15 (see chapter 4), blood volume in experimental eggs may therefore have been somewhat underestimated where embryonic growth was retarded at that age, but is unlikely to have affected blood volume measurements before that day.

Haematocrit (26.6%) and haemoglobin concentration (5.80 g/100 ml) of the control eggs on day 15 were somewhat smaller than those in Tazawa *et al.* (1971b) (27.6% and 7.98 g/100 ml, respectively). This may have been caused by a different strain or a slightly lower development temperature in the present study.

In contrast to the haemoglobin concentration, a small, but not significant increase in haematocrit was observed in the day 15 embryos. Although the haematocrit

correlated with haemoglobin concentration in the blood, the latter was reduced in the experimental eggs, indicating a lower haemoglobin concentration in the red blood cells. This mirrors results by Nakazawa and Tazawa (1988) in eggs partially coated with epoxy cement. These researchers explained their observations with an increase of bicarbonate concentration as a consequence of hypercapnia that caused the inflow of water into the red blood cells, resulting in an increased mean corpuscular volume of these cells. The same circumstances may very well apply here.

As the increase of haematocrit does not represent an increase in haemoglobin concentration and therefore in blood oxygen-carrying capacity, the response is not directly adaptive to hypoxia. Nevertheless, haematocrit levels rise in eggs with low shell conductance (Tazawa *et al.*, 1983, Nakazawa and Tazawa, 1987, 1988; Tazawa *et al.*, 1988), under hypoxic conditions (Xu and Mortola, 1989; Ruijtenbeek *et al.*, 2000) and in unturned eggs (Tazawa, 1980b) from day 16. An increase of haematocrit in response to hypoxia has been observed as early as day 14 (Dusseau and Hutchins, 1988). The failure to increase haematocrit in the eggs of the present study may therefore signal the lack of hypoxic stress to the embryos.

Capillary haematocrit similarly did not differ between control and the free side of experimental (wax/hole and wax/oxygen) eggs. However, the high values of capillary haematocrit in the measurements of this study may have obscured any such effect if it had existed. This is supported by the failure of the embryos to increase haematocrit values from day 10 to 14 in the chorioallantoic capillaries. During this period of incubation, venous haematocrit levels rise from 19.7% to 24.5% (Tazawa *et al.*, 1971b). They increase throughout development, peaking at around the time of internal pipping (Tazawa, 1984). Capillary haematocrit is usually lower than that in the major vessels (e.g. Sarelius and Duling, 1982; Lee *et al.*, 1985; Brudin *et al.*, 1986; Desjardins and Duling, 1987; Fisher *et al.*, 1992). However, capillary haematocrit values observed ($61.9 \pm 14.7\%$ to $74.0 \pm 4.7\%$) were considerably higher than those reported in the literature for venous blood. Raised blood cell concentrations were also observed by Wangensteen and Weibel (1982).

Instant circulatory arrest before fixation (using liquid nitrogen) was intended to prevent artificial haemo-concentration in the microvasculature, and to preserve blood volumes as found *in vivo* (cf Bur *et al.*, 1985). As nearly all microvessels were found to contain blood cells, this technique was successful in preserving the blood cells in the CAM at the time of fixation and agrees with Wangensteen and Weibel (1982) that

nearly all of the blood vessels are in use. However, there is an indication that blood plasma may have been lost in the preparation of the tissues, as the capillaries here were flat and almost completely filled with blood cells, whereas micrographs by Ausprunk *et al.* (1974) showed them to be round and more filled with plasma after fixation in situ with 2% formaldehyde and 2.5% glutaraldehyde. The high haematocrit values observed have therefore likely been caused after the sample had been removed from the egg, possibly during the thawing in the fixation solution. In the light of this, the reduction of capillary haematocrit under the wax has to be regarded with caution, but may indicate a lowered perfusion of the microvessels in these tissues. This is supported by the greater number of empty vessels observed here.

4.4.2.3 Blood volume in the chorioallantoic membrane

The wax side of the CAM had a slightly, if only rarely significant, greater volume of blood than the free side, despite the reduced density of low order vessels and capillaries. This contradiction may have been caused by a greater number of higher-order vessels, the dilation of smaller vessels or their growth in diameter (Wilting *et al.*, 1995), but this was not measured. Blood volume under the free side of the eggs did not differ from the control, which is consistent with the observation that the density of pre- and post-capillary vessels as well as the capillary surface area also did not deviate from the control. Dusseau and Hutchins (1988) and Strick *et al.* (1991) demonstrated that overall vessel density in the CAM of hypoxic chicken eggs can increase beyond control values. The failure to increase blood volume and vessel density under the free side of the egg, where it might be beneficial in compensating for the shell manipulation in experimental eggs, shows that changes to the blood volume are either not needed at this stage or not possible in absence of an immediate (local) hypoxic stimulus.

Strick *et al.* (1991) observed a reduced vascular density in the CAM of eggs in a hyperoxic environment (45% and 70% oxygen). This reduction of vessel density was equally not observed here, but is probably explained by the small increase in P_{O_2} under the shell by drilling holes (wax/hole treatment) or in ambient P_{O_2} (wax/oxygen treatment) as compared to the earlier study. It has to be noted, however, that blood volume in the membrane is not equal to blood flow through it. Oxygen uptake under the free side could be increased by increasing blood flow, even if this reduces the maximum saturation that is achieved. As the oxygen dissociation curve of haemoglobin is not linear in relation to P_{O_2} (Tazawa, 1978), a reduction of blood P_{O_2} may reduce oxygen

content proportionally less, so that the increase of blood flow through the free side could compensate at least somewhat for the loss of respiratory surface area. This would reduce gas space Po_2 under the shell, as was observed in chapter 3.

From day 10 to day 16 of incubation, the blood volume in the chorioallantoic membrane increased only slightly from 0.17 ± 0.07 ml to 0.26 ± 0.06 ml. The available literature yielded no comparable measurements of blood volume in the chorioallantoic membrane. DeFouw *et al.* (1989) calculated blood volume in the microvasculature from the vessel density and width on day 10 (0.93 µl cm^{-2}) and day 14 (1.25 µl cm^{-2}) in first to third order pre- and post-capillary vessels. These results were lower than values measured in the present study on day 10 (2.8 ± 1.2 µl cm^{-2}) and day 14 (3.5 ± 0.8 µl cm^{-2}), which was probably caused by their limitation to first to third order vessels, excluding capillaries and higher than third order vessels. The relative increase, however, was similar in both cases (1.4 vs 1.3 fold). Since DeFouw *et al.* (1989) observed no increase in the density of higher than third order vessels during this time, the change in blood volume measured may be solely attributable to changes in the density of smaller pre- and post-capillary vessels, as there was also no change in capillary surface area and diameter. However, this would require a larger increase in the density of these vessels than observed: The DeFouw *et al.* values would indicate that the first to third order vessels hold 33.2% and 35.7% of total blood volume, respectively. For a total increase in blood volume by a factor of 1.3, the volume in these vessels would require a multiplication factor of the vessel density of 1.90 and 1.84. This is somewhat higher than the changes in vascular density observed during this time period here (factor 1.52), and by Strick *et al.* (1991, approximately 1.5), but were similar to the approximately 1.8-fold increase observed by Kurz *et al.* (1995). The remaining increase in blood volume may be found in increased vascular diameters (lumen) as the blood vessels develop (growth by intercalation, Wilting *et al.*, 1995).

The 1.3-fold increase of blood volume in this study was considerably smaller than the 3.4-fold increase in blood flow rate through the allantoic arteries as described by Tazawa and Mochizuki (1977) between day 10 (1.2 ml min^{-1}) and day 16 (4.1 ml min^{-1}). This difference in the rate of increase may reflect an increased blood velocity through the chorioallantoic vasculature with age, resulting in a shorter transit time and diminished haemoglobin oxygen loading. Transit time calculated directly from these values would be 10 s at day 10 and 3.8 s on day 16 through the whole CAM. However, the main location for the respiratory gas exchange is in the capillary vessels.

The capillary volume per egg observed in this study (0.0124 ± 0.005 ml for all control eggs from day 10 to 14) equalled 7.4% on day 10 and 4.7% on day 14, respectively, of total blood volume in the CAM, holding proportionally less of the blood in later development. Assuming a 15% shunt between arterioles and venules (Piiper *et al.*, 1980), the 1.2 ml min^{-1} flow in the allantoic artery on day 10 provided by Tazawa and Mochizuki (1977) would equal a flow of 1.02 ml min^{-1} through the capillary bed. With a capillary blood volume of 0.0124 ml on day 10, transit time in the capillaries would amount to 0.73 s, which would drop to 0.29 s with an allantoic flow of 3.0 ml on day 14. These transit times are lower than that necessary for saturation but fit within the range observed in the yolk sac membrane of the 4 day old chicken embryo by Meuer and Bertram (1993). However, blood oxygen affinity of the bird embryo changes during incubation (Reeves, 1984), and these measurements are only conditionally useful. A better comparison are calculated contact times for blood in the capillaries of the chicken CAM (Tazawa and Mochizuki, 1976). They fall from 0.87 ± 0.21 s on day 10 to 0.57 ± 0.20 s on day 14. The 0.73 s transit time calculated for day 10 in the present study falls within this range, but by day 14, transit time (0.29 s) would not be sufficient for full saturation, although probably still more than needed for half saturation (0.11 ± 0.03 s on day 16, Tazawa *et al.*, 1976b). The comparatively short transit times through the capillary plexus obtained in the present study are probably a consequence of the low capillary blood volume measured.

Blood volume in the capillary plexus was considerably smaller than observed by Wangensteen and Weibel (1982) at day 16 (0.0485 ± 0.0106 ml) using a very similar technique. This difference is unlikely to be caused by the greater age of the embryos in the earlier study, as the capillary density observed under electron microscopy did not change over time. Instead, these small capillary volumes may be a consequence of desiccation – as suggested in the context of the high haematocrit values (62% – 71%). In this case, a "dilution" of the observed haematocrit by a factor of 2-3 would be sufficient to reach "normal" values of 20.5% ± 2.5% on day 10 to 27.6% ± 1.8% on day 14 (Tazawa *et al.*, 1971b). This would result in a capillary blood volume more similar to that study (0.037 ml) and only slightly less than the Wangensteen and Weibel value of 0.0485 ml. If, on the other hand, the high haematocrit values are a consequence of vessel congestion during fixation, the low measurement of capillary volume does not reflect an artefact of preparation, and the low values observed may be caused by different breeds or different age.

4.4.3 Capillary morphometry

4.4.3.1 Barrier thickness

In the control eggs chorioallantoic barrier thickness declined from day 10 to day 14 and agreed well with values from the literature (Fitze-Gschwind, 1973; Dunn and Fitzharris, 1979; Wangensteen and Weibel, 1982). There were indications for an acceleration of the invagination process in the experimental eggs (holes side), and a consistently greater distance between the air space and the capillary lumen under the waxed side than under the free side. The reduced number of capillaries and consequentially the longer diffusion distance under the wax may be responsible in part for the greater tissue barrier thickness in wax samples. However, the difference between the wax and the free side of the eggs remained even when only the shortest measurements (one per image) between the groups. This compensated for the longer diffusion distances caused by the lower capillary density under the wax. Although the difference between wax and free side was significant only on day 14 in the wax/oxygen experiment ($p = 0.0424$, t-test), the constancy of the pattern suggests a failure to reduce barrier thickness to the same level under the waxed eggshell. This is in contrast to observations by Burton and Palmer (1992) of an acceleration in the invagination of the capillary plexus in a globally hypoxic environment, and tendencies for a delay under hyperoxic conditions. The contrast between the present results and the study by Burton and Palmer may be attributable to the degree of hypoxia involved – while low P_{O_2} values may still allow for some oxygen uptake and thus may still stimulate capillary growth, total blockage of the pores and the resulting extreme hypoxia under parts of the shell could inhibit capillary maturation.

4.4.3.2 Diffusing capacity of the CAM

The chorioallantoic diffusing capacity (D_{CAM}) was calculated as the inverse of the sum of the inverses of the tissue diffusing capacity (D_{tissue}), plasma diffusion capacity (D_{plasma}) and the "blood diffusing capacity" (D_{blood}) as described in Wangensteen and Weibel (1982):

$$1/D_{CAM} = 1/D_{tissue} + 1/D_{plasma} + 1/D_{blood} \qquad (4.7)$$

The diffusing capacity of the tissue barrier was calculated from the harmonic mean thickness of the tissue barrier ($(\tau_h)_t$), the actual CAM surface area (S_{CAM}), the capillary surface area (S_{cap}) and the Krogh's diffusion constant of oxygen in tissue (2.48×10^{-8} cm^2 O_2 STPD min^{-1} kPa^{-1} (Weibel, 1973) according to

$$D_{tissue} = K \times [S_{CAM} + S_{cap}] / [2 \times (\tau_h)_t] \quad (4.8)$$

Similarly, D_{plasma} was obtained from the harmonic mean thickness of the plasma barrier ($(\tau_h)_p$), the capillary surface area and the same the Krogh's diffusion constant of oxygen in tissue for lack of a value through plasma, according to

$$D_{plasma} = K \times S_{cap} / (\tau_h)_p \quad (4.9)$$

assuming that erythrocyte surface area is equal to the capillary surface area. The "diffusing capacity" of the blood was obtained from the capillary haematocrit and the reaction rate for the reaction of oxygen with haemoglobin supplied by Tazawa *et al.* (1976b) for day 16 (4.55 ml O_2 ml-RBC^{-1} min^{-1} kPa^{-1}). Although the reaction rate for days 10 to 14 could be expected to be somewhat smaller than that for day 16, the latter value was acceptable because there was no clear relationship between the oxygenation velocity factor and the incubation age (Tazawa *et al*, 1976b).

The capillary volume was rather small in comparison to literature data, and the haematocrit level was very high (see section 4.4.2.3). For the calculation of the diffusion capacity of the chorioallantoic membrane it was therefore assumed that blood plasma had been lost during the preparation of the tissue and that the red blood cells that had remained in the vessels were the more appropriate base on which to calculate the diffusing capacity of the blood than the blood volume as described by Wangensteen and Weibel (1982).

As the capillaries were almost filled with blood cells, the harmonic mean plasma barrier was probably measured smaller than it is in the living embryo, increasing D_{plasma} above its actual value. However, plasma resistance to gas movement is comparatively small in relation to the other two variables involved (resistance of the tissue barrier and the haemoglobin oxygen binding), and this overestimation had therefore no great impact on the calculation of D_{CAM}.

The diffusing capacity increased from day 10 to day 14 in the hole/control ($p = 0.0042$, $F_{2,13} = 8.560$, ANOVA), but not from day 12 to 14 in the oxygen/control groups (tab. 4.2). It was significantly reduced under the wax side in comparison to the free side of the same eggs on day 14 in the wax/hole experiment ($p = 0.0002$, $n = 5$; paired t-test) and on days 12 ($p = 0.0011$, $n = 6$) and 14 ($p = 0.0002$, $n = 6$) in the wax/oxygen experiment. It was not increased under the free (hole or oxygen) side of the experimental eggs in comparison to the control groups, again pointing towards the observation that the embryos do not show any response to the treatment in the vasculature on the free side. The indication for an accelerated capillary invagination appears in this context to be more likely the random result of a small sample size.

Experiment	Untreated control eggs	Wax side of exp. eggs	Free side of exp. eggs	comparison wax/free side
Wax/hole day 10	0.025 ± 0.005 (5)	no data	0.025 ± 0.003 (4)	n.a.
Wax/hole day 12	0.024 ± 0.003 (6)	0.024 ± 0.006 (6)	0.024 ± 0.002 (6)	n.s.
Wax/hole day 14	0.031 ± 0.001 (6)	0.009 ± 0.002 (5)	0.028 ± 0.002 (5)	p = 0.0002
Wax/oxygen day 12	0.031 ± 0.001 (6)	0.020 ± 0.004 (6)	0.033 ± 0.001 (6)	p = 0.0011
Wax/oxygen day 14	0.027 ± 0.001 (5)	0.010 ± 0.001 (6)	0.025 ± 0.001 (6)	p = 0.0002

Tab. 4.2: Diffusing capacity of the CAM [ml O_2 min^{-1} kPa^{-1}]. Means and SD, sample size in brackets.

Failure to increase the chorioallantoic diffusing capacity in hypoxic eggs was observed by Metcalfe *et al.* (1979), whereas hyperoxia caused a reduction of D_{CAM} (Temple and Metcalfe, 1970). This supports the argument that chicken embryos can down-regulate vascular characteristics more easily than up-regulate them. The values observed in the present study are lower than observed by Wangensteen and Weibel (1982) on day 16 (0.051 ml O_2 min^{-1} kPa^{-1}), which may be attributed to the different

developmental age, as haematocrit increases during incubation and the tissue barrier thickness may further decline.

4.4.3.3 Chorionic epithelial thickness

The thickness of the chorionic epithelium, the outer ectodermal layer of the chorioallantois, increases from day 4 to day 12 linearly, thereafter gradually until day 19 (Fitze-Gschwind, 1973). The present data for days 12 (5.7 μm) and 14 (8.9 μm) resemble values by Wangensteen *et al.* (1970/71; 8 mm) and Tazawa and Ono (1974; 6 to 10 μm), but are lower than Fitze-Gschwind's data (10.3 and 11.4 μm, respectively, interpolated linearly), although they show the same trend. The increase in epithelial thickness is a consequence of the capillary invasion and a morphological change of the epithelial cells in this context. As the capillary density in the wax side samples is smaller than in the hole side and the control samples, the total number of capillaries is not related to the increase in epithelial thickness, since this is greater under the wax. Instead, the morphological change of the tissue *per se* is responsible. However, the reason for the increased tissue volume under the wax is unclear and may be related to the diameter of the capillaries that is also increased. The latter could be interpreted as a mechanism to facilitate blood flow, which would be in contrast to the other measures such as reduced vessel density that appear to limit blood flow to this hypoxic part of the CAM.

4.4.4 Conclusion

The comparison of vascular density between the free and the occluded side of the egg showed that the chorioallantoic membrane reacts to the local reduction of shell permeability with the reduction of the circulatory system in that area. However, a diffusion/perfusion match was not achieved, as there was still a substantial amount of vasculature under the waxed side. Further, vessel density under the free side was not increased locally, highlighting the difficulty to increase the vascularity locally. The results of this study indicate that the development of the chorioallantoic membrane is at its maximum in the normoxic, undisturbed embryo, as suggested by Metcalfe *et al.* (1979) and Burton and Palmer (1992). This is supported by the inability of the embryo to accelerate CAM growth under hypoxic conditions caused by a partly occluded eggshell without compensatory holes or increase of environmental P_{O_2}.

Since the haemoglobin concentration and the haematocrit of the experimental embryos were not increased in comparison to the control eggs up to day 15, the local variation of shell conductance imposed on the embryo may not have caused a whole system hypoxia in the presence of a diffusion/perfusion mismatch. This could possibly be achieved by an increased blood flow through the chorioallantoic area under the free shell, maintaining overall oxygen uptake.

The effect of the increased oxygen environment on the CAM was similar to that of the holes, supporting the argument that oxygen is the main factor influencing CAM development.

5. Embryonic growth and respiration

5.1 Introduction

The previous chapter investigated the adaptability of the circulatory system in the avian chorioallantoic membrane (CAM) to local changes in eggshell conductance. Adapting the perfusion in the CAM to match the regional diffusive gas flow through the shell benefits embryonic gas exchange and hence embryonic growth. The following section investigates how successfully adaptations in the CAM can compensate for the variation in the shell conductance.

Embryos can be expected to maximise growth within the confines of the egg, as survival chances improve with increasing hatchling mass (Perrins, 1996, in Great Tit). For example, a large embryo and consequently a large hatchling mass may improve tolerance for starvation and temperature stress (Rhymer, 1988, in Mallards; Thomas and Brown, 1988, in Canada Geese). As the hatchling matures, the size advantage may decrease as the difference to other hatchlings gradually declines (Thomas and Brown, 1988, in Canada Geese; Burke *et al.*, 1997, in broiler chicken; Smith and Bruum, 1998, in Starlings), but low body size and energy reserves after hatching endanger the hatchling during a vulnerable period.

Many factors that regulate embryonic growth and hatchling size are beyond the control of the embryo. Firstly, hatchling size is frequently correlated to egg size (O'Connor, 1979, in European Swifts; Birkhead and Nettleship, 1982, in thick-billed Murres; Hepp *et al.*, 1987 in Wood ducks; Rhymer, 1988, in Mallards; Thomas and Brown, 1988, in Canada Geese; Smith and Bruum, 1998, in Starlings). In birds, egg size varies between individuals of the same species and between eggs from the same hen depending on age (Adegbola and Olatke, 1988; Ahn *et al.*, 1997), season (Birkhead and Nettleship, 1982), body condition (Hepp *et al.*, 1987), and nutrition (Romanoff and Romanoff, 1949). Larger eggs permit stronger growth, because they frequently have a greater energy content in the yolk (Romanoff and Romanoff, 1949; Hepp *et al.*, 1987) as well as physically more space for the growing embryo. However, Xu and Mortola (1988) found a better correlation of embryo size with shell conductance than with egg size (fresh egg mass) in chicken. Shell conductance correlates with egg mass to a power of less than one (Ar *et al.*, 1974; Ar and Rahn, 1978, 1985). It may also vary widely even between eggs of the same size, thus potentially limiting embryos in eggs with

greater resistance to gas movement in growth and development (Tullett and Deeming, 1982; Burton and Tullett, 1983; Meir and Ar, 1996; Meir et al, 1999).

The effect of low eggshell conductance is similar to incubation under low oxygen partial pressure (P_{O_2}) that reduces embryonic growth and survival (Stock and Metcalfe, 1987; Xu and Mortola, 1989). Oxygen availability exerts a strong selection pressure onto the embryo, as oxygen consumption is correlated to body size, given a constant correlation between mass and metabolism (Burton and Tullett, 1983). Oxygen consumption rate rises further with production costs of metabolites and new tissue, although the energy bound in any new tissue cannot be calculated from the gas exchange. Accordingly, metabolic activity changes according to the developmental stage, and oxygen consumption of fast growing tissue scales with mass differently from mature tissue (C. Vleck *et al.*, 1979). Respirometric analysis can therefore record growth indirectly.

In precocial species such as the chicken, oxygen consumption reaches a plateau (from day 17, Romijn and Lokhorst, 1951; Burton and Tullett, 1983), or even declines from a maximum (after 75% of incubation) in other species, which may be explained as a consequence of the reduced growth rate of the embryo (C. Vleck *et al.*, 1979; D. Vleck *et al.*, 1980). During the plateau phase, oxygen availability may become limiting: an increase in ambient P_{O_2} at this stage increases oxygen consumption, embryonic growth and survival (Metcalfe *et al.*, 1981; Metcalfe *et al.*, 1984; Stock and Metcalfe, 1987; Tullet and Burton, 1987; Tazawa *et al.*, 1992, Christensen *et al.*, 1997, but see Williams and Swift, 1988). However, some authors argue oxygen availability is not limited, as internal oxygen partial pressure can be adapted to increase diffusion across the shell barrier (C. Vleck *et al.*, 1979), and that the embryo has to protect itself from an excess of reactive oxygen species (Ar and Mover, 1994).

While the embryo cannot alter oxygen environment and shell conductance, it can exert some control over the gas exchange by adapting the perfusion of its chorioallantoic membrane. If the embryo does not adjust blood flow to local differences in shell conductance, the variable shell conductance may cause a diffusion/perfusion mismatch. This can cause functional hypoxia, comparable to a low overall shell conductance and low ambient P_{O_2}. As hypoxic blood from areas with a reduced shell conductance mixes with saturated blood, the oxygen content of the mixed blood declines, and the gas exchange may be reduced in comparison to control embryos. This would consequently reduce the growth rate.

This study evaluates the ability of the chicken embryo to maintain its oxygen uptake under conditions of regional differences in shell conductance, and if this is correlated with the growth performance. For this purpose, eggshells were half covered with wax to create an area of low (to zero) gas exchange. Two different forms of compensation for the loss of respiratory surface area were chosen: some eggshells were perforated on the opposite side to the wax, to increase gas exchange (section 2.4). As there was some indication that lateral movement of gases under the shell and therefore the effect of the holes is low (chapter 3), other eggs were kept in a environment with increased oxygen partial pressure to increase oxygen availability more uniformly across the section of free shell (section 2.3). The ability of embryos in treated eggs to maintain gas exchange at the normal level was tested against a control and compared to embryo mass. An accelerated decline in mass-specific oxygen consumption and the correlation between the shell conductance and the oxygen uptake (adjusted for egg size) were considered to indicate oxygen limitation. Water content of the embryos was considered as a measure for embryonic maturation, as it declines with embryonic development (Romanoff, 1967; Starck and Ricklefs, 1998), and compared to numbers of pipped eggs and yolk re-absorption. The yolk reserves of the embryo were observed as a further indicator for the "fitness" of the embryo, as any surplus energy in the yolk that the embryo does not use for growth and maintenance during incubation may be stored and used for hatching and in the first few days thereafter (Romanoff, 1960; Christensen *et al.*, 1996). It was investigated if embryos from treated eggs lose yolk reserves because of increased maintenance costs, e.g. for increased heart activity. Finally, the importance of the developmental stage for the ability to adapt to and compensate for the variable shell conductance was investigated in the third series of experiments with wax/hole treatment at different times of incubation.

5.2 Material and Methods

5.2.1 Incubation and treatment groups

Shell permeability was locally reduced in experimental eggs by covering them with wax to 50% along one side (pole to pole, cf. fig 2A). Three series of experiments were conducted. In the Series A - wax/oxygen treatment, the experimental eggs were then incubated in a dry environment with increasing oxygen fraction from 22.75% oxygen on

day 10 to 31.0% oxygen on day 20 (cf. section 2.3), while the control eggs remained untreated. In the Series B - wax/hole treatment, the experimental eggs received 4 to 7 holes of 0.6 mm diameter drilled through the shell in a ring around the centre of the wax-free side (section 2.4). The control eggs also received the holes, but those were then filled up with Araldite ®. In both series, treatment started on day 10.

In the last series of experiments, the wax/hole shell treatment was applied between days 1 and 12 of incubation to observe the importance of the timing of shell manipulation.

A detailed description of incubation conditions and experimental protocol is given in chapter 2.

5.2.2 Survival rate

Embryos found dead during respiratory measurements or at the opening of the egg on day 15 were described regarding to approximate age, length and CAM development, and then discarded. Embryos that died before treatment of the shell were excluded from the analysis of survival rates.

5.2.3 Data analysis

Embryo mass at hatching changes linearly with egg mass (Ar and Rahn, 1985). Eggshell conductance is also correlated to egg mass, to the power of 0.78 between species (Ar *et al.*, 1974; Ar and Rahn, 1978, 1985), although this correlation may not always be strong within a species (Visschedijk *et al.*, 1985). Embryonic respiration is correlated with egg mass by a similar factor between species (0.78, Rahn *et al.*, 1974; 0.73, Ar and Rahn 1985). The results of the undisturbed control eggs in the present study agree with these observations: fresh egg mass correlated with shell conductance ($r = 0.447$, $p < 0.01$, $n = 32$), wet ($r = 0.616$, $p < 0.001$) and dry ($r = 0.539$, $p < 0.01$) embryo mass, and wet ($r = 0.548$, $p < 0.01$) and dry ($r = 0.602$, $p < 0.001$) yolk mass on day 15. The slope of the log/log curve of wet embryo mass over fresh egg mass was 0.71, and egg mass accounted for 37% of the variability in embryo mass (fig. 5.1). Embryo mass, however, did not correlate with eggshell conductance.

The range of egg masses was small (43.8 to 74.7 g), and the respiratory measurements as well as embryo and yolk mass have therefore been normalised for egg mass to the power of one by dividing each with the individual fresh egg mass and

multiplying with average fresh egg mass, which equalled 61.03 ± 5.82 g in Series A (wax/oxygen treatment), 60.71 ± 5.85 g in Series B (wax/hole late incubation experiments), and 58.15 ± 4.74 g for Series C (wax/hole treatment at different days).

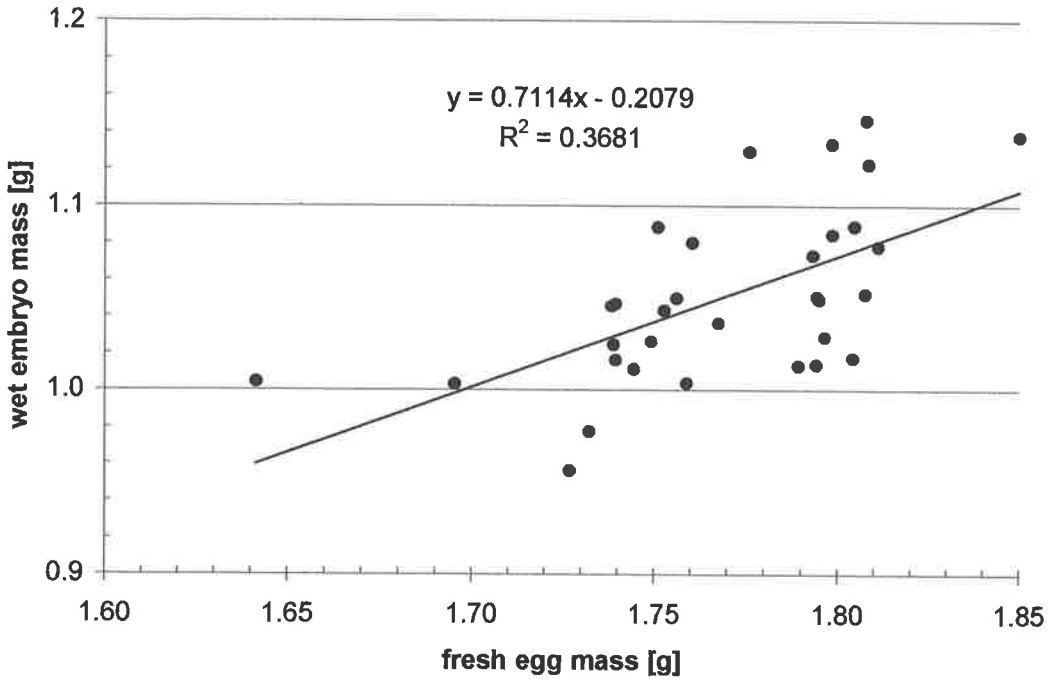


Fig. 5.1: Correlation between wet embryo mass on day 15 and fresh egg mass in the undisturbed control (n = 32). Log-log curve.

Unless defined otherwise, all data are presented as means and standard deviation (SD). Procedural control and experimental groups were compared using a two-tailed Student's t-test where data matched normal distribution. Correlations between variables followed Pearson product moment correlation. Multiple comparisons of group means with the undisturbed control used Dunnett's method (Dunnett, 1955). Comparisons of groups over time used analysis of variance (ANOVA) or MANOVA for repeated measurements. Pipping and survival frequencies were compared individually between control and experimental groups of the same age or age at treatment with a two-tailed Fisher's exact test of independence (Sokal and Rolf, 1995), and between all controls and experimental groups in a Series with the χ^2 test. Significance was accepted at $p < 0.05$, but values of $p < 0.10$ may be listed as approaching significance.

5.3 Results

5.3.1 Series A: Wax/oxygen treatment

5.3.1.1 Embryonic growth

The increase of oxygen content in the environment appeared to compensate the chicken embryos for the loss of conductive surface caused by covering half of the shell with wax. Embryos under this treatment showed no significant decrease in growth rate over the course of incubation compared to the controls. Wet and dry embryo mass in both treatments increased from day 12 (3.39 ± 0.61 g and 0.26 ± 0.05 g, respectively) to day 20 (30.43 ± 2.82 g and 5.96 ± 0.67 g, respectively) (fig. 5.2).

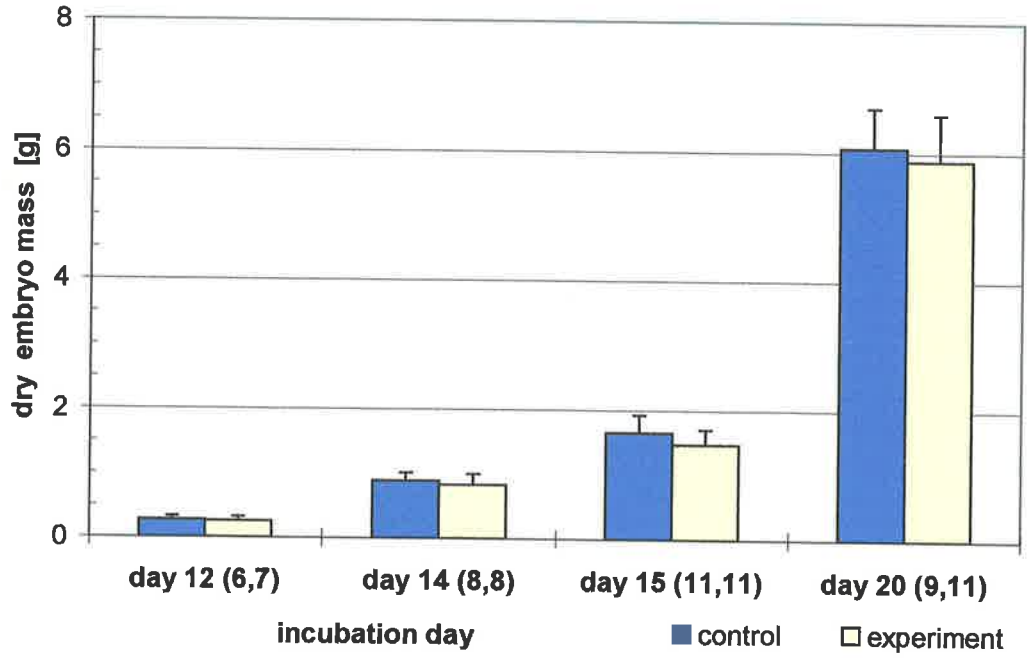


Fig. 5.2: Dry mass of embryos (normalised for fresh egg mass) in wax/oxygen experiments from day 12 to 20 after shell treatment on day 10. Means and SD, sample size in brackets.

Water content of the embryos also did not differ between control and experimental groups. It declined during development from 92.2% ± 0.4% on day 12 to 80.4% ± 1.8% on day 20 ($p < 0.0001$, $r = -0.958$, $n = 71$) (tab. 5.1).

terminated	Control	Wax/oxygen
day 12	92.19 ± 0.38	92.25 ± 0.38
day 14	89.21 ± 0.60	89.34 ± 0.72
day 15	86.38 ± 1.20	87.01 ± 0.91
day 20	80.50 ± 1.07	80.28 ± 2.33

Tab. 5.1: Water content of embryos in wax/oxygen treatment [%]. All eggs were treated on day 10 and terminated on the day specified.

A similar number of embryos had pipped in control and experimental group on day 20 (Fisher's exact test). Of the nine surviving embryos in the control group, two embryos had pipped internally and three had pierced the shell and commenced external pipping. Of the eleven embryos in the experimental group one was pipping internally, two externally. The yolk sac was withdrawn into the body cavity in four embryos of each group.

The amount of remaining yolk declined during incubation from 16.2 ± 1.8 g wet and 7.8 ± 0.8 g dry mass on day 14 to 10.2 ± 1.9 g wet and 5.1 ± 0.8 g dry mass on day 20 (fig. 5.3). Prior day 15, samples were difficult to obtain as the yolk sac ruptured easily. There were no consistent differences of the yolk dry mass between control and experimental eggs. On days 14 and 20, yolk mass did not differ between control and experimental groups. However, there was a significant reduction in the wet and dry mass of remaining yolk (corrected for fresh egg mass) in the experimental group on day 15 ($p_{wet} = 0.004$, $p_{dry} = 0.006$, $n_{control} = 10$, $n_{experiment} = 11$, t-test) (fig. 5.3).

Water content of the yolk did not differ between treatment groups of the same age. It declined during incubation from 52.4% ± 2.4% at day 14 to 49.9% ± 3.3% at day 20 ($p = 0.0006$, $r = -0.458$, $n = 52$). Only one sample (54.5%) was obtained for day 12. On day 20, wet yolk mass correlated negatively with wet embryo mass (both normalised for fresh egg mass) in the control group ($p < 0.0019$, $r = -0.8774$, $n = 9$), but not in the experimental group ($r = -0.0906$, $n = 11$).

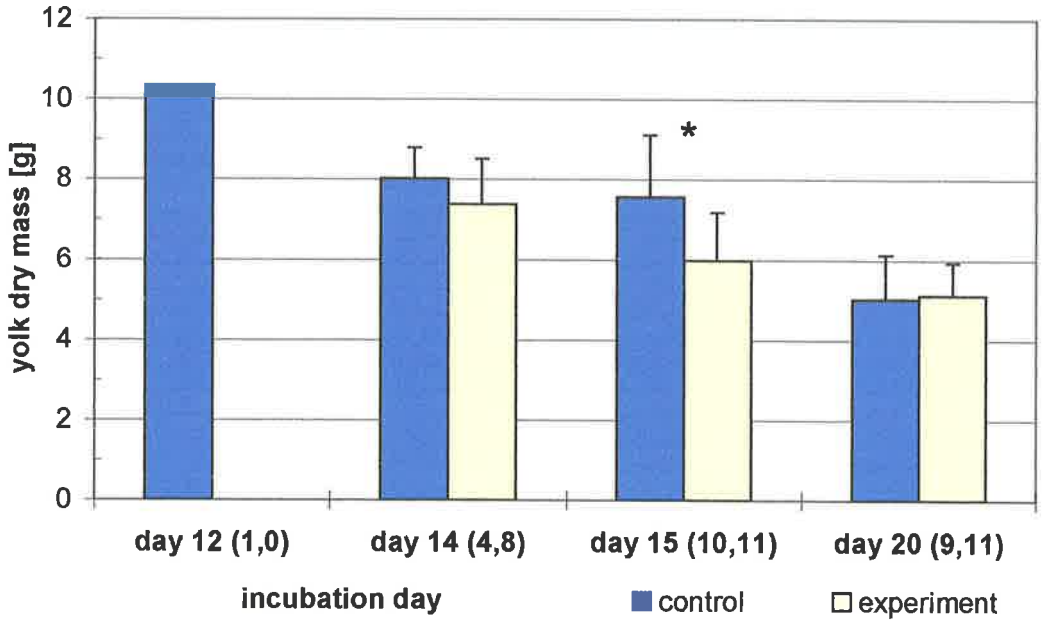


Fig. 5.3: Dry yolk mass (normalised for fresh egg mass) in wax/ oxygen experiments from day 12 to 20 after shell treatment on day 10. Means and SD, sample size in brackets. *indicates significant differences.

5.3.1.2 Gas exchange

Oxygen consumption rose from 0.089 ± 0.027 ml min^{-1} on day 12 to 0.480 ± 0.139 ml min^{-1} on day 20 and did not differ between the control and experimental groups at any time (fig. 5.4a). Similarly, carbon dioxide production and respiratory exchange ratio did not differ between treatments (fig. 5.4b,c).

Similarly, mass-specific $\dot{V}O_2$ and $\dot{V}CO_2$ did not differ between control and experimental groups of the same age, but were reduced on day 20 as compared to days 12 and 14 ($F_{2,46} = 16.0276$, $p < 0.0001$, ANOVA). There was no difference between days 12 and 14 (Tukey HSD) (fig. 5.5a,b). Within the groups, $\dot{V}O_2$ and $\dot{V}CO_2$ were not significantly correlated with shell conductance.

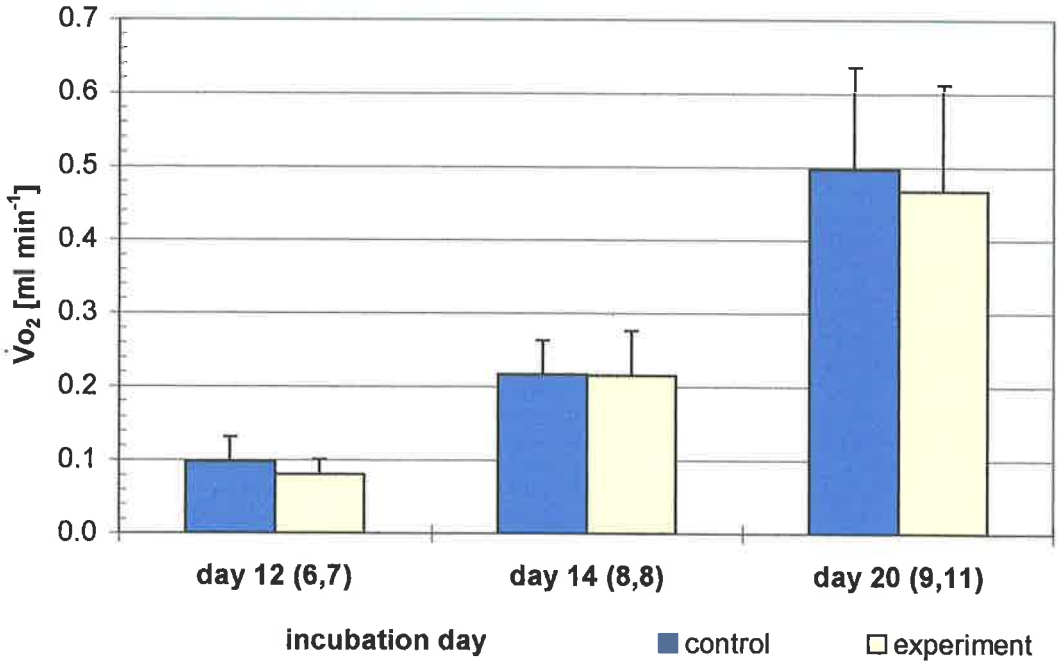


Fig. 5.4a: Oxygen consumption of embryos in wax/oxygen treatment (normalised for fresh egg mass). Means and SD, sample size in brackets.

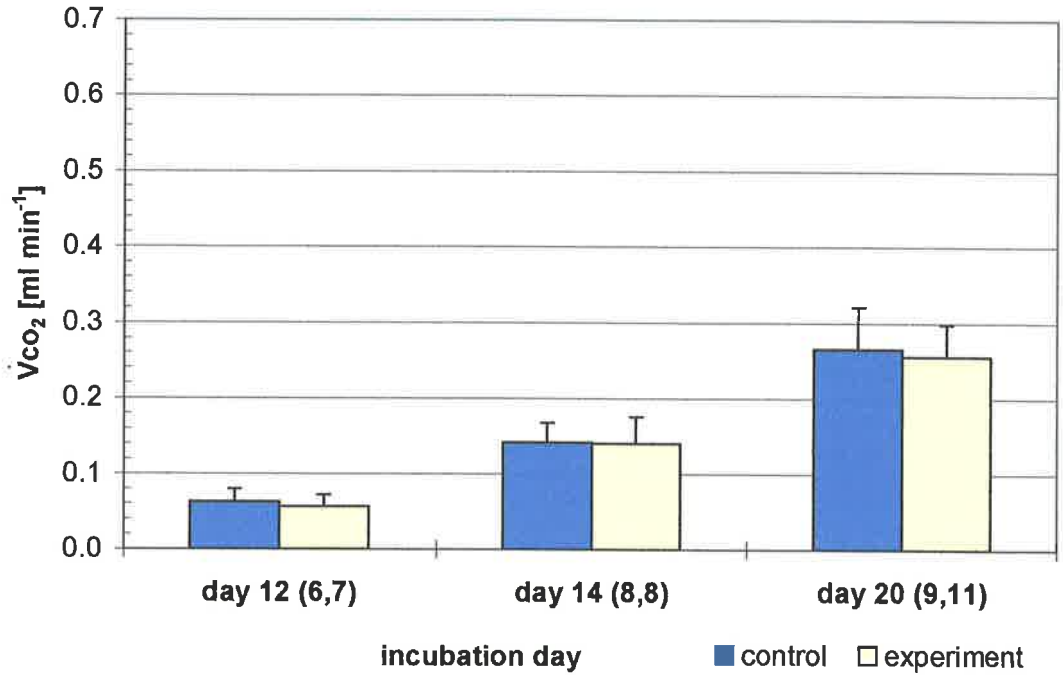


Fig. 5.4b: Production of carbon dioxide by embryos in wax/oxygen treatment (normalised for fresh egg mass). Means and SD, sample size in brackets.

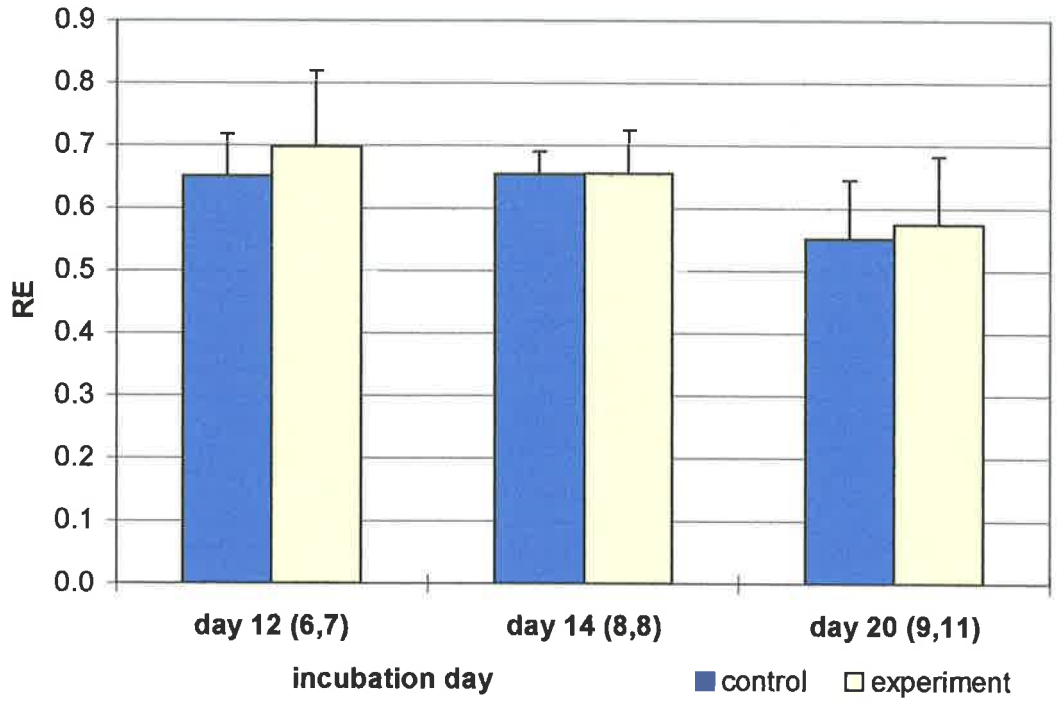


Fig. 5.4c: Respiratory exchange ratio of embryos in wax/oxygen treatment. Means and SD, sample size in brackets.

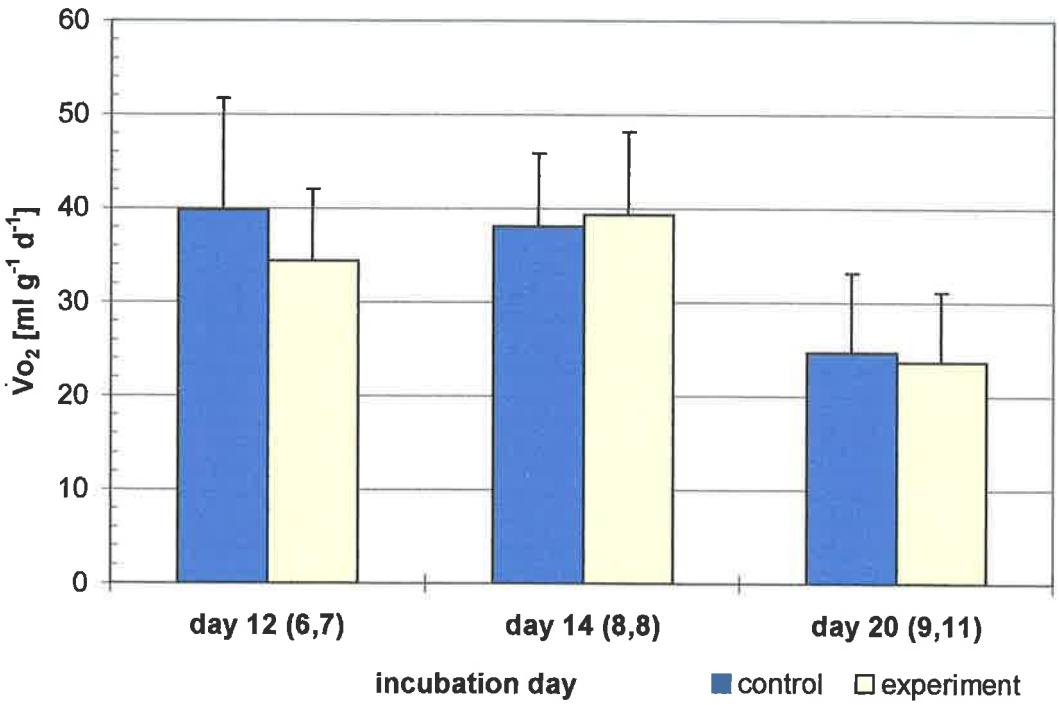


Fig. 5.5a: Mass-specific oxygen consumption of embryos in wax/oxygen treatment. Means and SD, sample size in brackets.

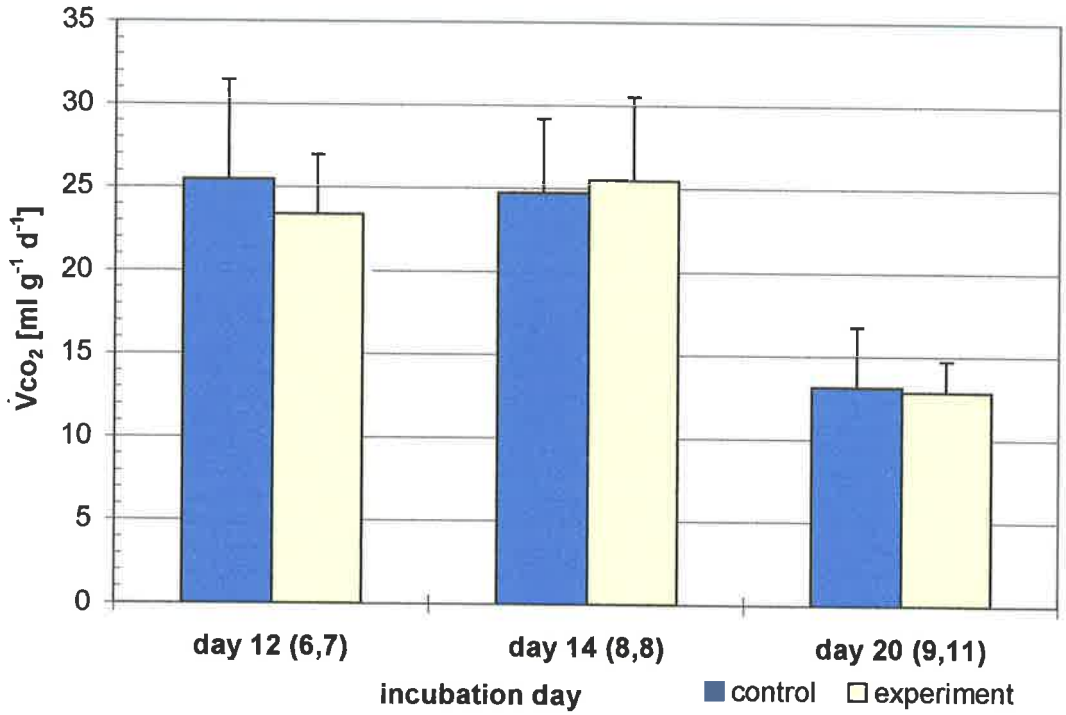


Fig. 5.5b: Mass-specific production of carbon dioxide by embryos in wax/oxygen treatment. Means and SD, sample size in brackets.

5.3.2 Series B: Wax/hole treatment - late incubation

5.3.2.1 Embryonic growth

Embryos in the undisturbed control eggs grew exponentially from day 10 (1.58 ± 0.42 g wet mass) until day 15 (11.82 ± 1.09 g) of incubation (fig. 5.6).

In a series of experiments with shell treatment on day 8, there was no significant effect of shell treatment on wet and dry embryonic mass on days 10, 12 and 14. Day 15 was the earliest time at which the difference in embryonic mass between experimental and hole/control eggs became evident ($p = 0.005$, t-test) (fig. 5.7).

The relative water content of the undisturbed embryos declined from 93.2% ± 0.3% on day 10 to 86.8% ± 1.0% on day 15 ($p < 0.0001$, $r = -0.890$, $n = 118$). Similarly, it declined in the hole/control eggs from 93.0% ± 0.2% on day 10 to 85.6% ± 1.6% on day 15 ($p < 0.0001$, $r = -0.819$, $n = 22$) and in the experimental embryos from 92.9% ± 0.3% to 87.2% ± 1.0% ($p < 0.0001$, $r = -0.896$, $n = 23$). Water content did not differ between hole/control and experimental groups on day 10 to 14 (fig. 5.8), but on day 15 embryonic water content in the experimental group was higher than in the hole/control group ($p = 0.047$, t-test).

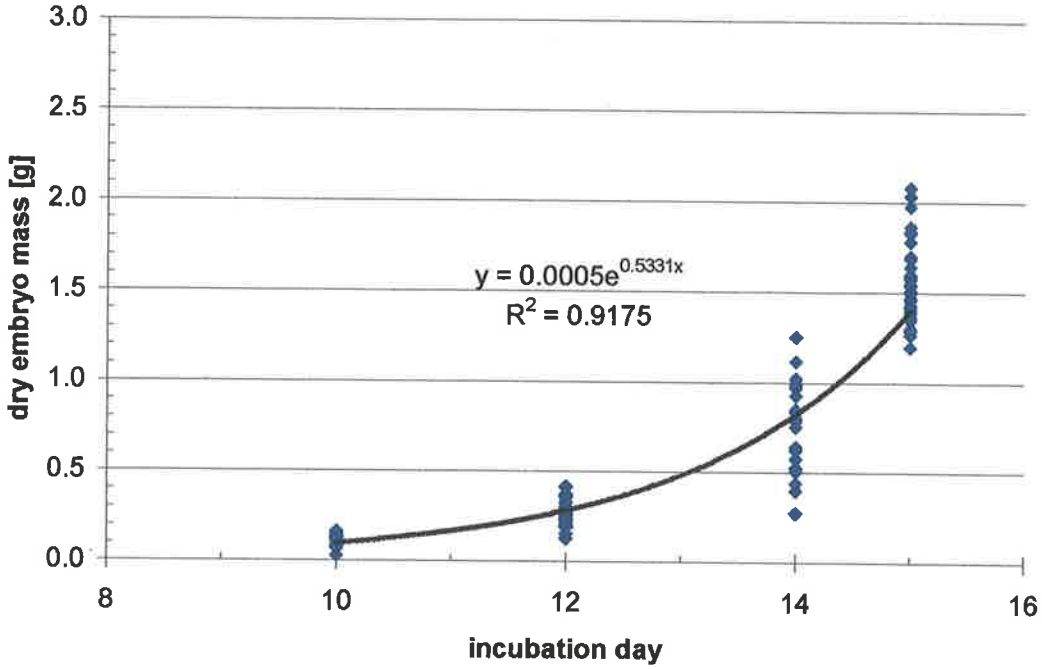


Fig. 5.6: Dry embryo mass (normalised for fresh egg mass) of undisturbed control eggs from day 10 to day 15.

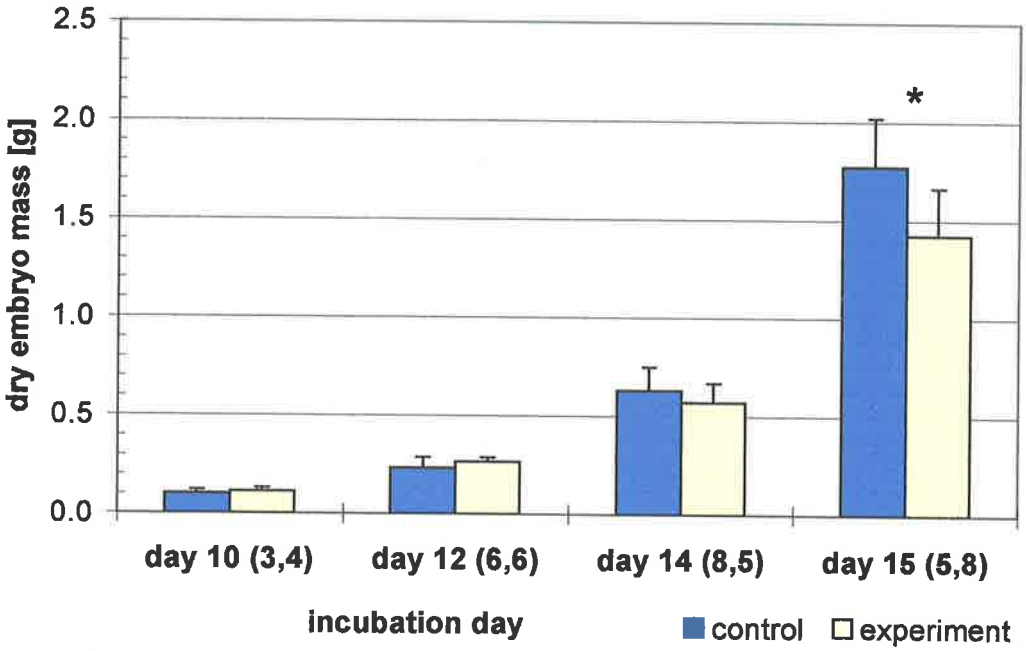


Fig. 5.7: Dry embryo mass (normalised for egg size) in wax/hole experiments from day 10 to day 15. All eggs manipulated on day 8. Values for day 15 derived from Series C. Means and SD, sample size in brackets. *indicates significant differences.

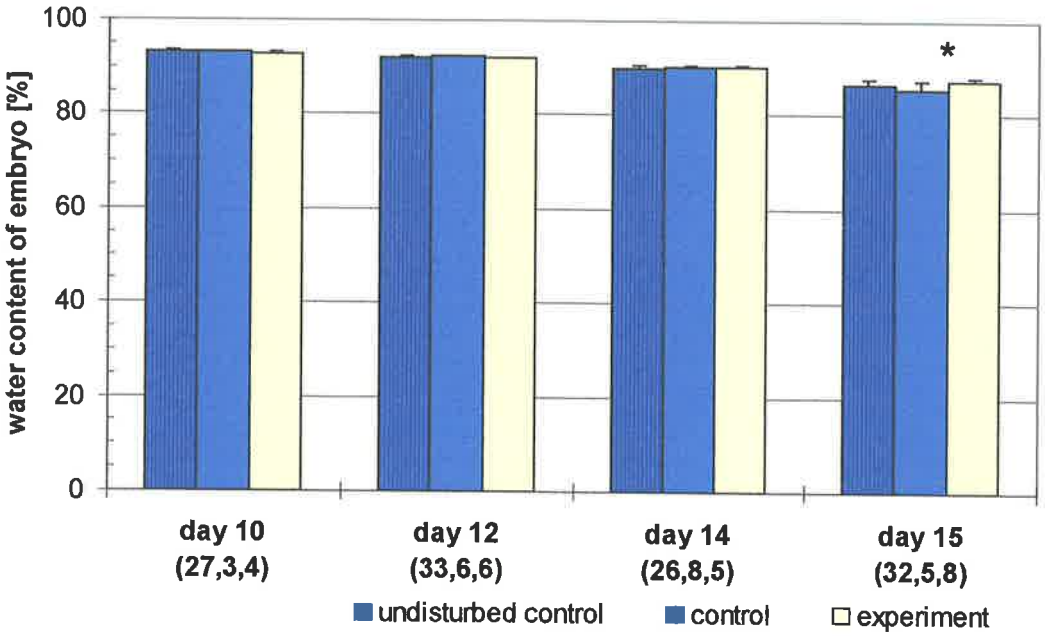


Fig. 5.8: Water content of embryos from day 10 to day 15 of incubation, after shell treatment on day 8. Means and SD, sample size in brackets. *indicates significant differences.

As young embryos showed no effect of shell treatment, a second set of experiments (wax/hole treatment on day 10) continued observation until the end of incubation (days 12, 14, 16, 18, and 20). The effect of the shell manipulation on embryonic growth became evident only in the last quarter of incubation. On days 12 to 16 there was no difference in wet or dry embryo mass between hole/control and experimental eggs. However, comparisons on days 18 and 20 show marked differences between the treatments groups (fig. 5.9). On day 18, wet mass of control embryos was significantly greater (20.94 ± 2.54 g) than that of experimental embryos (17.75 ± 2.13 g) ($p = 0.008$, t-test), and dry control embryos (3.99 ± 0.57 g; 5.09 ± 1.09 g) weighed significantly more than their experimental counterparts (3.33 ± 0.46 g; 4.07 ± 0.58 g) on day 18 and 20, respectively ($p = 0.013$; $p = 0.012$, t-test).

From day 12 to day 18 there was no difference in embryonic water content between experimental and hole/control groups. It declined steadily throughout incubation from 91.8% ± 0.3% on day 12 to 80.0% ± 1.6% on day 20 in the control ($r = -0.954$, $p < 0.0001$, $n = 38$) and to 81.2% ± 1.1% on day 18 in the experimental group ($p < 0.0001$, $r = -0.962$, $n = 40$), where it subsequently increased to 83.0% ± 1.3% on day 20 (fig. 5.10). The difference on the day 20 may have been caused by the altered water

vapour conductance of the eggshell in the experimental group (87.30 ± 13.57 mg d^{-1} kPa^{-1}), which was significantly lower than in the control group (104.28 ± 21.73 mg d^{-1} kPa^{-1}) ($p = 0.040$, t-test). This may have led to the reduced water content in the control embryos (80.0% ± 1.6%) compared to the experimental embryos (83.0% ± 1.3%) on this day ($p < 0.001$, t-test). However, the water loss in the control group (16.8% ± 2.8%), although significantly greater than in the experimental group (11.4% ± 1.8%, $p < 0.0001$, t-test), is unlikely to have caused such a strong effect on the water content of the embryo (*cf.* Simkiss, 1980; Davis *et al.*, 1988).

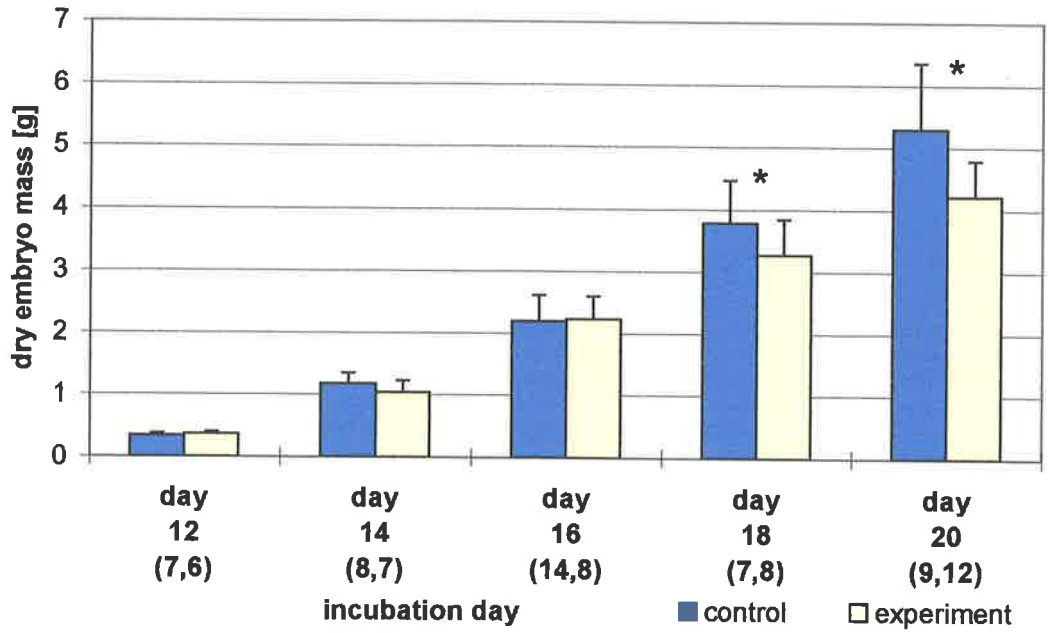


Fig. 5.9: Dry mass of embryos (normalised for fresh egg mass) during the second half of incubation after wax/hole treatment on day 10. Means and SD, sample size in brackets. * indicates significant differences.

At the end of the day 20 experiment, similar numbers of embryos were pipping internally: two of the nine control embryos and three of the twelve experimental embryos (Fisher's exact test). The yolk sacs were completely internalised in six and eight embryos, respectively.

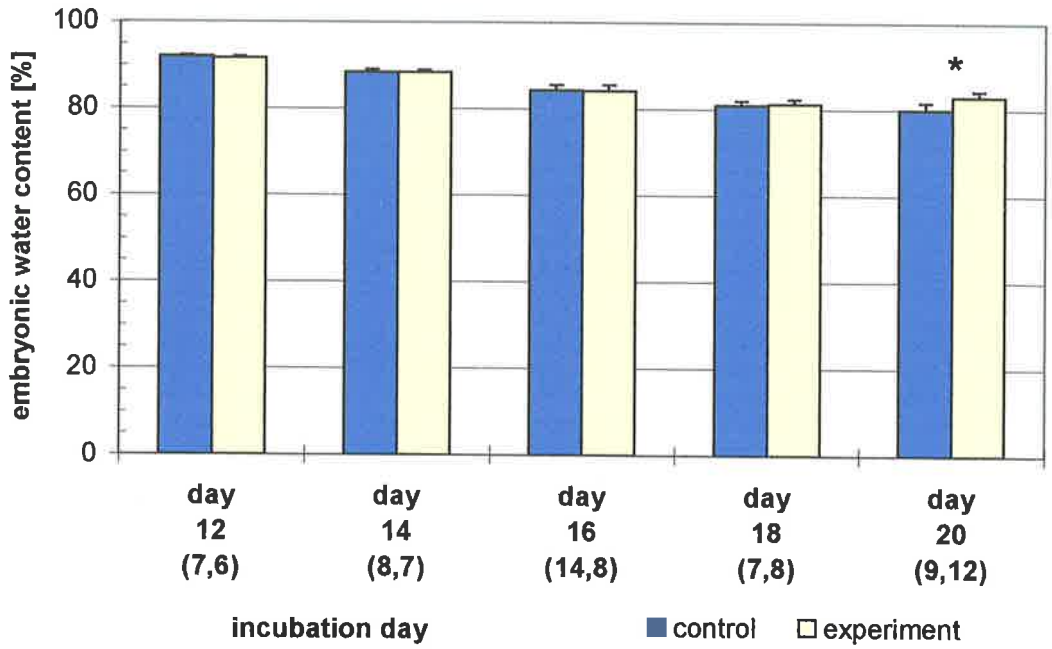


Fig. 5.10: Water content of embryos during the second half of incubation after wax/hole treatment on day 10. Means and SD, sample size in brackets. * indicates significant differences.

The amount of yolk left in the yolk sac declined slightly from day 12 to day 20 (fig. 5.11) in hole/control ($p = 0.0104$, $r = -0.413$, $n = 37$) and experimental eggs ($p = 0.0625$, $r = -0.268$, $n = 49$). Remaining wet mass of yolk did not differ between the hole/control and experimental groups at any age, but there remained significantly more dry yolk in the experimental group on day 20 (7.4 ± 0.6 g) than in its same-aged control group (6.0 ± 1.2 g) ($p = 0.004$, $n_{control} = 12$, $n_{experiment} = 9$, t-test). On day 20, wet yolk mass correlated negatively with wet embryo mass (both normalised for fresh egg mass) in the control group ($p < 0.0179$, $r = -0.7582$, $n = 9$), but not in the experimental group ($r = -0.4056$, $n = 10$).

Yolk water content was greater on day 12 ($61.3\% \pm 5.1\%$) than on the other days ($54.6\% \pm 3.1\%$ to $51.7\% \pm 4.1\%$), and declined only slightly throughout the second half of incubation ($p < 0.001$, $r = -0.440$, $n = 82$). It did not differ at any time between experimental and control groups.

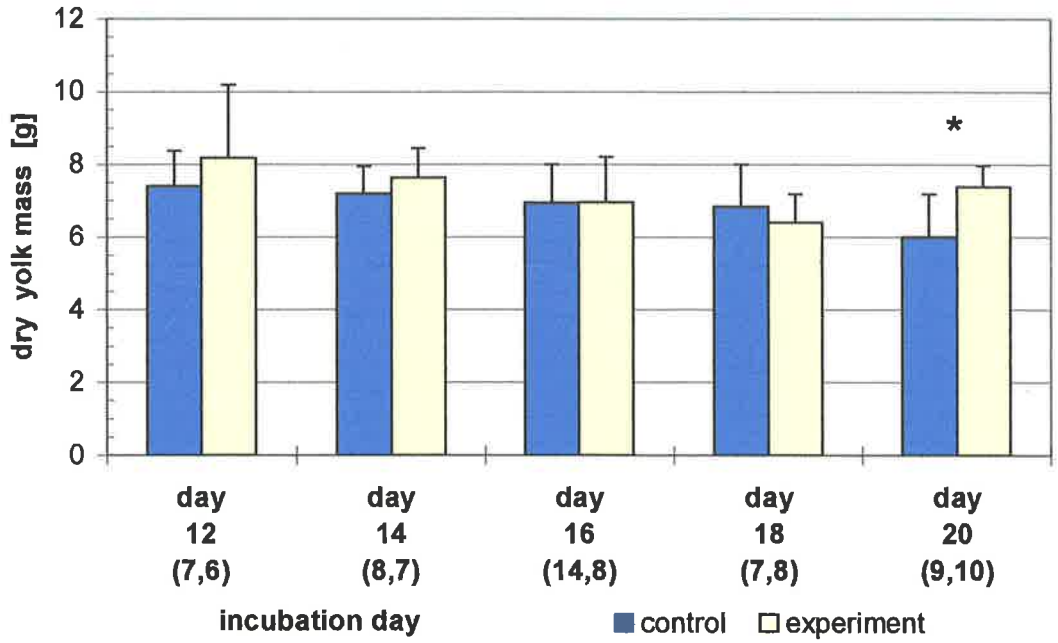


Fig. 5.11: Dry mass of remaining yolk (normalised for fresh egg mass) during the second half of incubation after wax/hole treatment on day 10. Means and SD, sample size in brackets. * indicates significant differences.

5.3.2.2 Gas exchange

The pattern of gas exchange during the second half of incubation was followed in eggs with wax/hole shell treatment on day 10. Corresponding to the increasing body mass, $\dot{V}O_2$ (fig. 5.12a) and $\dot{V}CO_2$ (fig. 5.12b) (corrected for fresh egg mass) increased in the second half of incubation. Oxygen consumption continued to increase until day 20 in both experimental and control eggs, but after day 16 the increase was no longer significant (Tukey HSD). The carbon dioxide production of the control embryos showed the same pattern, whereas the experimental embryos showed no significant increase in $\dot{V}CO_2$ after day 14. From day 16, control embryos showed a significantly greater $\dot{V}O_2$ (day 16: $p = 0.040$, $n_{contr/exp} = 8/14$; day 18: $p = 0.003$, $n_{contr/exp} = 8/11$; day 20: $p = 0.037$, $n_{contr/exp} = 9/11$, t-test) and $\dot{V}CO_2$ (day 16: $p = 0.047$, day 18: $p = 0.003$, day 20: $p = 0.050$, t-test) than the experimental embryos.

The mass-specific $\dot{V}O_2$ and $\dot{V}CO_2$ declined during the second half of incubation (fig. 5.13a,b). Mass-specific $\dot{V}O_2$ was lower in the experimental group than the control group on day 16 ($p = 0.090$, $n = 14$ and 8, respectively) and on day 20 ($p = 0.004$, $n =$ 11 and 9, respectively, t-test). This was repeated in the $\dot{V}CO_2$ production on day 16 ($p =$

0.028) and day 20 (n = 0.004, t-test), in spite of the reduced body mass of the wax/hole embryos. The respiratory exchange ratio of the embryos was generally higher in

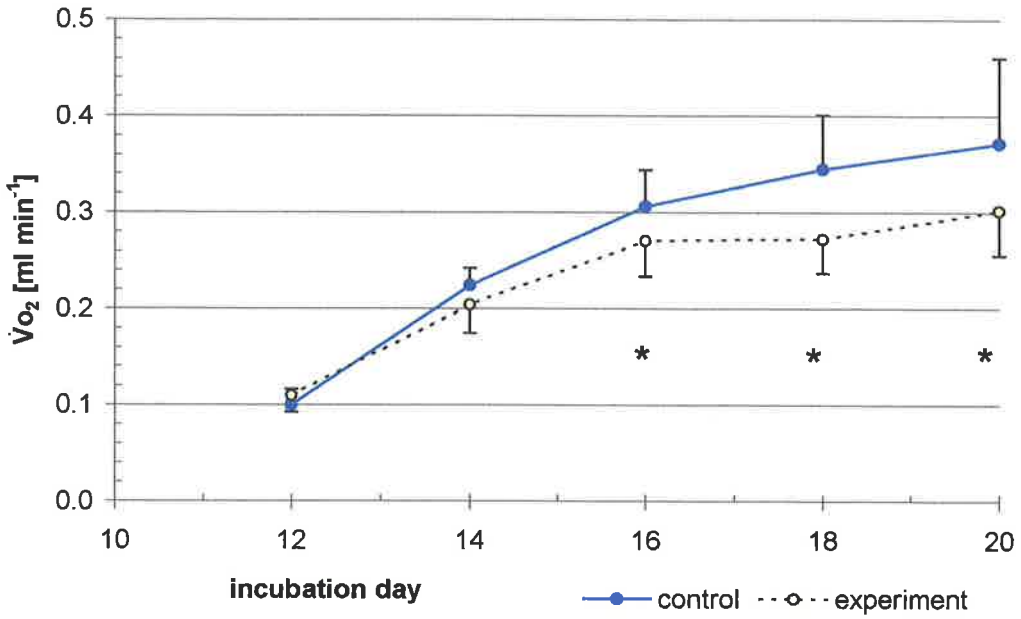


Fig. 5.12a: Oxygen consumption of embryos in wax/hole experiments during the second half of incubation (normalised for egg size). Means and SD, 6 to 14 different eggs averaged per data point. *indicates significant differences.

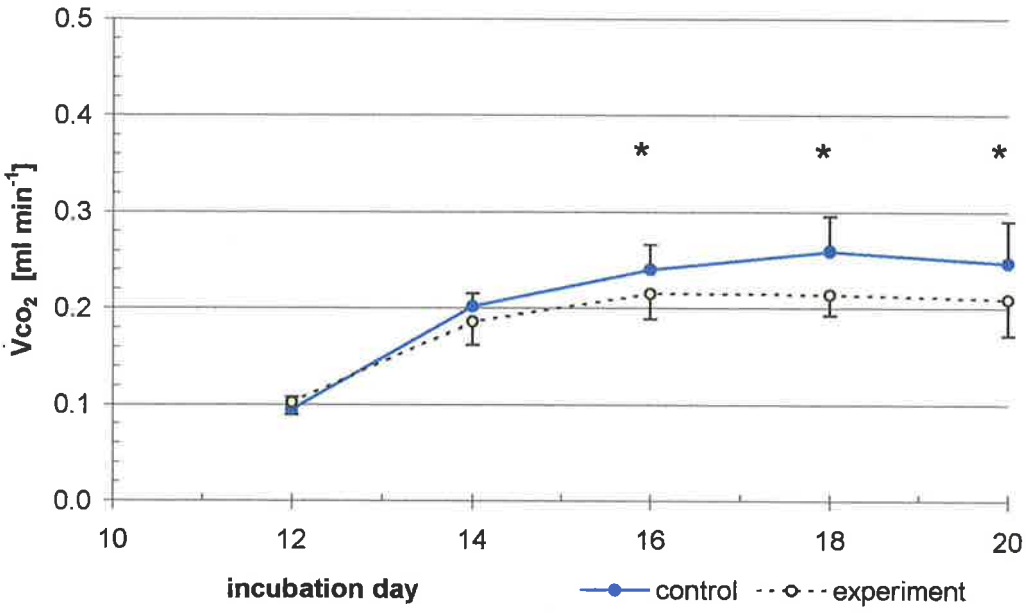


Fig. 5.12b: Production of carbon dioxide by embryos in wax/hole experiments during the second half of incubation (normalised for egg size). Means and SD, 6 to 14 different eggs averaged per data point. *indicates significant differences.

the experimental eggs than in the control eggs, but this difference approached significance only on day 18 (p = 0.079, t-test) (fig. 5.13.c).

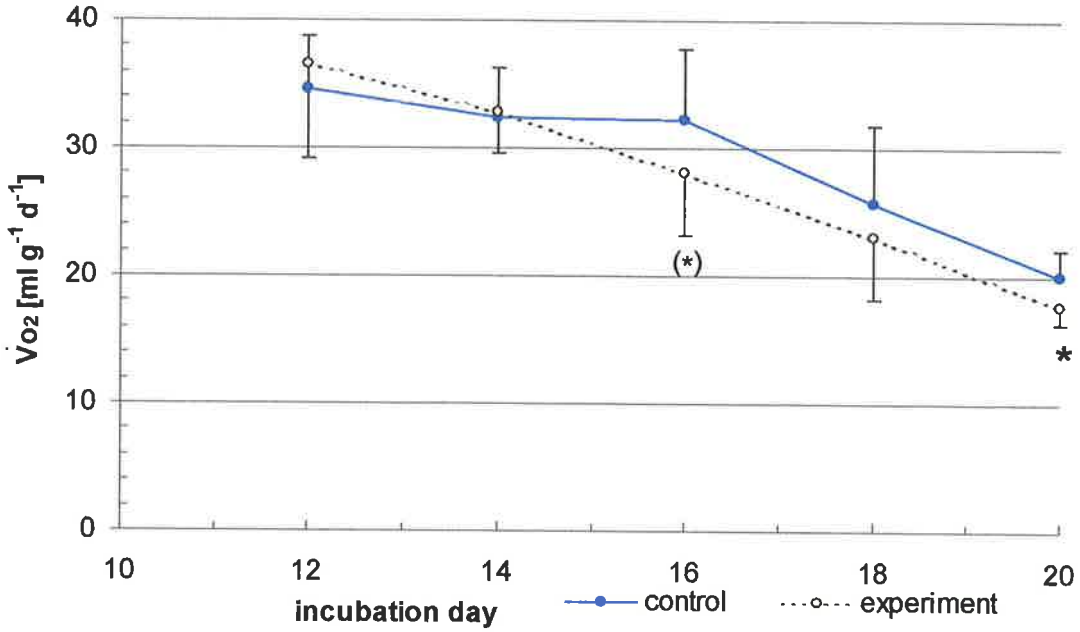


Fig. 5.13a: Mass-specific oxygen consumption of embryos in wax/hole experiment during the second half of incubation. Means and SD, 6-14 eggs averaged per data point, all different eggs. *indicates significant differences.

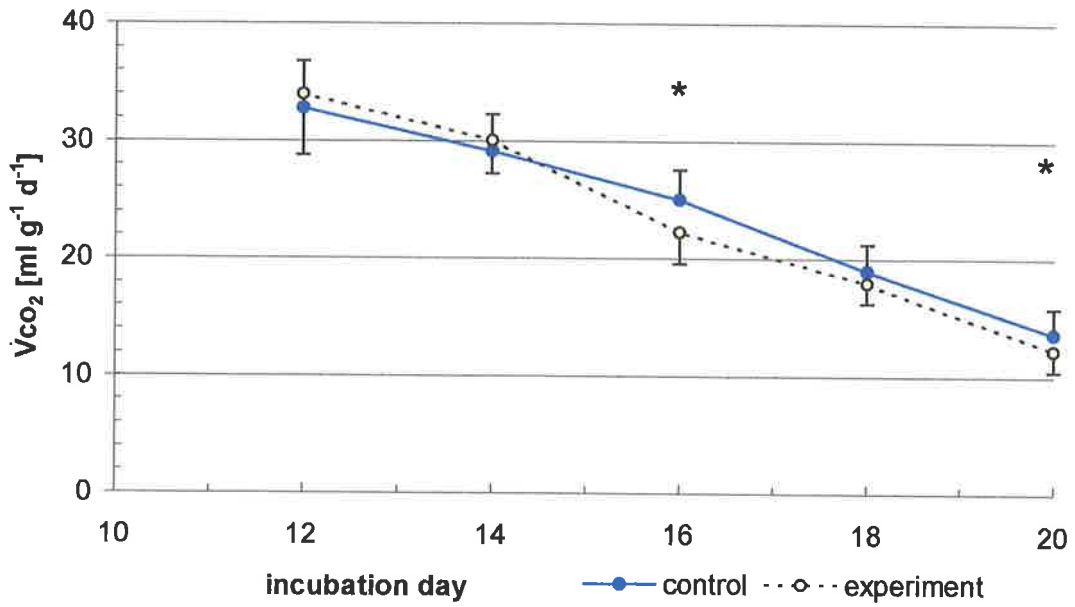


Fig. 5.13b: Mass-specific carbon dioxide production by chicken embryos in wax/hole experiments during the second half of incubation. Means and SD, 6 to 14 different eggs averaged per data point. *indicates significant differences.

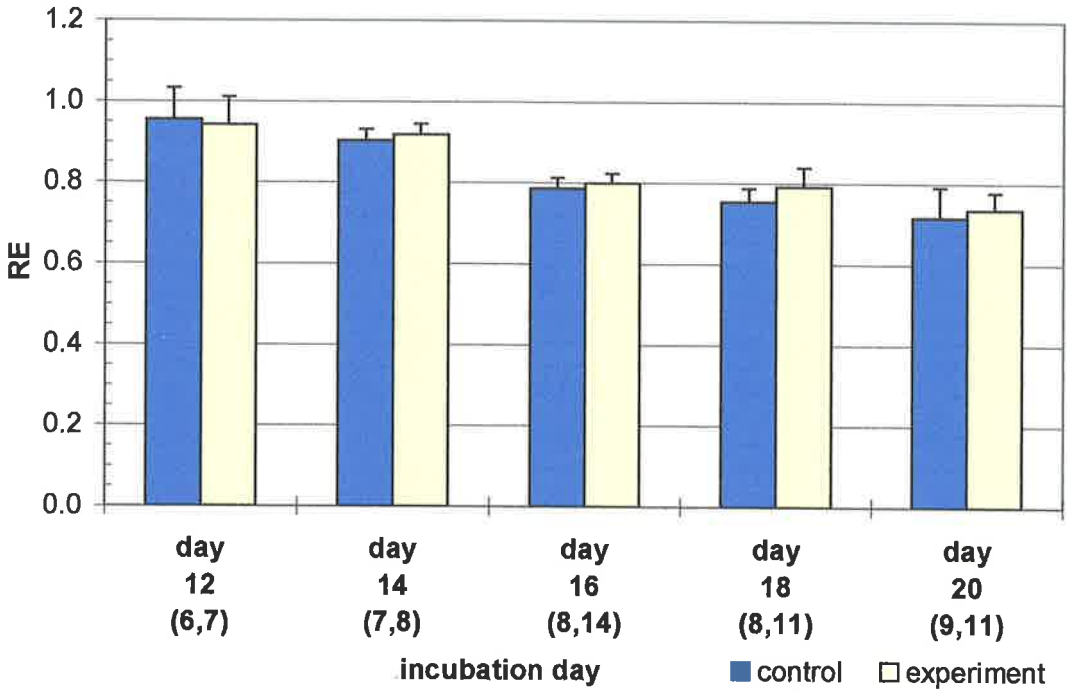


Fig. 5.13c: Respiratory exchange ratio of embryos in wax/hole experiments during the second half of incubation. Means and SD, sample size in brackets.

There was no correlation between oxygen consumption and eggshell conductance in the control groups (day 12 to day 20), except on day 14 ($p = 0.043$, $r = 0.778$, $n = 7$). The experimental groups showed such a positive correlation more frequently: on day 12 ($p = 0.019$, $r = 0.828$, $n = 7$), day 16 ($p = 0.004$, $r = 0.728$, $n = 14$) and day 20 ($p = 0.032$, $r = 0.658$, $n = 11$). This was confirmed when the sample size was increased by pooling the measurements from either all control or all experimental groups of the different treatment groups at the relevant day (e.g. $\dot{V}O_2$ measurement of day 12 from groups terminated on day 12, 14, 16, 18 and 20). In this case, $\dot{V}O_2$ did not correlate on any day with shell conductance in the pooled control data, but correlated at all days with shell conductance in the pooled experimental groups (fig. 5.14a-e).

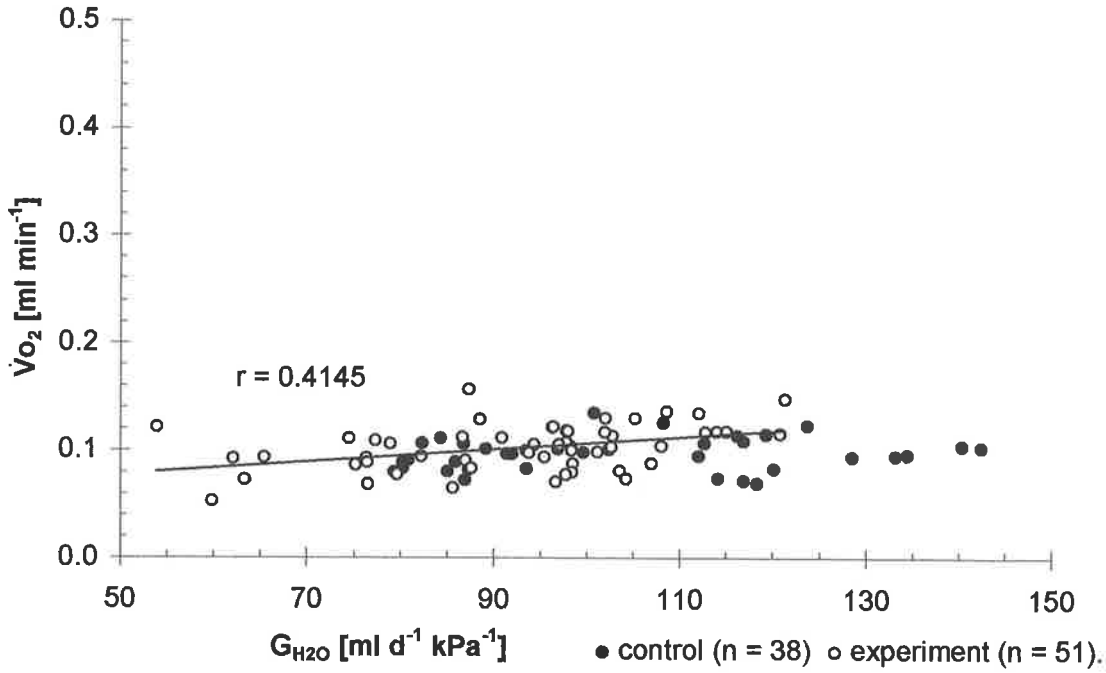


Fig. 5.14a: Oxygen consumption correlated with G_{H2O} in wax/hole experimental eggs, but not control eggs,on day 12.

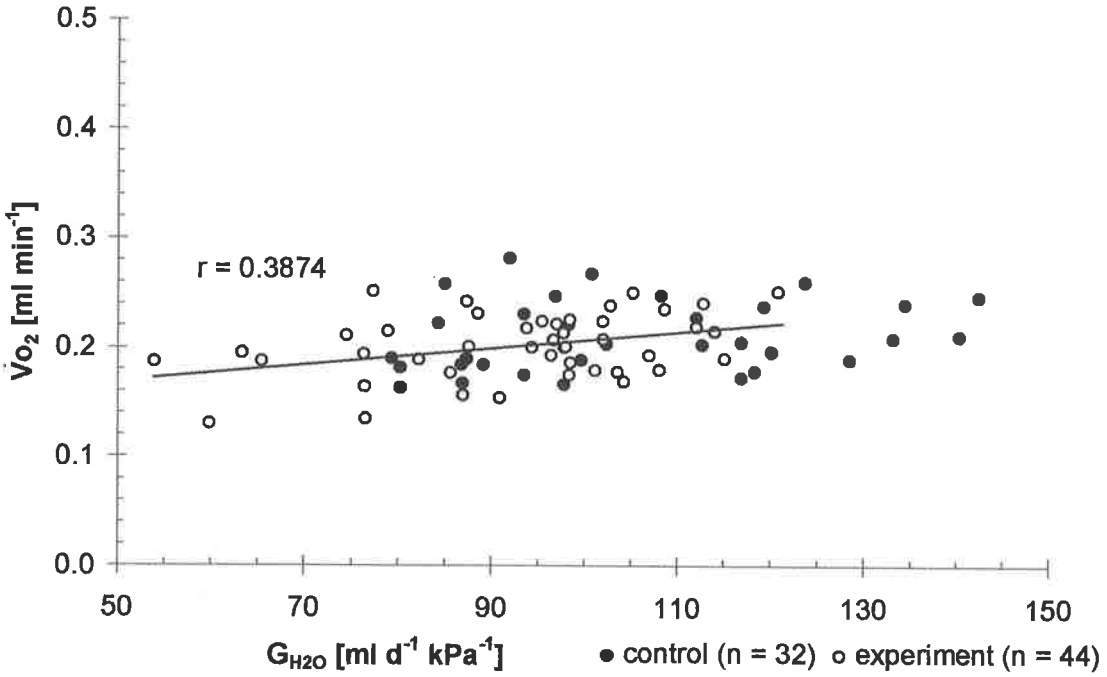


Fig. 5.14b: Oxygen consumption correlated with G_{H2O} in wax/hole experimental eggs, but not control eggs, on day 14.

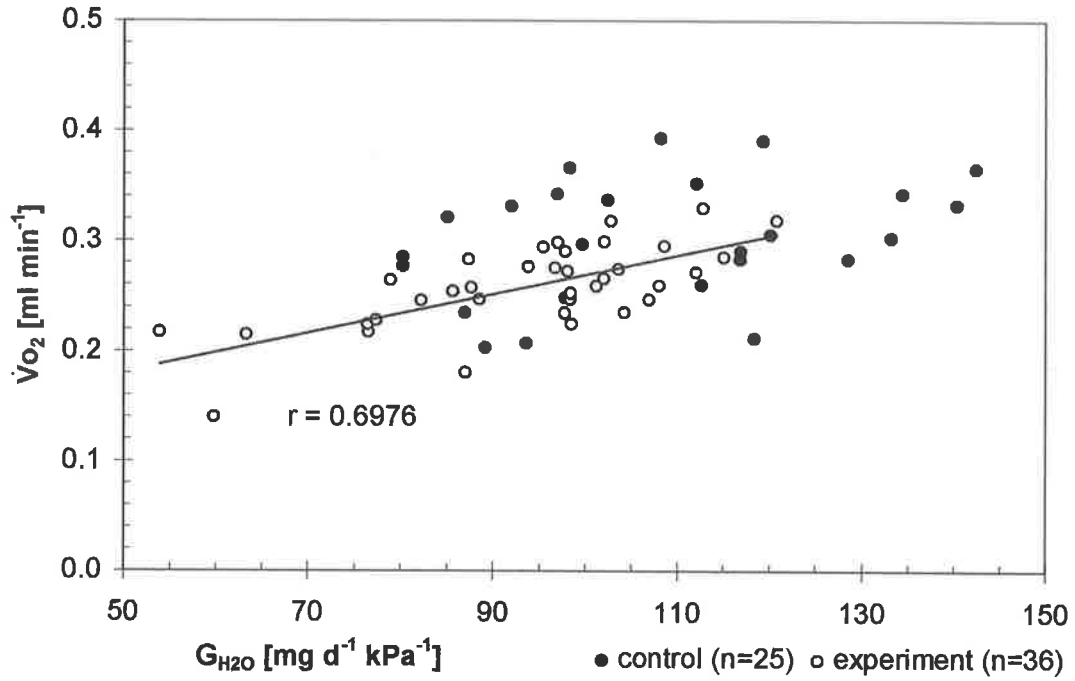


Fig. 5.14c: Oxygen consumption correlated with G_{H2O} in wax/hole experimental eggs, but not control eggs, on day 16.

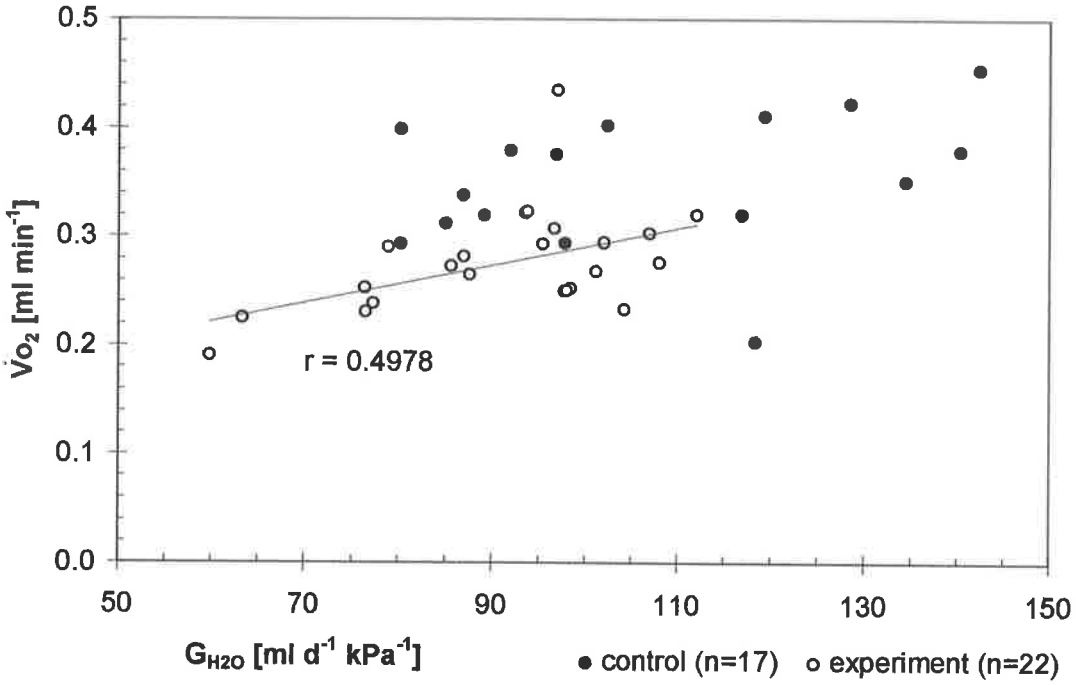


Fig. 5.14d: Oxygen consumption correlated with GH2O in wax/hole experimental eggs, but not control eggs, on day 18.

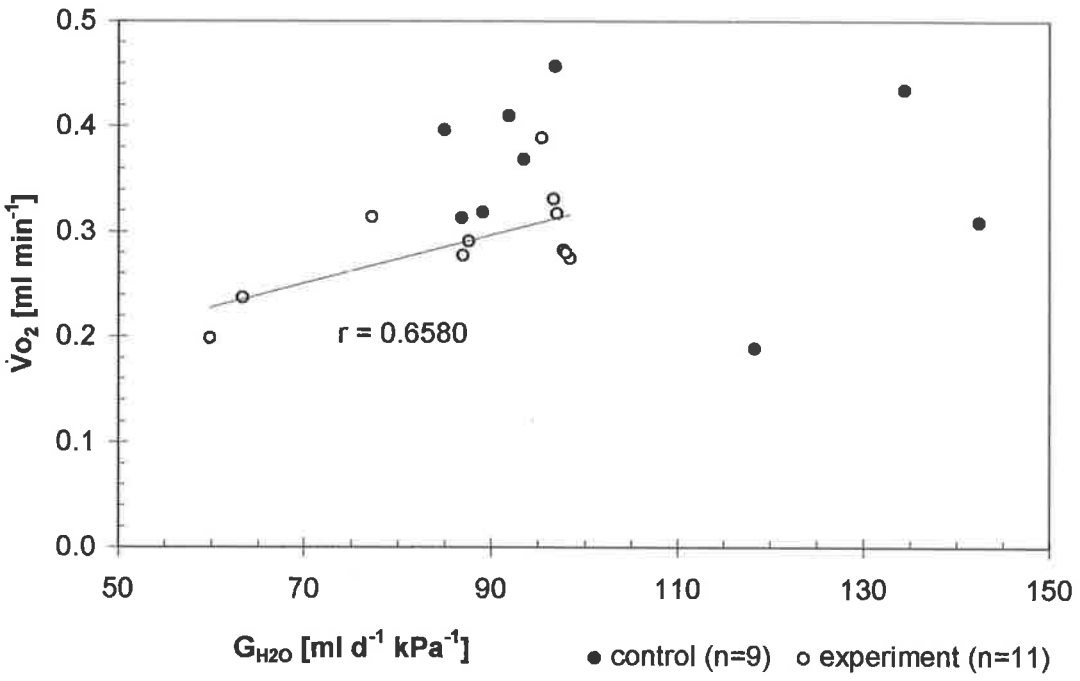


Fig. 5.14e: Oxygen consumption correlated with G_{H2O} in wax/hole experimental eggs, but not control eggs, on day 20.

Shell conductance was positively correlated to fresh egg mass in both pooled control groups ($r = 0.497$, $n = 38$) and pooled experimental groups ($r = 0.500$, $n = 51$) ($p = 0.0015$ and $p = 0.0002$, respectively) (fig. 5.15). Oxygen consumption was therefore compared to mass-specific shell conductance. There was no positive correlation between $\dot{V}O_2$ and mass-specific shell conductance in any of the control groups, single or pooled. However, the experimental group on day 16 ($p < 0.01$, $r = 0.724$, $n = 14$) and the pooled experimental groups on days 12 ($p < 0.01$, $r = 0.363$, $n = 51$), 14 ($p < 0.05$, $r = 0.360$, $n = 44$) and 16 ($p < 0.01$, $r = 0.624$, $n = 36$) maintained the positive correlation between oxygen consumption and shell conductance when normalised for fresh egg mass, indicating a true correlation between shell conductance and $\dot{V}O_2$ independent of egg size.

The oxygen consumption in the wax/hole experiments was compared to the data from the wax/oxygen treatment terminated on day 20. The $\dot{V}O_2$ of the hole/control group did not differ from values in the wax/oxygen experiment. However, $\dot{V}O_2$ of the experimental group in the wax/hole treatment was significantly reduced in comparison to the control and experimental groups in the wax/oxygen treatment throughout the second half of incubation ($p < 0.0001$, $F_{3,36} = 10.4115$, ANOVA) (fig. 5.16a).

Mass-specific $\dot{V}O_2$ on day 20 was also significantly reduced in the wax/hole experimental group when compared to the wax/oxygen treatment ($p = 0.0492$, $F_{3,36} =$

2.88, ANOVA), while the hole/control eggs showed no difference to the wax/oxygen treatment (fig. 5.16b).

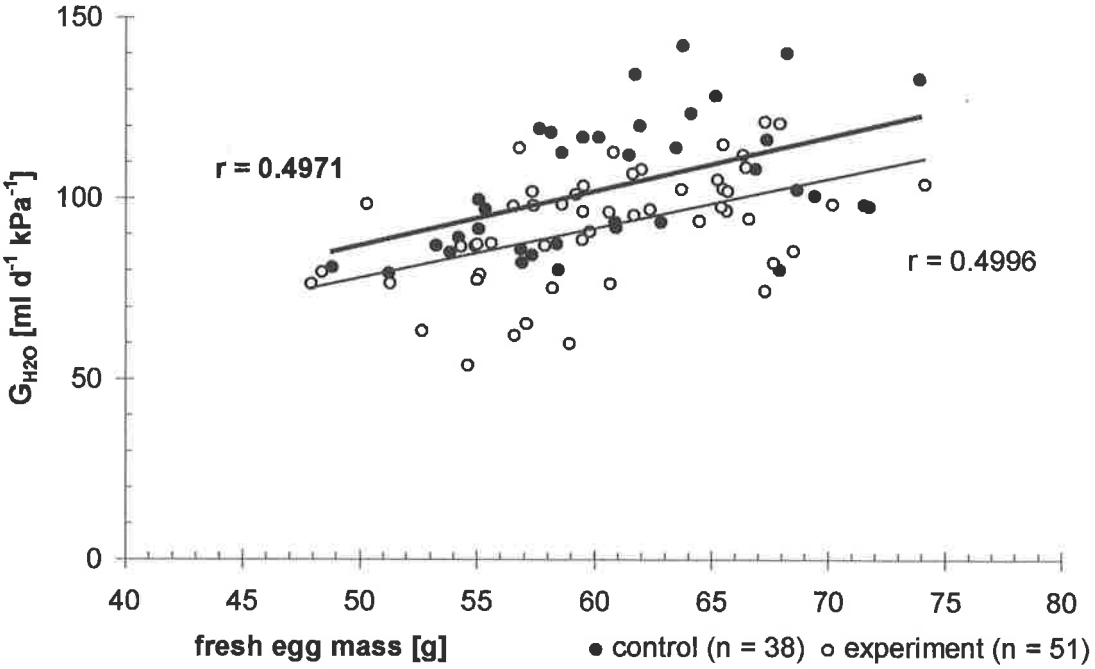


Fig. 5.15: Correlation between fresh egg mass and experimental shell conductance in wax/hole experiments.

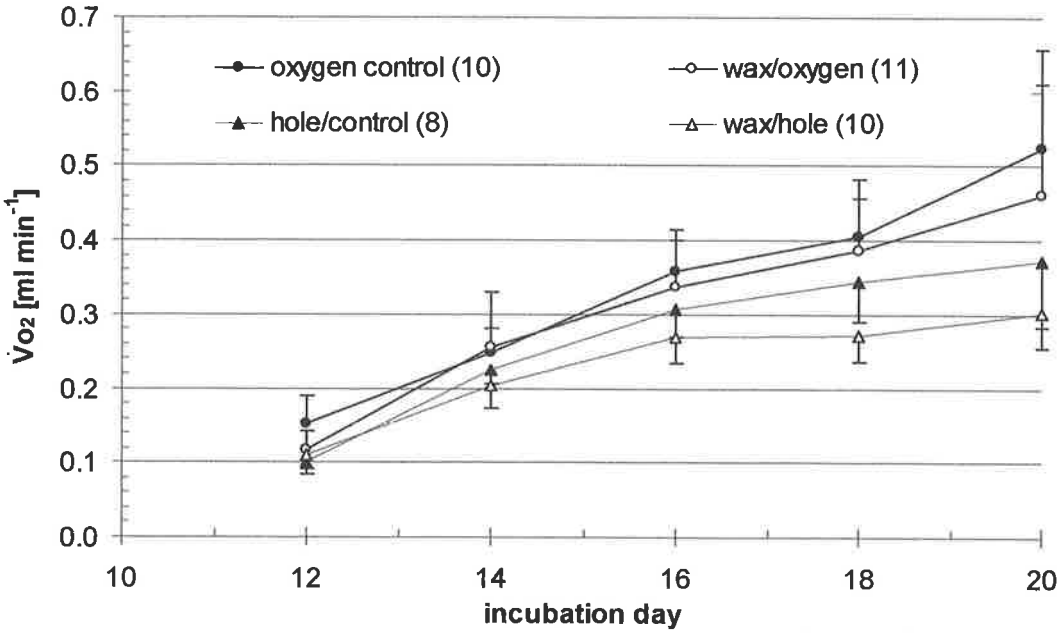


Fig. 5.16a: Comparison of oxygen consumption in wax/oxygen (Series A) and wax/hole treatment (Series B, sample size averaged), normalised for 60.7 g fresh egg mass. Means and SD, sample size in brackets.

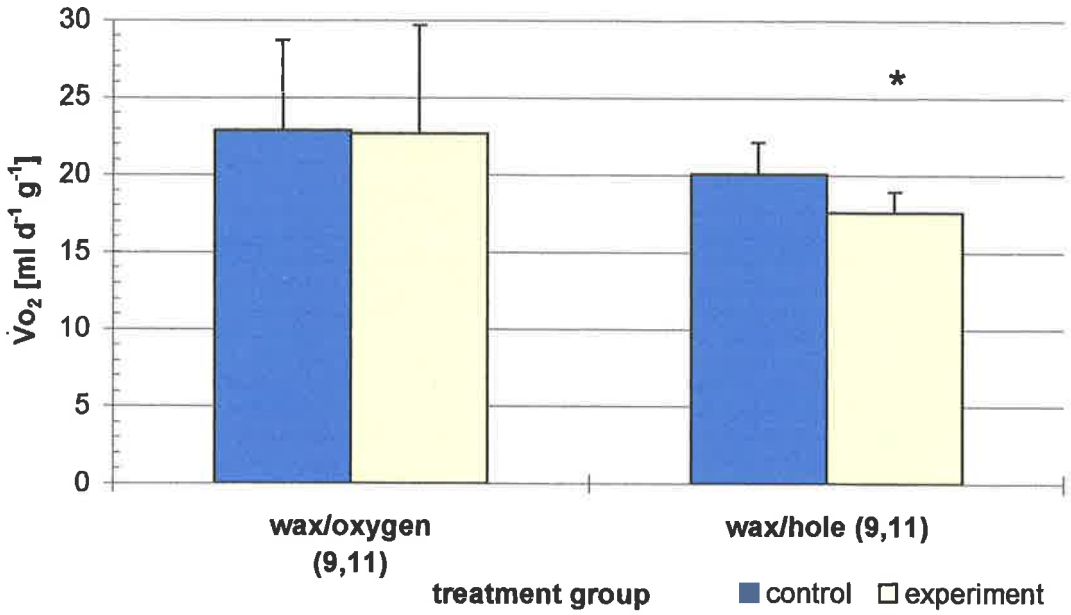


Fig. 5.16b: Comparison of mass-specific oxygen consumption on day 20 in wax/oxygen and wax/hole treatment. Means and SD, sample size in brackets. *indicates significant differences.

5.3.3 Series C: Importance of treatment-timing

5.3.3.1 Embryonic growth

The effect of the shell manipulation on subsequent embryonic growth correlated with the stage of development at the time of treatment. Chicken embryos did not survive the treatment in eggs if they were manipulated at the beginning of incubation (day 1) (see below, section 5.3.4). In the experiments on days 4 to 7 of incubation, chicken growth until day 15 was not significantly reduced in comparison to hole/control eggs. After shell manipulation on days 8 to 12, wet embryo mass was negatively affected by the treatment in experimental (wax/hole) eggs that were manipulated on days 8 ($p = 0.009$), 10 ($p = 0.076$) and 12 ($p = 0.088$, t-test), and dry embryo mass was reduced on day 8 ($p = 0.029$) and day 12 ($p = 0.043$) (fig. 5.17).

No group differed significantly from the undisturbed control eggs at day 15 of incubation in wet (11.16 ± 1.03 g) and dry (1.48 ± 0.21 g) embryo mass (multiple means comparison, Dunnett's Method). Since the CAM attaches to the inner shell membrane from day 10 and only then is fixed in place and permanently exposed to the effect of the shell manipulation, the data may be grouped into those experiments before (days 4 - 8)

and after (days 10 - 12) CAM attachment. Embryonic dry mass did not differ between the hole/control and experimental eggs in the early group ($p = 343$, $n_{contr} = 27$, $n_{exp} = 21$, t-test), but was significantly reduced in eggs with shell treatment after attachment ($p = 0.028$, $n_{contr} = 15$, $n_{exp} = 20$, t-test).

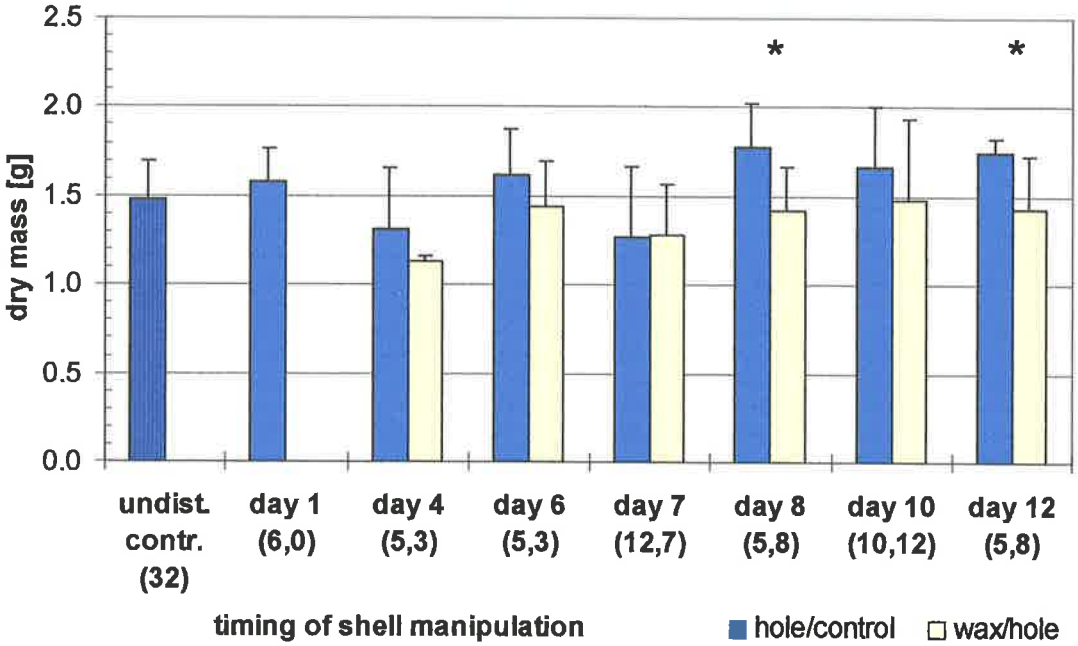


Fig. 5.17: Dry embryo mass (normalised for fresh egg mass) on day 15 after wax/hole treatment at different ages. Means and SD; sample size in brackets. * indicates significant differences.

Embryonic water content of the undisturbed control on day 15 was 86.8% ± 1.0% and was negatively correlated with dry embryo mass ($r = -0.85$, $p < 0.001$, $n = 32$). Of the procedural control and experimental groups only the hole/control group on day 7 (88.4% ± 2.2%) differed from the undisturbed control significantly ($p < 0.05$, Dunnett's Method). Water content of the experimental embryos was generally higher than that of the control, but did not differ statistically except for the experiment on day 8, where the wax/hole embryos had a significantly greater water content than the hole/control group ($p = 0.047$, $n_{contr} = 5$, $n_{exp} = 8$, t-test) (fig. 5.18). The timing of shell manipulation was negatively correlated with the water content in embryos of the hole/control ($p < 0.05$, $r = -0.363$, $n = 42$) and wax/hole groups ($p < 0.05$, $r = -0.301$, $n = 41$).

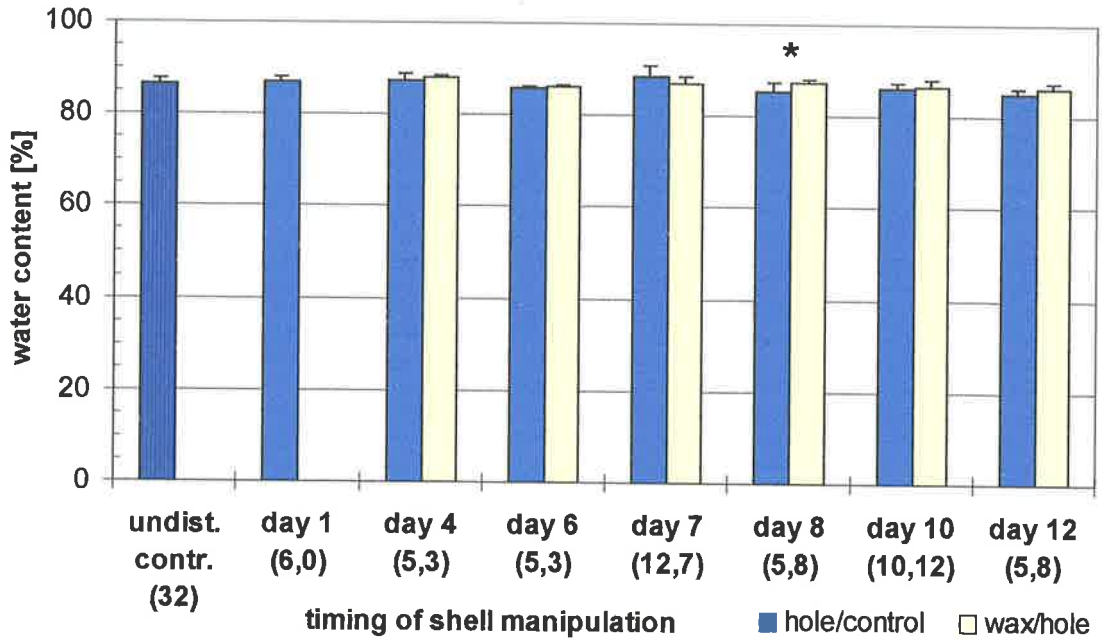


Fig. 5.18: Water content of embryos on day 15 after wax/hole treatment at different ages. Means and SD; sample size in brackets. * indicates significant differences.

The amount of wet (14.9 ± 2.2 g) and dry (6.9 ± 0.9 g) yolk mass in the yolk sac of the undisturbed control group on day 15 showed no correlation with embryonic mass. Only wet and dry yolk mass in the experimental groups on day 4 (8.7 ± 0.6 g and 3.7 ± 0.4 g, respectively) and day 10 (9.0 ± 5.3 g and 3.7 ± 0.4 g, respectively) differed significantly from the undisturbed control ($p < 0.05$, Dunnett's method).

The remaining yolk (dry mass) at termination was not influenced by the timing of the shell manipulation. The amount of yolk left in the yolk sac was often less in the experimental group than in the control group (fig. 5.19), but this was significant only in the day 10 treatment ($p = 0.022$, $n_{contr} = 10$, $n_{exp} = 12$, t-test). In the same experiment, the water content of the yolk was higher in the experimental (57.7% ± 3.5%) than in the control group (51.9% ± 1.6%) ($p < 0.001$, t-test). Elsewhere no difference in the yolk water content was found between hole/control and experimental group of the same age. Water content of the yolk in the undisturbed control was 53.6% ± 2.3%. Only yolk water content in wax/hole day 12 (62.1% ± 10.1%) was significantly different from that in the undisturbed control ($p < 0.05$, Dunnett's method).

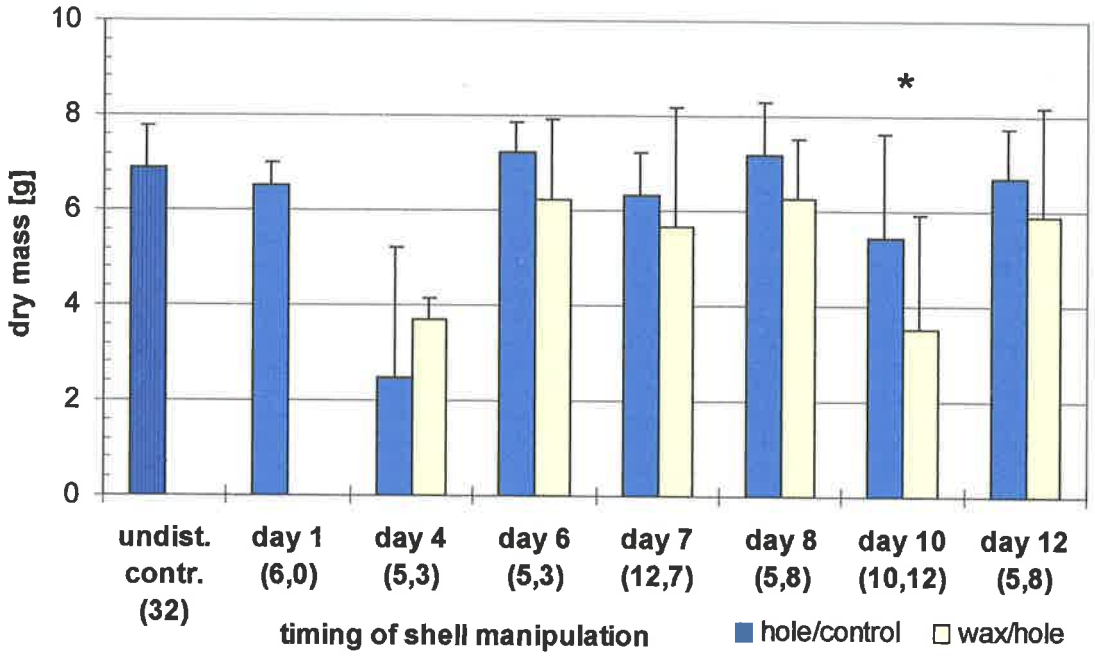


Fig. 5.19: Dry yolk mass on day 15 (normalised for fresh egg mass) after wax/hole treatment at different ages. Means and SD; sample size in brackets. * indicates significant differences.

Experimental shell conductance was not correlated to wet or dry embryo mass in any of the groups, nor to water content of the embryo or yolk.

5.3.3.2 Gas exchange

The eggs showed few differences in gas exchange after wax/hole treatment of the shell at various times of development. Prior to shell manipulation, $\dot{V}O_2$ and $\dot{V}CO_2$ showed no difference between control and experimental eggs, as intended for the experiment. On the two days (days 10 and 13) where eggs from all groups were compared, there were no differences in $\dot{V}O_2$ and $\dot{V}CO_2$ between control and experimental groups (fig. 5.20a,b). The RE showed a great variation between experiments, from 0.72 to 1.08 (a fault of the CO_2 analyser calibration), but there was no consistent difference between control and experimental eggs.

On the day directly after shell treatment, gas exchange ($\dot{V}O_2$ and $\dot{V}CO_2$) did not differ between the control and experimental groups in most experiments. The exception was the experimental group manipulated on day 10, where $\dot{V}O_2$ and $\dot{V}CO_2$ were significantly reduced on day 11 compared to the control group ($p_{\dot{V}O_2} = 0.028$, $p_{\dot{V}CO_2} = 0.017$, $n_{control} = 5$, $n_{experiment} = 6$, t-test). As there was further no correlation of the time of shell treatment with $\dot{V}O_2$ or $\dot{V}CO_2$, the immediate reaction of the embryo to the shell treatment

was probably not influenced by age of the chicken embryo at the time of shell manipulation..

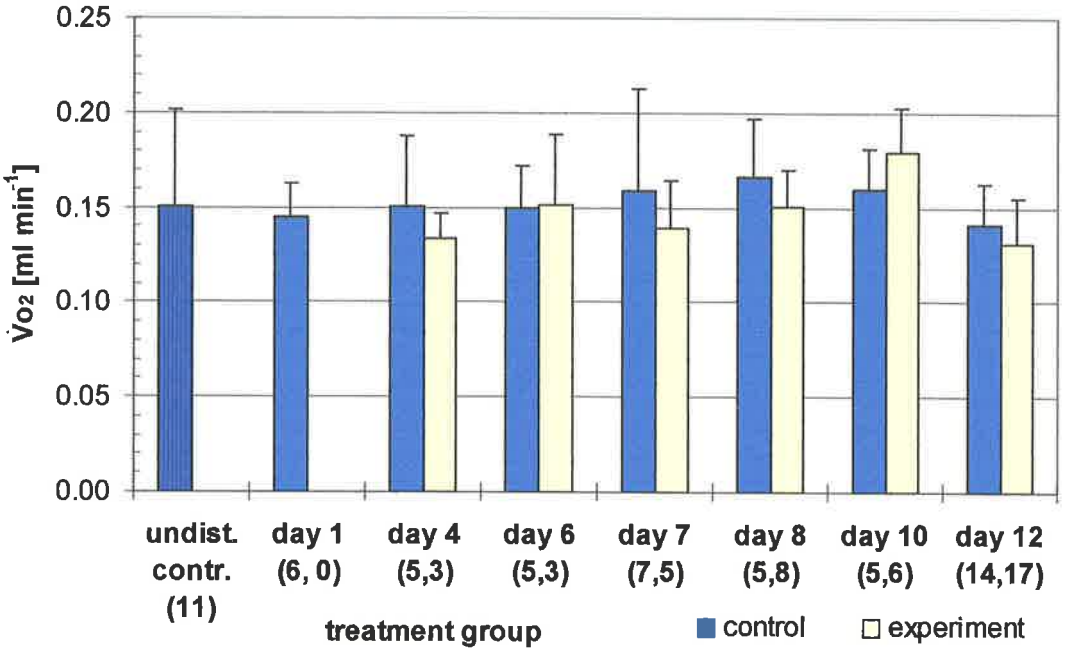


Fig. 5.20a: Oxygen consumption of embryos on day 13 (normalised for fresh egg mass) after wax/hole treatmeant at various days. Means and SD, sample size in brackets.

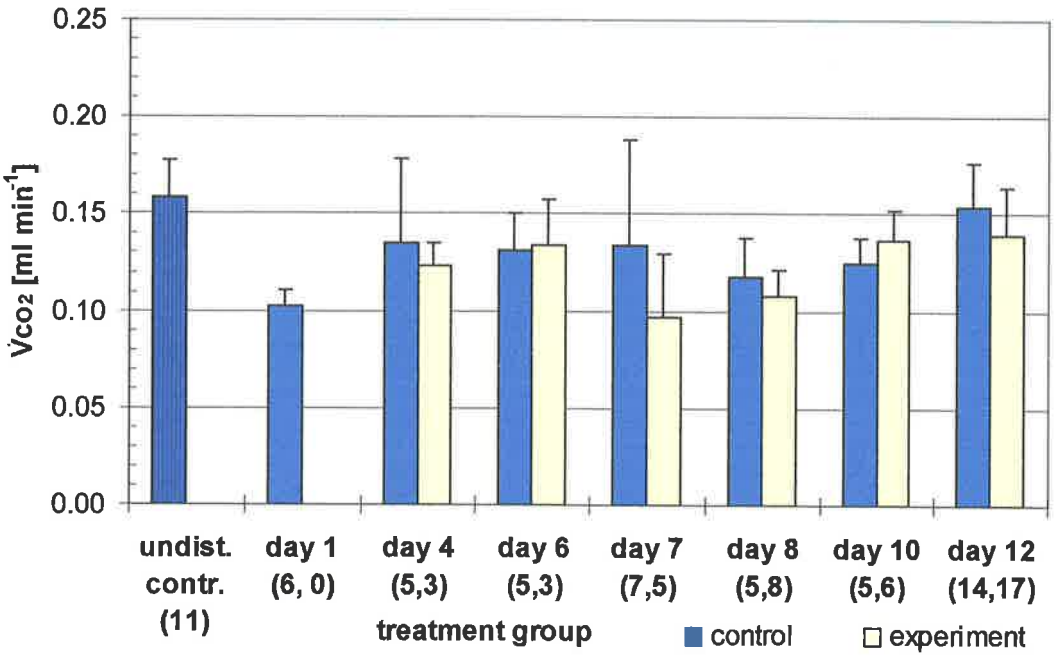


Fig. 5.20b: Production of carbon dioxide by embryos on day 13 (normalised for fresh egg mass) after wax/hole shell treatment at various days. Means and SD, sample size in brackets.

While sample size was often small, these results are confirmed by the analysis of the combined groups before (days 4 to 8) and after (days 10 and 12) CAM attachment (fig. 5.21a,b). In the measurements on day 13, there were no differences in $\dot{V}O_2$ between

hole/control and experimental groups of the same age-at-treatment groups, and no differences between all experimental eggs treated before (days 4-8) and those after (days 10-12) CAM attachment. Although such a difference between age-at-treatment groups was statistically significant for $\dot{V}CO_2$, this is likely to be a consequence of faulty CO_2 analyser calibration, as the same difference was found between the control groups.

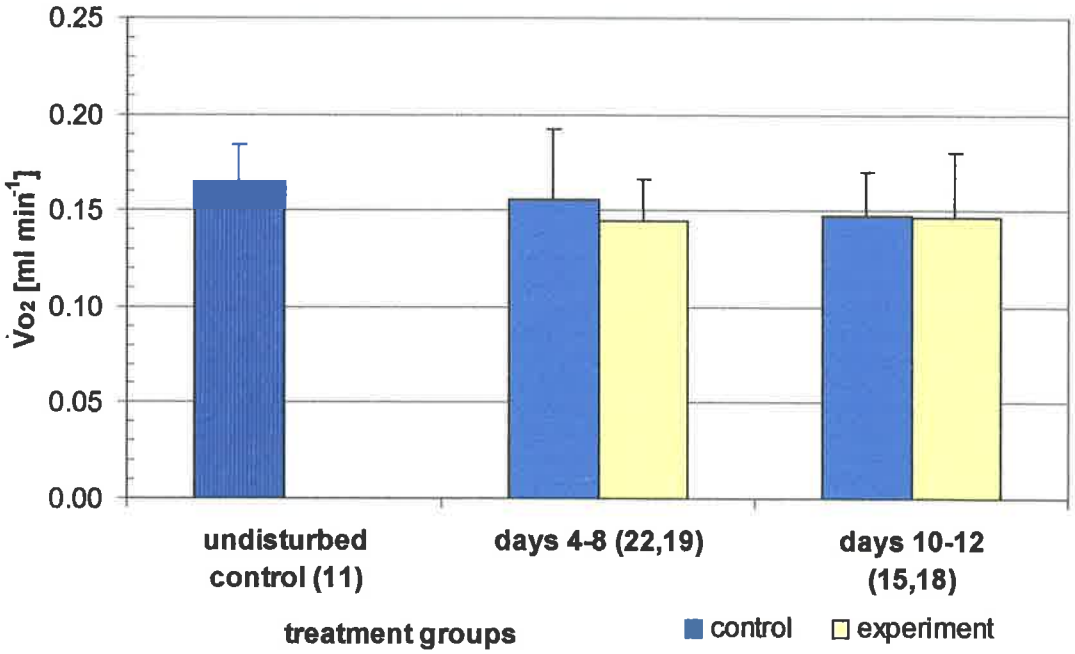


Fig.5.21a: Oxygen consumption of embryos on day 13 (normalised for fresh egg mass) in combined groups after wax/hole treatment at various days. Means and SD, sample size in brackets.

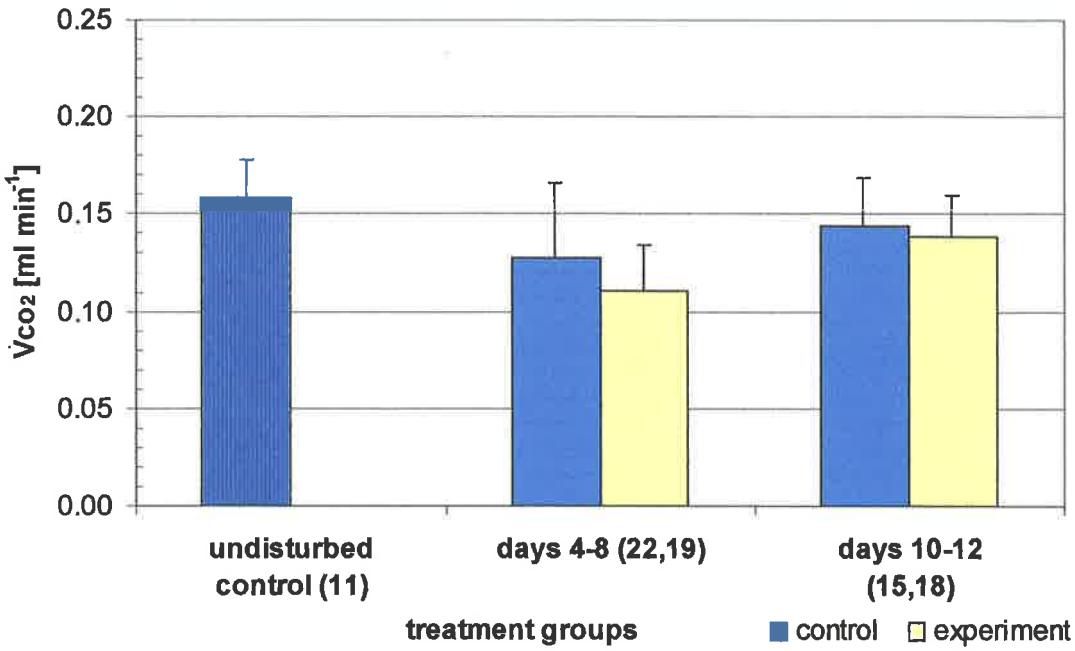


Fig. 5.21b: Production of carbon dioxide of embryos on day 13 (normalised for fresh egg mass) in combined groups after wax/hole treatment at various days. Means and SD, sample size in brackets.

5.3.4 Survival

The survival rate of the embryos in the wax/oxygen treatment (Series A) was generally very high, and the experimental eggs showed no greater mortality than the control group. Survival rates lay between 81.8% and 100% (tab. 5.2).

Termination on	oxygen/ control	wax/oxygen experiment
day 12	85.7 (7)	87.5 (8)
day 14	100.0 (8)	100.0 (8)
day 15	100.0 (11)	100.0 (11)
day 20	81.8 (11)	100.0 (11)

Tab. 5.2: Survival rate [%] in wax/oxygen experiments after shell treatment on day 10, terminated between days 12 and 20. Sample size in brackets.

The survival rate of eggs in Series B (wax/hole treatment on day 10) varied greatly from 45 to 100% at termination between days 12 and 20. Only on day 14 was there a significant difference in survival rate of hole/control (100%) and experimental

embryos (68.4%; p = 0.040, Fisher's exact test) (tab. 5.3). Survival rate seemed to decline from day 16 of incubation in experimental and hole/control groups (fig 5.22). In undisturbed control eggs, the survival rate was reduced on day 20 (tab. 5.3).

Termination on day	control group	experimental group	Undisturbed control
12	85.7 (14)	72.2 (18)	96.0 (25)
14	100.0 (15)	*68.4 (19)	89.5 (19)
16	72.7 (11)	82.4 (17)	97.5 (40)
18	57.1 (14)	52.4 (21)	100.0 (7)
20	45.0 (20)	54.5 (22)	64.9 (37)

Tab. 5.3: Survival rate [%] in all wax/hole experiments after treatment on day 10, terminated between days 12 to 20. Sample size in brackets. *indicates significant differences between control and experimental group.

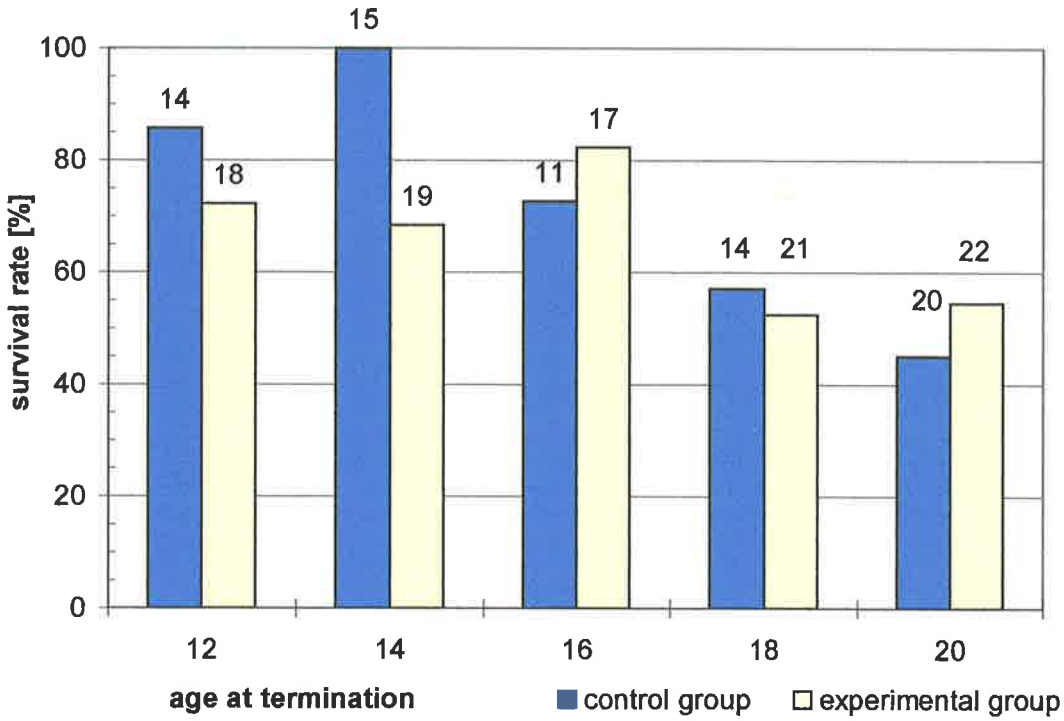


Fig. 5.22: Survival rate in all experiments after wax/hole treatment on day 10, labels show sample size over each column.

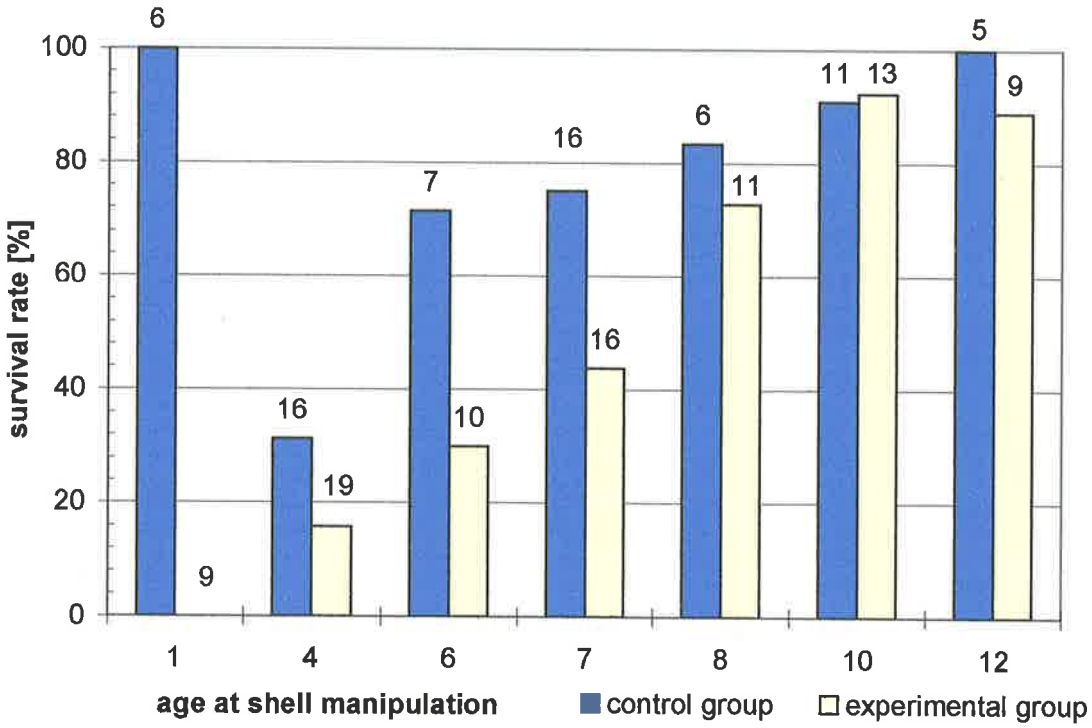


Fig. 5.23: Survival rate [%] until day 15 in experiments with wax/hole treatments at different ages. Sample size over each column.

The importance of the embryo's age at shell manipulation was observed in Series C. In a comparison of survival rates up to day 15 of incubation, the survival rate of the embryos was the greater the later the eggshell was manipulated (see fig 5.23). Mortality was different between individual control and experimental groups only on day 1, where no experimental embryo survived the shell treatment. In general, mortality was higher in all the experimental groups than in the hole/control groups ($p = 0.002$, χ^2 test).

5.3.5 Location of the aircell

The manipulation of the shell permeability had no influence on the location of the aircell. In all but one egg of all groups was the aircell found at the blunt end. However, aircells in the experimental groups were often skewed towards the side of greater shell conductance (i.e. towards the holes drilled into the shell).

5.4 Discussion

5.4.1 Series A: Effect of the wax/oxygen treatment

The wax/oxygen treatment allowed experimental embryos to grow normally under very great differences in regional oxygen availability (7.6 ± 1.3 kPa between *wax* and *holes*, section 3.3.1). Although the wax cover over half the shell reduced local shell permeability and therefore gas exchange to practically zero (chapter 3), oxygen uptake and embryo mass (wet and dry) in wax/oxygen treated groups did not differ from the control groups throughout the second half of incubation (fig. 5.4, 5.2), indicating similar growth curves in the treatments. This was supported by similar mass specific oxygen uptake rates in the experimental and control embryos (fig. 5.5a,b). The level of hyperoxia applied in this study was therefore not high enough to increase embryonic growth and respiration above that of the control, as reported for a 60% oxygen environment by Temple and Metcalfe (1970) and Stock and Metcalfe (1987). Further, it did not cause the control embryos to increase oxygen uptake in the acute exposure to hyperoxia during the respiratory measurement (Visschedijk *et al.*, 1980; Stock and Metcalfe, 1987; Ar *et al.*, 1991). On the other hand, growth and gas exchange of the experimental embryos were not suppressed below control values, as described in other reports where parts of the egg had been covered with a gas impermeable material (Tazawa *et al.*, 1971a; Metcalfe *et al.*, 1981; Tazawa *et al.*, 1988). The absence of significant correlations between shell conductance and oxygen consumption further indicates that the embryos were not oxygen stressed in these experiments (cf Burton and Tullett, 1983). The increase of P_{O_2} in the atmosphere was therefore sufficient to compensate for the loss of the pore area under the wax, but was not enough to lift the metabolism above that of normoxic control measurements (hole/control, fig. 5.16a), thus meeting the design of the experiment.

The fitness potential of the experimental embryos was further not diminished by an elevated energetic cost of development. If gas exchange is limited by the shell conductance, metabolic costs may increase if the embryo reacts with an increase in blood flow. However, oxygen consumption of experimental embryos did not differ from control embryos at a similar body mass, indicating that embryos of the experimental groups did not incur significant additional metabolic costs. In agreement with this, relative heart mass, as potential indicator of stroke volume, and heart rate did not increase in eggs with partly covered shell (Laughlin, 1978; McCutcheon et al., 1982).

Heart rate even decreased in early incubation in a 10% oxygen environment (Akiyama et al., 1999). Under hypoxic conditions, anaerobic metabolism could theoretically offer an alternative solution for growth compensation (Bjønnes *et al.*, 1987). However, avian embryos show almost no anaerobic metabolism (Ar *et al.*, 1991), and the lack of increased consumption of yolk as indicated by the similar wet mass of remaining yolk (10.2 ± 1.9 g) in control and experimental eggs on day 20 of incubation, supported this.

The decrease in yolk mass observed in treated eggs on day 15 (fig. 5.3) could be seen as support of an earlier increase in energy demand due to morphological adaptation to the regional differences in shell conductance in the phase of rapid chorioallantoic maturation (days 6-14). However, accelerated growth and increased vascularisation above control values were not observed in the microscopical analysis of the CAM on day 15 (fig. 4.6). Further, the difference in yolk mass on that day was not carried on into the later samples, and is therefore more probably the consequence of a random effect. Lastly, if the experimental shell manipulation required a greater relative energy output to maintain growth, one could expect a negative correlation between the mass of remaining yolk at the end of incubation with embryo mass, possibly stronger than that found in the control group, which was not observed. Experimental embryos thus avoided the loss of yolk reserves that are important for post-hatching growth and survival (Rhymer, 1988; Thomas and Brown, 1988).

There was also no effect of the shell treatment on the maturity of the embryos prior to hatching. The initiation of pipping and yolk re-absorption, as well as embryonic water content on day 20 (80.4% ± 1.8%), did not differ between the treatments, indicating a similar level of embryonic maturity. Although the embryos in this study were not allowed to hatch, one may speculate that this would occur simultaneously in the two treatments. In wild birds, the ability to prevent a delay in hatching would improve the survival chances of the embryo (Drent, 1975). Laughlin (1978) observed a delayed pipping and hatching in eggs covered to ca. 30% and 60% with PVC tape that did not cover the aircell, and some extension of the pipping stage in covered eggs. In the present report, however, similar numbers of eggs had externally pipped in the control (3 of 9) and experimental group (2 of 11) on day 20. This stage was therefore not delayed. Further, internal pipping numbers were similar in both groups (2 of 9 and 1 of 11, respectively), contrasting Visschedijk's (1968b) observation of the shortening of the internal pipping stage by 7.6 h, when the aircell shell was covered with paraffin. The difference may be explained by the fact that the aircell was only partly blocked in the

present study, and aircell P_{O_2} was not reduced in experimental eggs on day 20 (section 3.3.1). Oxygen partial pressure in the aircell is a stimulus for the external pipping of the chick (Visschedijk, 1968c), and hatching could thus be expected to be simultaneous. However, hatching might have been affected by a high P_{CO_2} in the egg caused by the wax cover over half the aircell (Visschedijk, 1968c), which was not investigated.

5.4.2 Series B: Effect of wax/hole treatment through incubation

The chicken embryos in the wax/hole treatment showed no difference in embryonic mass (dry or wet) and gas exchange before day 15 (fig. 5.7, 5.9, and 5.12). They were thus able to compensate for the great regional variation of shell permeability during a time of very rapid, exponential growth (fig. 5.6). However, the embryos were affected by the wax/hole treatment from day 15, after three quarters of incubation, when gas exchange of the precocial embryo is close to maximal, and plateaus thereafter (C. Vleck *et al.*, 1979). During this phase, the respiratory system is normally at its maximum efficiency, and oxygen availability may become limiting in undisturbed eggs (Tullett and Deeming, 1982; Burton and Tullett, 1983). A further strain, as imposed by the modified shell conductance, prevented the experimental embryos from maintaining oxygen consumption at the control level, and consequently, the development of the embryo was affected in the last quarter of incubation: in comparison to the control, gas exchange was reduced from day 16 (fig. 5.12) and embryo mass from day 18 (fig. 5.9), The decline of the embryonic growth rate was reflected in the decrease of the mass-specific oxygen consumption and carbon dioxide production of the experimental groups from day 16 (fig. 5.13a,b). It was lower than in the control groups in spite of the reduced body mass of the experimental embryos. Lastly, limitation of oxygen availability in the experimental eggs was indicated by the correlation of oxygen consumption with the shell conductance (fig. 5.14). However, this did not increase mortality of the experimental embryos above the hole/control (fig. 5.22).

There were few, if any, differences in the respiratory exchange ratio between hole/control and wax/hole experimental groups (fig. 5.13c), which confirms results by Stock and Metcalfe (1987), who found that the RE was not affected under hypoxia or hyperoxia. The RE measured in the present experiments was unfortunately affected by an unrecognised non-linearity of the carbon dioxide analyser and at times (cf. fig. 5.4) lay below the more typical values of 0.65 to 0.70 during the second half of incubation (Romijn and Lokhorst, 1951). This made comparisons between measurements at

different days difficult. Comparisons were, however, valid for measurements on the same day. Correspondingly, carbon dioxide production mirrored the results of the oxygen consumption and was reduced in the experimental group from day 16.

An extended utilisation of energy reserves in order to compensate for the altered shell permeability was not observed: the remaining dry yolk mass on day 20 was greater in the experimental eggs after wax/hole treatment (7.4 ± 0.6 g) than in the control eggs (6.0 ± 1.2 g) (fig. 5.11), and remaining yolk did not correlate negatively with experimental embryo mass. The smaller experimental embryos had consumed less yolk during incubation than the control, their growth limited by another factor (presumably hypoxia) so that their was no clear relationship between embryo mass and energy reserves in this group.

Similar to the wax/oxygen experiment, the maturity of the embryos in the experimental groups was not affected by the shell treatment. External pipping commenced on day 20 in a similar fraction of eggs in the experimental (3/12) and control (2/9) eggs, and the re-absorption of yolk was similar between the groups (8/12 and 6/9, respectively). Water content of experimental embryos was mostly similar to that of the control groups: it declined during incubation from 92% on day 12 to 81% on day 18 (fig. 5.10). This is comparable to values in the literature (Romanoff, 1967; Ar *et al.*, 1987). Only on day 20 was there a significant difference between the control (80%) and the experimental group (83%). This could indicate a decelerated maturation of the embryo (Starck and Ricklefs, 1998), but as it represents an increase in water content of wax/hole embryos from day 18 to day 20, this interpretation is not well founded. Stock and Metcalfe (1987) observed similarly higher water content in hypoxic embryos and attributed it to the hypoxia-induced increase in circulating and extravascular fluids as documented by Grabowski (1966). The difference in embryonic water content observed on day 15 (87.2%) after shell treatment on day 8 (fig. 5.8) is the only incidence of this kind at an earlier age (cf Series C) and may be attributed to the same pathology. The different water content was not caused by low shell conductance or differences in water loss rate between the groups, as body composition of embryos is maintained over a wide range of water loss (Hoyt, 1979; Simkiss, 1980; Davis *et al.*, 1988).

In contrast to the water content of the embryo, the water content of the yolk on day 20 (51.7%) did not differ between experimental and control groups. This confirms observations by Hoyt (1979) and Burton and Tullett (1985) that the water content of the

yolk is maintained at its normal level in eggs with variable shell conductance, presumably to facilitate absorption into the embryo's body cavity.

5.4.3 Comparison of the wax/oxygen and wax/hole treatment

The two treatments "wax/oxygen" and "wax/hole" clearly affected the embryos differently: while wax/oxygen treated embryos were able to develop normally, wax/hole treated eggs reduced gas exchange and growth rate during the last quarter of incubation. The mechanism involved that allowed the hyperoxic environment to compensate the embryo for the loss of half its pore area where the artificial pores could not, may be founded on three bases:

Firstly, the impact of the holes was locally limited (see chapter 3), as lateral gas movement under the shell was small, whereas the increase in oxygen partial pressure was more homogeneous. Holes in the aircell have shown to increase hatchability (Meir and Ar, 1996; Meir *et al.*, 1999; but see Visschedijk, 1968a), but their impact declines rapidly with distance (Meir *et al.*, 1999). Unfortunately, their impact on embryonic growth was not reported by these authors. Over the equatorial area, where shell conductance is at its lowest (Seymour and Visschedijk, 1988), the holes may have caused a greater change to local P_{O_2}, but their influence would have been locally more restricted than that of holes into the open space of the aircell.

The reduction of growth in the last quarter of incubation after wax/hole treatment is a similar response to that of embryos in low conductance eggs (Tullett and Deeming, 1982; Burton and Tullett, 1983), or in eggs with part of the shell covered (Tazawa *et al.*, 1971a), while embryos in eggs under hypoxic conditions (Xu and Mortola, 1989) may be affected earlier. These results argue that the increase in shell conductance caused by the holes could not be utilised sufficiently by the embryo in late incubation. However, average shell water vapour conductance of the experimental eggs was lower by 11% than that of the control eggs (102.8 mg H_2O d^{-1} kPa^{-1}), although the difference between the same-age experimental and control groups was significant only in the day 20 experiment. In undisturbed eggs with a natural variance in shell conductance, this difference may cause a similarly sized reduction of embryonic gas exchange and body mass at the end of incubation (Burton and Tullett, 1983). In the present study, this effect may have contributed to the difference between the treatments that were designed to test the effects of a diffusion/perfusion mismatch.

Secondly, oxygen loading of the blood was probably greater under increased P_{O_2} than under the holes (Metcalfe *et al.*, 1981). The increased P_{O_2} in the environment would lead to a P_{O_2} under the free shell of experimental eggs equal to the control only if there was no lateral movement under the wax, and the wax-half was not involved in respiration. However, some lateral movement of oxygen must have occurred. This would result in an average P_{O_2} under the free shell higher than in control eggs, which may allow for faster oxygenation and better oxygen uptake. In undisturbed eggs, blood saturation is 88% on days 10 to 16 of incubation, and it is reduced to 84.5% on day 18 (Tazawa and Mochizuki, 1976). This level may be elevated in hyperoxic eggs. Lateral diffusion would also increase oxygen uptake under at least part of the waxed area, which causes the effective respiratory exchange area to be larger than in the wax/hole treatment, and decreases the proportion of de-oxygenated blood from the waxed side mixing with oxygenated blood.

Lastly, it might be hypothesised that blood flow through the CAM may have been augmented more under hyperoxia than under normoxia (Ar *et al.*, 1991). However, the increase in blood flow observed by these researchers was not significant, and it was suggested the 16 day chicken embryo is an oxygen conformer. Further, chorioallantoic blood vessel density is known to decrease under hyperoxic conditions (Strick *et al.*, 1991), invagination of the capillary plexus is delayed (Burton and Palmer, 1992), and the diffusing capacity of the CAM is reduced (Temple and Metcalfe, 1970), suggesting that perfusion is reduced rather than increased under hyperoxic conditions. This makes a stimulatory effect of a hyperoxic environment unlikely and rather favours Ar and Mover's (1994) interpretation of guarding against too much oxygen.

5.4.4 Series C: Importance of treatment-timing

Changes in the timing of shell treatment had little effect on the development of the chicken embryo. The greatest impact was observed on the survival of the embryo after an early shell manipulation (days 1 to 7). This was probably related to the growth of the CAM. Embryos experience very low oxygen conditions during the third and fourth day of development, when the area vasculosa is still the only respiratory surface area (Lomholt, 1984). Only the area of shell directly in contact with the membrane contributes to gas exchange/oxygen uptake (Ackerman and Rahn 1980; Lomholt, 1984). Correspondingly, eggs waxed before on day 1 did not survive this bottleneck, when half of the upper egg surface (and thus probably half of the area vasculosa) was separated

from the air. From day 4, the commencement of turning must have caused the complete covering of the gas exchange area at times. Although bird embryos are capable of surviving anoxia for a limited time (Tran *et al.*, 1996; Akiyama *et al.*, 1999; Di Carlo and Litovitz, 1999), the repeated anoxic phase may have caused the high mortality. The periodic anoxia continued until day 7 while the CAM still covered only up to half of the egg (Ackerman and Rahn, 1980). The rise in survival rate with delayed shell treatment may be explained by the increased size of the CAM, which would reduce the amount of time in total anoxia and level the maxima and minima of oxygen availability.

Another explanation for the increased mortality of young embryos after early shell manipulation may be found the in the number of infections with pathogens. Although not investigated here, access of microbes to the embryo may have been facilitated by the drilling of holes in the shell, possible furthered by the wet shell membranes at that age (Kutchai and Steen, 1971; Seymour and Piiper, 1988). This explanation, if true, would imply that the loss of part of the respiratory surface area and the variability of regional oxygen partial pressure were not associated with the differences in embryonic survival.

Embryo mass, water content, and yolk reserves showed differences between control and experimental eggs in the later manipulated groups: embryo mass on day 15 differed in groups with shell treatment on days 8 to 12 (fig. 5.17), embryonic water content on day 8 (fig. 5.18), and yolk mass on day 10 (fig. 5.19). Earlier manipulated groups, however, showed no differences in these variables. These ostensible effects of the timing of shell treatment are probably a consequence of the higher mortality in the early manipulated groups, leading to a small sample size and the lack of statistical significance. It would rather appear that the timing of treatment had no strong influence on the development of the embryos. Further, CAM attachment does not appear to constitute a watershed of adaptability in CAM development. This would be supported by the lack of difference in the gas exchange of control and experimental groups before and after CAM attachment (fig. 5.21).

Although the timing of shell manipulation was negatively correlated with the water content in embryos, it is difficult to find an explanation for this relationship, as hole/control and wax/hole groups were similarly affected. It may mean that the earlier manipulation slowed the maturation of the embryos to some extent. As this included the control group, this cannot be a consequence of the altered shell conductance, but must be related to another aspect of the technique. Correspondingly, shell conductance did

not correlate with water content of the embryo or yolk. This was expected, as the water content in these compartments is highly regulated (Hoyt, 1979; Simkiss, 1980; Davis *et al.*, 1988).

5.4.5 Conclusion

The wax/oxygen treatment compensated the chicken embryo for the loss of half of its respiratory surface area. As this treatment concentrated on oxygen availability to the embryo, it supports the suggestion of Tullett and Burton (1987) that oxygen, not carbon dioxide is important for the regulation of embryonic growth.

In contrast, the wax/hole treatment restricted growth of the experimental embryos in late incubation. Their growth performance mirrored that of individuals with low shell conductance, as embryonic growth and respiration were only affected late in development (cf Burton and Tullett, 1983, 1985), rather then that of hypoxic embryos, where growth rates may be retarded earlier (Xu and Mortola, 1989).

The mechanism of how the normal growth until day 14 was achieved could involve increased chorioallantoic blood flow, but the increase is potentially only small (Ar *et al.*, 1991). An active increase of the chorioallantoic diffusing capacity, for example by an increase of vessel density or a reduction of the diffusion distance, was not observed. It appears that the development of the chorioallantoic circulation cannot be accelerated, as suggested by Metcalfe *et al.* (1979), and the normal growth of young wax/hole embryos was more likely the consequence of an over-capacity of the respiratory system. The undisturbed young embryo may not fully exploit its respiratory gas exchange capacity, as it maintained gas space P_{O_2} above the assumed minimum of late incubation (Tullett and Deeming, 1982; Rahn *et al.*, 1987). In experimental eggs, oxygen partial pressure averaged over the hole side of the eggs were reduced to levels below the norm at this stage of development, but within the physiological limits of the species (Vleck *et al.*, 1979; Tazawa *et al.*, 1988). This maintained oxygen flow across the shell, while its uptake into the blood remained at the control level until day 15. As the haemoglobin concentration and therefore the blood oxygen capacity (section 4.3.3.1) did not increase in experimental eggs during this period, the compensation for the altered shell conductance appeared passive, and the embryo appeared to rely rather on the appropriate adaptation of its shell conductance than on the adaptation of its circulatory system. As the possibly only active means of adaptation, it would be of interest to examine haemoglobin oxygen affinity, blood P_{O_2} and blood flow through the

chorioallantoic membrane more closely. This may help to explain how the embryos in the present study were able to maintain normal growth until day 20 (wax/oxygen treatment) or until day 15 (wax/hole treatment) despite the apparent lack of adaptation to the regional differences in shell conductance.

6. General Summary and Conclusion

Background

Previous studies have shown that eggshell conductance of the bird egg and blood flow in the underlying chorioallantoic membrane (CAM) can differ regionally, for example between the blunt end and the equatorial region of eggs. It was also observed that the perfusion of the CAM is changed in response to various conditions of barometric pressure and gas concentrations on the whole egg level.

My aim was to create a gradient of oxygen availability across the eggshell, and to test the ability of the chicken embryo to match the growth and the perfusion of the chorioallantoic membrane locally to the diffusive conductance of the eggshell that had been widely altered on a regional level. This study concentrated on vessel density, blood volume, and barrier thickness in the chorioallantoic membrane, as this is the respiratory organ during the phase of strongest growth of the embryo and in the final phase of incubation. The consequences for embryonic gas exchange and growth were observed to evaluate the ability of the embryo to compensate for the partial loss of its gas exchanger.

Oxygen gradient in the egg

The regional changes to the gas conductance of the eggshell were achieved by waxing half of the egg from pole to pole and enhancing shell conductance on the opposing side with the drilling of four to seven holes. The effect of this treatment on the shell conductance was evaluated by measuring the water loss rate of the eggs and the oxygen partial pressure (P_{O_2}) under the shell. A modified equilibration technique was developed to measure regional P_{O_2} under the shell by sampling gas in a 0.05 ml chamber sealed to the shell.

Lateral conductance for oxygen in the compound shell membranes was found to be very low, so that even the blockage of only a few pores would cause a substantial reduction of P_{O_2} under the shell. Consequently, the fall in P_{O_2} under the area of the equilibration chamber (0.25 cm radius) averaged 2.1 kPa through the second half of incubation. Shell treatment that altered regional shell conductance (wax/hole treatment) therefore created a very large gradient of oxygen partial pressure in the gas spaces of the egg. Oxygen partial pressure under the waxed half was reduced to 3.8 kPa in late incubation, reaching venous levels. The drilling of holes through the shell, designed to raise local P_{O_2} above that of the controls, succeeded for young embryos until day 14,

therefore covering the period of rapid vessel proliferation and maturation of the chorioallantoic membrane. Thereafter, however, Po_2 under the shell in the holes-area declined below control values.

Growth and vascularisation of the chorioallantoic membrane

To measure the growth of the surface area of the CAM, eggs were marked with a grid-system of longitudes and latitudes. On day 6 of incubation, they were waxed to one half on either the blunt end, the pointed end or one side. Eggs were frozen solid in liquid nitrogen on day 8 and day 10, the shell was pealed off and the outline of the membranes traced on a standardised Sanson-Flamsteed projection on paper. The relative surface area of the CAM was not reduced under the waxed side, except for the group waxed on the pointed end and opened on day 8. Relative surface area of the CAM on day 8 (56.3% ± 4.5%; mean and SD) and on day 10 (81.9% ± 7.8%) was not increased in comparison to literature values, indicating the inability of the embryo to accelerate chorioallantoic growth in eggs with a greatly reduced shell conductance. Chorioallantoic growth and development are probably already at their maximum in the normoxic, undisturbed embryo.

To evaluate the perfusion of the CAM under a gradient of oxygen availability, one half of each experimental egg was waxed while oxygen availability was increased under the free shell by incubation in a high oxygen environment (wax/oxygen treatment) or by drilling holes through the shell (wax/hole treatment). The effect of increasing oxygen in the environment on the CAM was similar to that of the holes, supporting the argument that oxygen is the main factor influencing CAM development.

The length-density of arterioles and venules (≤ 25 μm) in the waxed sections was reduced from days 10 to 15 under the wax side of experimental eggs by 15% to 33% in comparison to the free side. There was no difference between the values from the free side of experimental eggs and control eggs. The timing of shell treatment had no effect on the length density on day 15.

The density of capillaries in the CAM and the barrier thickness of the endothelial tissues were obtained using morphometric methods on transmission electron micrographs from CAM sections obtained on days 10, 12 and 14 of incubation. Capillary density declined under the wax in comparison to the free side, but there was no difference between the free side of experimental eggs and the control eggs. Capillary surface area of the control treatment ranged from 76% to 91% of the egg surface and did

not change over time. The harmonic mean thickness of the tissue barrier in the control treatment declined from day 10 (2.5 ± 0.9 μm) to day 14 (0.6 ± 0.2 μm). This process appeared to be accelerated in the experimental eggs compared to the control. The harmonic mean thickness of the plasma barrier did not change over time and averaged 0.15 ± 0.05 μm for all controls. It was greater under the wax (0.48 ± 0.60 μm) than under the free side (0.18 ± 0.10 μm).

Capillary volume did not change from day 10 to day 14. It was significantly reduced under the waxed side in comparison to the free side, which again did not differ from the control. As this volume was rather small in comparison to literature data and the capillary haematocrit greatly raised (62% to 74%), it is assumed that plasma was lost during the processing of the material. However, haematocrit values were reduced in the wax samples, possibly indicating the lowered perfusion of these microvessels. Capillary diameter and the thickness of the endothelial tissue were somewhat increased under the wax, the structure of the epithelial layer was less well defined and the number of leucocytes was increased, indicating a general change to the tissue under the wax. Total chorioallantoic blood volume increased in undisturbed control eggs from days 10 (0.20 ± 0.09 ml) to 16 (0.26 ± 0.06 ml). The values obtained from parts of the CAM after a quick freeze in liquid nitrogen were very similar, indicating that cutting the frozen tissue did not cause a large loss of blood from the vessels. Similar to the control eggs, blood volume in the CAM of experimental eggs increased over time. Blood volume under the wax was frequently raised above the values from the free side, which in turn did not differ from the control eggs. However, haemoglobin concentration in a group of wax/hole eggs (4.96 ± 0.92 g/100ml) was lower than in the control eggs (5.80 ± 0.44 g/100ml) on day 15. The timing of the shell treatment had no effect on blood volume on day 15 or the difference between waxed and hole side.

The chicken embryo appeared unable to adapt its chorioallantoic circulatory system to the oxygen gradient created by wax/oxygen and wax/hole treatment, to match its perfusion to the diffusion through the shell. While the decline of the vessel density under the waxed area may constitute an adaptation to the limited oxygen availability in this area, it did not go far enough to match perfusion with diffusion, as the latter was reduced to almost zero.

Growth and respiration of the embryo

Although there was no observation of adaptation under the free side to compensate for the waxed side, the changes to the shell permeability had little impact on the young embryo. The maturation of the embryos was not affected, and no additional costs of development were incurred. The high oxygen environment compensated the embryo fully for the loss of half of its respiratory surface area. No differences were observed between the experimental and the control eggs in either embryo growth or respiration at any time in the second half of incubation. In the wax/hole experiments, a difference between wet and dry mass of embryos in control and experimental eggs was observed earliest on day 15. From day 16, embryonic respiration was reduced by between 12% to 21% in the experimental eggs. Oxygen consumption was found to correlate with overall shell conductance in these experimental eggs, but not in the control eggs, highlighting the metabolic limitation to experimental embryos in the last days of incubation that was probably caused by the low lateral diffusion under the shell and the consequently low effect of the holes.

Conclusion

It was found that the respiratory system of the bird embryo can adapt to local differences in the shell conductance in a limited way: it appears that blood flow in the CAM can be down-regulated, but not increased locally. This could indicate that compensatory vessel growth is not necessary nor possible under natural conditions. As early embryos did not reduce gas exchange and growth, while there was no apparently active adaptation to the altered shell conductance, the respiratory system of the bird embryo appeared to possess inherent reserves to compensate passively for changes to oxygen availability, at least during the earlier stage of incubation. Adaptation and compensation may not have been needed at this stage, when vascular proliferation was still active. Late in incubation oxygen uptake suffered in wax/hole eggs, but as the vessel growth phase was over, the options to compensate for partial loss of the gas exchanger may have been limited. Rather than adapting its respiratory organ, the bird embryo may be relying on the appropriate adaptation of its shell conductance.

Future research directions

In absence of a change to the chorioallantoic diffusing capacity under the free shell, the mechanism by which oxygen uptake under the free side was increased to compensate

for the lack of uptake under the wax remains unclear. The early normal growth of the wax/hole embryos and their failure to increase haemoglobin concentration and haematocrit up to day 15 may indicate that these embryos did not suffer a whole system hypoxia in the presence of a diffusion/perfusion mismatch.

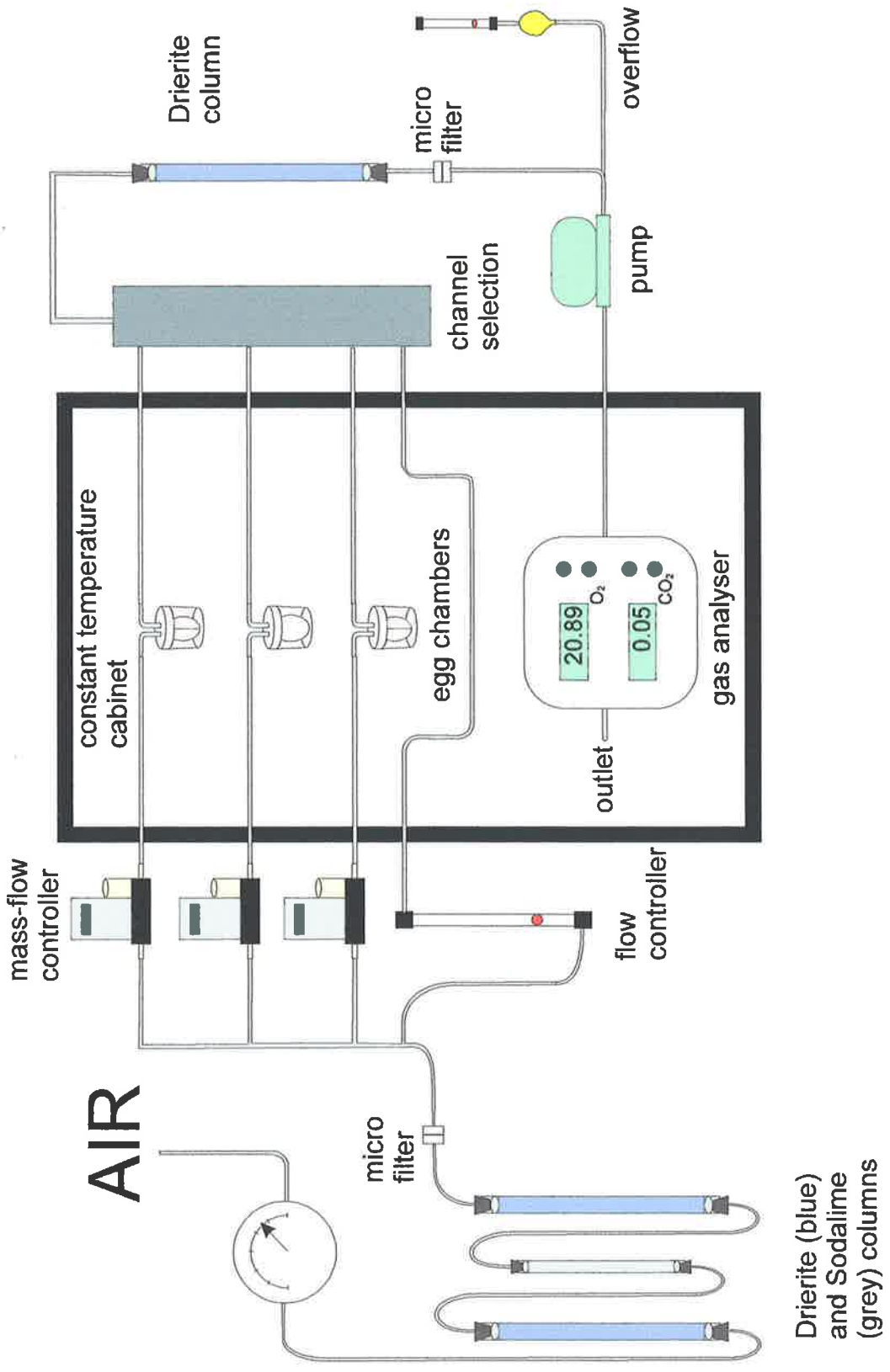
Average P_{O_2} in the gas spaces on the hole side of the eggs was reduced, and oxygen diffusion across the shell was therefore increased. However, the reduction of P_{O_2} under the shell would imply that a full oxygen saturation of the blood could not be obtained, unless the oxygen affinity of the haemoglobin was increased. Further, oxygen flow across the inner barrier would decrease according to Fick's equation, as the diffusing capacity of the chorioallantoic membrane was not increased in the treatment. Oxygen uptake under these conditions could only be maintained if the embryo lowered average blood P_{O_2}. This may be accomplished by lowering venous P_{O_2}, or by reducing the transit time of the blood through the capillary plexus. As the oxygen saturation of haemoglobin does not change linearly with P_{O_2}, a reduction of blood P_{O_2} may reduce oxygen content proportionally less. Further, the haemoglobin oxygenation velocity declines with increasing saturation of the haemoglobin, so blood oxygen loading may be reduced by a smaller factor than the transit time, which increases oxygen transport in proportion to the blood flow.

The present work investigated the adaptive capacity of the circulatory system in the chicken CAM and showed that this organ has little morphological adaptability to local changes in shell conductance and oxygen availability. There were indications that research into the short-term adaptability of the chorioallantoic membrane of bird eggs, i.e. the flexibility of blood flow rates and transit times in the CAM, may yield rich results that help to explain observations in the present study. Changes to the blood flow through the organ would offer the embryo a way to react to short-term blockage of the egg surface, for example in contact with the brood patch of the brooding parent. Future research may find that the oxygen availability in the area of the egg in direct contact with the metabolically active skin of the brood patch is reduced. In the early phase of chorioallantoic growth, the CAM is limited to this relatively hypoxic area, as it is floating directly under the brood patch. This may be one reason why the growth of the gas exchanger is at maximum under natural conditions. When the CAM covers the egg completely, the shell surface area away from the brood patch offers better gas exchange opportunities, and blood flow may be directed accordingly.

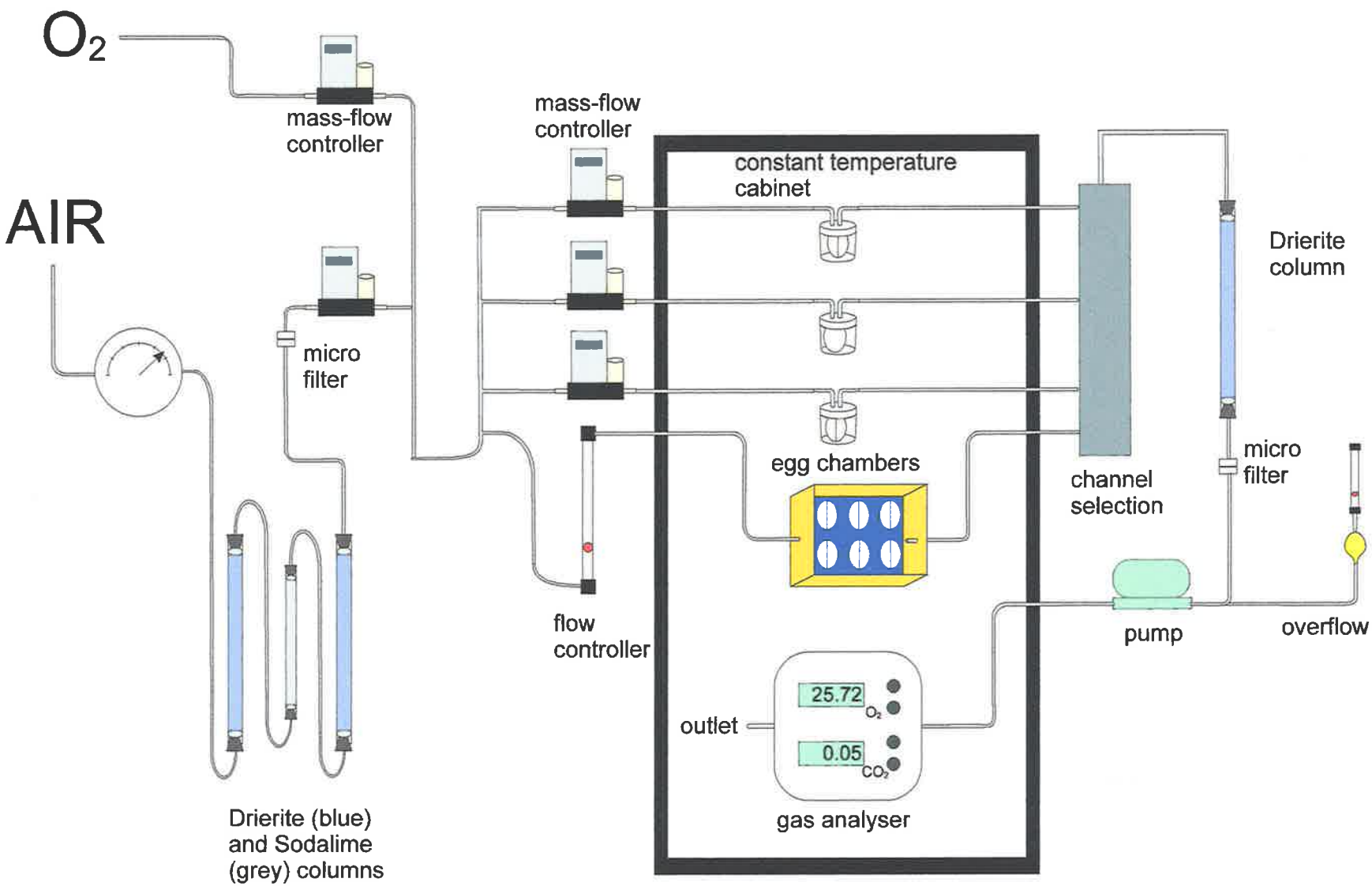
7. Appendices

7.1 Respirometry system

Normoxic configuration



Hyperoxic configuration



7.2 Solutions for fixation and staining

7.2.1 Light microscopy - fixation

0.1 M Phosphate Buffered Saline (PBS), pH 7.4

- Solution 1 (0.2 M)
  28.4 g Na_2HPO_4 anhydrous dissolved in 1 l distilled water
- Solution 2 (0.2 M)
  31.21 g $NaH_2PO_4.2H_2O$ dissolved in 1 l distilled water
- Add enough distilled water to 202.5 ml solution 1 and 47.5 ml solution 2 to make up 500 ml.

2.5% Glutaraldehyde

- add 10 ml of 25% glutaraldehyde to 90 ml PBS
- refrigerate
- solution won't last for much more than one week - make up new!

➔ take tissue sample, still attached to shell (will curl up otherwise), and fix in 2.5% glutaraldehyde solution for $2 < x < 24$ hr (best: 3-4 hr) - the longer fixation, the more difficult to separate tissue from shell afterwards.

➔ rinse tissue with PBS and distilled water, each for 10 min. Float in distilled water and separate CAM from shell using fine forceps.

7.2.2 Light microscopy - staining

Haematoxylin

- 4 g Haematoxylin
- 80 g Ammonium Alum (Aluminium Ammonium Sulphate)
- 100 ml absolute alcohol
- 800 ml distilled water
- 0.2 g/l sodium iodate (ripener)

➔ dissolve alum in water by heating. Dissolve haematoxylin in alcohol and add to alum solution (before adding to alum, take off the heat and add alcohol slowly)

➔ bring mixture rapidly to the boil, take off heat, cool, and add sodium iodate

Eosin

- stock I: 1 % Eosin Y in water
- stock II: 1 % Phloxine B in water

➔ Eosin staining solution:

- 100 ml stock I
- 10 ml stock II
- 750 ml of 95 % ethanol
- 4 ml acetic acid

➔ mix

Staining timetable for samples

- 3 min dist. water
- 2 min haematoxylin
- wash in tap water
- 1 min ammonia water (0.5%)
- wash in tap water
- quick dip into 1 % HCl
- wash quickly in tap water
- 1 min ammonia water (0.5%)
- wash in tap water
- 1 min eosin
- wash in tap water
- 2 min abs. alcohol
- 1 min abs. alcohol
- 1 min abs. alcohol
- 3x5 min Histoclear

➔ coverslip with DePeX (Gurr ®, BDH Chemicals, Kilsith, Victoria, Australia) mounting medium

7.2.3 Transmission electron microscopy – processing tissue samples

➔ First Fixation in 2.5% glutaraldehyde in 0.1 M Phosphate buffer as in section 7.2.1

➔ rinse tissue with PBS and distilled water, each for 10 min. Float in distilled water and separate CAM from shell using fine forceps.

➔ cut sample for TEM (no more than 1x2 mm) and transfer back into glutaraldehyde solution.

➔ Post-fixing in OsO_4

Millonigs Osmium Tetroxide

- 10 ml phosphate buffer
- 2 ml dist. water
- 0.1 g glucose
- 0.1 g OsO_4

➔ clean dark bottle and all material used with alcohol. Don't touch anything with bare hands. Make up with chilled solution. Score ampullae with diamond pencil, place in bottle of other components, shake vigorously till ampullae breaks.

➔ leave in cold till dissolves (24h). Store cold.

➔ post fix (in 1% Osmium Tetroxide in phosphate buffer) at 4°C for 1 hr.

➔ rinse twice in phosphate buffer at 4°C.

From here after: use plastic pipettes to avoid glass splinters near tissue.

Dehydration and embedding of samples

➔ rinse samples in 50% alcohol.
➔ wash samples in

	for	
50% alcohol	10 min	
70%	10 min	
95%	10 min	
100%	10 min	
100% over $CuSO_4$	10 min	2x
100% alcohol:propylene oxide 1:1	10 min	
propylene oxide	15 min	2x
propylene oxide: Taab resin 2:1	overnight*	
propylene oxide:Taab resin 1:2	6 - 8 h*	
resin	20 h	

(* time can be extended in fridge)

➔ embed in fresh resin (TAAB ® resin embedding kit T004; TAAB Laboratory Equipment, Great Britain).

➔ in the moulds, allow a thin bottom layer of resin to settle for 3 h at 60°C.

➔ fill up mould with resin and transfer samples. Lay out samples flat and smooth.

➔ polymerise in oven at 60°C for 48 h.

7.2.4 Transmission electron microscopy – cutting and staining of sections

7.2.4.1 Thick sections

➔ trim the hardened resin blocks with razor blades to from a trapezium shaped cone around the specimen.

➔ cut thick sections (0.5 mm) of specimen with a glass knife. Stain sections with Adam Lockert method.

Sol. 1: Methylene Blue Stain

- Methylene Blue 0.13 g
- Azure II 0.02 g
- Clycerol 10 ml
- Methanol 10 ml
- 0.15 M phosphate Sörensen buffer pH 6.8 30 ml
- Dist. Water 50 ml

Sörensen Phosphate Buffer pH 6.8

Sol. A:

- Na_2HPO_4 $2H_2O$ 1.1876 g
- Dist. Water 100 ml

Sol. B:

- KH_2PO_4 0.908 g
- Dist. Water 100 ml

➔ mix 49.2 ml of Sol. A with 50.8 ml of Sol. B for pH 6.8

Sol. 2:

- Basic Fuchsin 0.125 g
- Dist. Water 50 ml

Sol. 3:

- Sodium Tetraborate 0.125 g
- Dist Water 50 ml

➔ Working Fuchsin stain: Mix equal volumes of Sol. 2 and 3. Does not keep more than 3 h. Make up in drops.

➔ dry sections on slide over hot plate. Stain for 3 min with Methylene Blue (Sol. 1) on hot plate, rinse with dist. water and dry.

➔ stain for 2 min with working Fuchsin stain on hot plate, rinse with dist. water. Wipe excess water with filter paper and dry slides quickly on plate. Mount with DePeX (Gurr â, BDH Chemicals, Australia Pty. Ltd. Kilsyth. Vic. 3137) and cover slip. Stain is not permanent (2-3 months). Store in cool, dark place.

7.2.4.2 Thin sections

➔ select useful samples from thick sections under light microscopy.

➔ re-trim the blocks.

➔ cut thin sections (pale gold) from these blocks with a diamond knife and mount on copper/palladium grids (mesh size 200).

➔ stain grids with Uranyl Acetate and Lead Citrate.

Uranyl Acetate

- make up fresh before use.
- saturate uranyl acetate (ca ¾ ml powder) in 10 ml of 70% alcohol.
- leave on rotator for about 1 h to dissolve.
- centrifuge on high for 15 min.
- millepore filter

➔ place a few drops of solution onto dental wax "boats" in petri dish with some 70% alcohol.

➔ float grids face down on drops. Stain for 20 min in dark.

➔ dip grids 8-10 times each into one beaker with 70% and three beakers with dist. water and dry on filter paper.

Lead Citrate

- Lead nitrate 1.33 g
- sodium citrate 1.76 g
- dist. water 30 ml
- mix in 50 ml volumetric flask: shake vigorously for 1 min and intermittently for 30 min.
- add 8.0 ml of fresh 1 N NaOH and dilute to 50 ml with dist. water and mix by inversion. Stain (pH 12.0) is stable for 6 months.
- centrifuge for 15 min on high before use.

➔ place 10 to 20 pellets of NaOH to one side into a petri dish.

➔ pipette a few drops of the stain into the other side of the dish and float on the grids.

➔ stain for 15 min in the light.

➔ dip grids 8-10 times each into four beakers with dist. water and dry on filter paper.

7.3 Photometry

7.3.1 Haemoglobin Reagent - Drabkin's solution

- 1.0 g sodium bicarbonate
- 0.200 g potassium ferricyanide
- 0.050 g of potassium cyanide

➔ dissolve in enough distilled water to make one litre. Store in a brown bottle out of direct light.

7.3.2 Model by Lothian (1958)

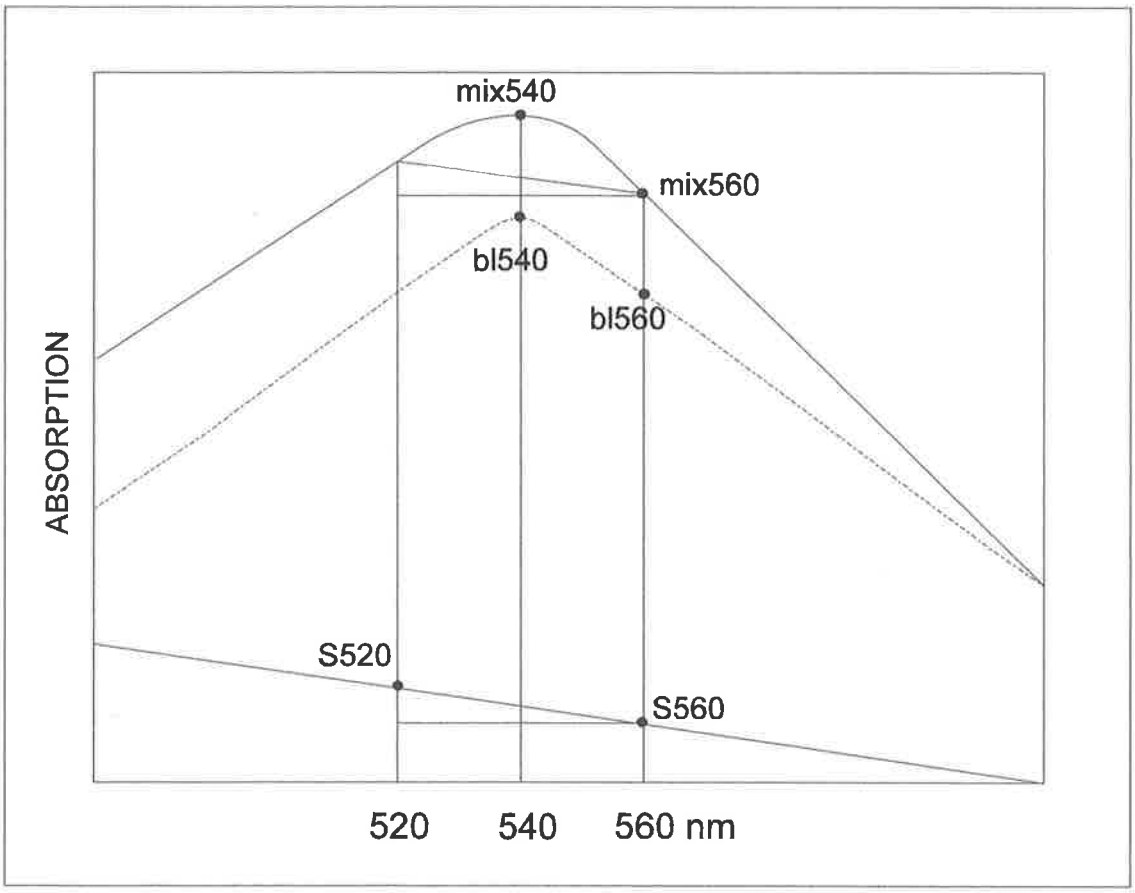
Definitions

bl_λ = absorption of pure blood at wavelength λ

mix_λ = absorption of mixed solution (CAM) at wavelength λ

S_λ = absorption of irrelevant solutes at wavelength λ

r = ratio of pure blood absorption at maximum (540 nm) over shoulders (520 nm and 560 nm)



➔ absorption measurement at a wavelength of 540 nm yields a local maximum of absorption for the blood (cyanomethaemoglobin) and for the mixed solution of blood and other chorioallantoic solutes.

➔ absorption measurements at equal distance yield equal absorption values for pure blood at 520 nm and 560 nm.

r is defined as the ratio of pure blood absorption at maximum (bl_{540}) over shoulder (bl_{560}) and is obtained from pure blood samples:

$$r = bl_{540}/bl_{560} \quad (7.1)$$

In the CAM tissue samples, bl_{540} and bl_{560} are unknown and need to be calculated from the absorption of the mixed solution (mix_{540} and mix_{560}). The difference between mix_λ and bl_λ is equal to the absorption of irrelevant solutes and defined as S_λ, so that

$$bl_\lambda = mix_\lambda - S_\lambda \quad (7.2)$$

and

$$r = (mix_{540} - S_{540}) / (mix_{560} - \mathbf{S_{560}}) \quad (7.3)$$

$$S_{540} = S_{560} + (S_{540}-S_{560}) \quad (7.4)$$

And therefore

$$r = (mix_{540} - (\mathbf{S_{560}} + (S_{540}-S_{560})) / (mix_{560} - \mathbf{S_{560}}) \quad (7.5)$$

Because $bl_{520} = bl_{560}$ and $S_\lambda = mix_\lambda - bl_\lambda$ (equation 7.2), and because the absorption of the irrelevant solutes is assumed to change linearly with λ,

$$S_{540} - S_{560} = ½ (mix_{560} - mix_{520}) \quad (7.6)$$

We can therefore solve equation (7.5) for S_{560}:

$$\mathbf{S_{560}} = (mix_{540} - ½ (mix_{560} - mix_{520}) - r \times mix_{560}) / (1 - r) \quad (7.7)$$

This allows the calculation of S_{540} from combining equations (7.4) and (7.6), so that

$$S_{540} = S_{560} - ½ (mix_{560} - mix_{520}) \quad (7.8)$$

Finally, bl_{540} is calculated as the difference of mix_{540} and S_{540}, and compared to the regression of blood absorption over blood concentration that was established for pure blood samples.

For example, on day 15 r = 1.166 and the regression established for a solution of blood in 1.2 ml Drabkin's solution was $bl_{540} = 0.0343 \times$ [µl blood] ($R^2 = 0.988$).

7.4 Relationship between oxygen uptake and Po2 under the shell

The relationship between oxygen uptake in the chorioallantoic membrane ($\dot{M}_{O2}$) and P_{O_2} under the shell, required for the calculation of K_{O_2} in the proposed model for lateral diffusion, was described with a regression equation based mainly on empirical data. Area-specific $\dot{M}_{O_2}$ on day 19 in the present study was 0.31 mmol d^{-1} cm^{-2}. At the same day, oxygen partial pressure under the free shell averaged 15.4 kPa, so that the maximum point for the regression was (15.4/0.31). As the minimum P_{O_2} measured under the wax was 3.8 kPa in late incubation and probably represented venous level, second data point (3.8/0) was established. Further points were calculated from Tazawa *et al.* (1992), who observed a 25% reduction of $\dot{M}_{O_2}$ after one hour in 15% oxygen environment, and a 57% reduction of $\dot{M}_{O_2}$ in 10% oxygen on day 18. Using the $\dot{M}_{O_2}$ and P_{O_2} under the free shell as observed in the present study, an average area-specific oxygen conductance of the eggshells was calculated in Fick's equation:

$$0.31 \text{ mmol } d^{-1} \text{ cm}^{-2} = G_{O_2} \times (19.8 \text{ kPa} - 15.4 \text{ kPa})$$
$$G_{O_2} = 0.070 \text{ mmol } d^{-1} \text{ cm}^{-2} \text{ kPa}^{-1}$$

This value was inserted into further calculations using 75% and 43% of $\dot{M}_{O_2}$ at 15% and 10% oxygen, respectively, to calculate P_{O_2} under the shell in these environments:

$$0.31 \times 0.75 = 0.070 \times (14.2 - P_{O_2}\text{in})$$
P_{O_2}in at 15% oxygen = 10.9 kPa

$$0.31 \times 0.43 = 0.070 \times (9.5 - P_{O_2}\text{in})$$
P_{O_2}in at 10% oxygen = 7.6 kPa

Oxygen partial pressures below 3.8 kPa could not occur naturally, if venous P_{O_2} is equal to 3.8 kPa. However, as they occurred in the first step of the model, further data points were introduced to flatten the curve for low values, where $\dot{M}_{O_2}$ was zero for P_{O_2} of 0, 1, 1.9 and 2.5 kPa.

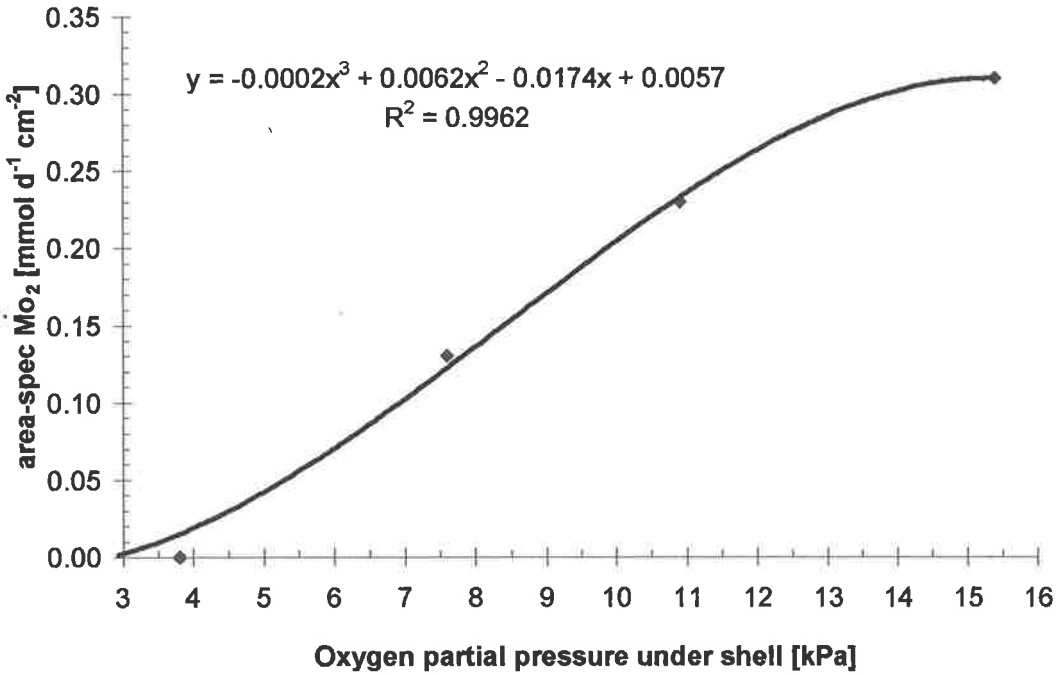
Data points

P_{O_2} [kPa]	$\dot{M}_{O_2}$ [mmol d^{-1} cm^{-2}]	based on
3.8	0.0	measurement (Fig. 3.4)
15.4	0.31	extrapolation (Fig. 3.6)
7.6	0.13	Tazawa et al. (1992)
10.9	0.23	Tazawa et al. (1992)

Regression equation

$R^2 = 0.996$

$\dot{M}_{O_2} = -0.000244\ P_{O_2}{}^3 + 0.006170\ P_{O_2}{}^2 - 0.017433\ P_{O_2} + 0.005674$



Change of area-specific oxygen consumption rate in relation to the P_{O_2} under the shell.

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