Supplementary Material

Supplementary Data

3.1 *In ovo* O_2 measurements during incubation using microelectrodes

- Experimental setup is depicted in **Supplementary Figure 1**.
- O_2 concentrations from within the egg gas cell, obtained during incubation of unfertilized eggs can be found in **Supplementary Data Table 1**.
- O_2 concentrations from within the egg gas cell, obtained during incubation of fertilized eggs (and their EggID) can be found in **Supplementary Data Table 2**.
- Scripts (MatLab) used for performing the calculations are submitted as 'Scripts for O2 dynamics incubation [eggID]'. The [eggID]:s are NE3, NE4, NE5, NE6, NE7 and PN2.

3.2 HIF protein levels

- The calculations of fold-change were performed as follows: Densitometry was performed on bands corresponding to target proteins HIF-1α, HIF-2α, and the loading control β-Actin for each sample. Normalized intensity values were generated by dividing target protein intensity by loading control intensity. Fold change was subsequently calculated by dividing normalized intensities across samples. Fold change values were averaged across three independent replicates.

3.3 Exposing early avian development to normoxia

- Experimental setup is depicted in **Supplementary Figure 2**.
- Additional images of the morphological state of embryos in the experiment is found in **Supplementary Figures 6-7**.
- Data from the experiments with drilled holes at 21% can be found in **Supplementary Data Table 3**.
- Data from the experiments at hyperoxia (40%) and hypoxia (10%) can be found in **Supplementary Data Table 4**.

3.4 Quantification of the O_2 diffusion coefficient of eggshell

3.4.1 Direct quantification of D_{O2} of eggshell using microelectrodes

- Experimental setup is depicted in **Supplementary Figures 3-4**.
- O_2 concentrations in the egg gas cell, obtained during manipulations of surrounding O_2 concentrations (in the bell jar) in unfertilized eggs can be found in **Supplementary Data Table 5**.

- Scripts (MatLab) used for performing the calculations are submitted as 'Scripts for O2 dynamics incubation [eggID]' and 'Scripts for geometry evaporation etc. The [eggID]:s are NE3, NE4, NE5, NE6, NE7 and PN2.

For these comparisons, a $K(O_2)$ value is available, and G = K x A. In these publications the K value has been determined for when half the ambient $P(O_2)$ has been reached. With unit conversion, we can then similarly as for the indirect above calculate a $D(O_2)$ value. However, since A is now both in the numerator of the $D(O_2)$ equation (from the G term derived from K) and in the denominator we can remove it. Instead of the molecular weight (M) for water, $M(H_2O)$, we use $M(O_2)$. Since the K value is stated in seconds already, there is no need to convert to days (which was in in the units of G previously).

Note that Kayar et al. 1981 performed their experiments at approximately 1600 m, which gives a pressure of approximately 720 torr instead of 760 torr.

$K(O_2)$ (cm^3 O_2 STP sec^{-1} cm^{-2} $torr^{-1}$)
Molar volume of a gas at STP = 22.4 L
$M(O_2)$ = 31.988 g $moles^{-1}$

Unit conversion of K:

$$K = \frac{K(STP) \times 0.001 \times 31.988}{22.4} \quad (g\ sec^{-1}\ cm^{-2}\ torr^{-1})$$

Since G = K x A:

$$\frac{K \times \mathrm{A} \times \Delta \mathrm{P(O2)}}{M(O2) \times 0.0001} = \frac{\Delta N}{\Delta t}\ (moles\ sec^{-1}) \times A$$

$D(O_2)$ can then be calculated:

$$D(O2) = \frac{\Delta N \times A \times z}{\Delta t \times A \times \Delta C} = \frac{\Delta N \times z}{\Delta t \times \Delta C}$$

Publication	K x 10^{-6} (cm^3 O_2 STP sec^{-1} cm^{-2} $torr^{-1}$)	ΔP(O2) (torr)	Temp. (°C)	Calc. ΔN/Δt x 10^{-6} (moles sec^{-1})	Calc. ΔC (moles m^{-3})	Calc. $D(O_2)$ x 10^{-8} (m^2 sec^{-1})
Kayar et al. 1981	0.12	67.5	37	3.62	3.68	z_1: 0.03 z_2: 0.04
	0.94	67.5	37	28.33	3.68	z_1: 0.23 z_2: 0.31
Wangensteen et al. 1970/71	3.2	75	37	107.14	3.88	z_1: 0.83 z_2: 1.11

3.4.2 Indirect quantification of D_{O2} of eggshell based on water vapor

- Egg parameters and weight loss over time can be found in **Supplementary Data Table 6**.
- Temperature and RH% during the evaporation experiments (experiments 6 and 7) can be found in **Supplementary Data Table 7**.
- Empirical data over the increase in gas-cell volume over incubation can be found in **Supplementary Data Table 8**.
- Weight loss over time and eggs is plotted in **Supplementary Figure 8**.
- Scripts (MatLab) used for performing the calculations are submitted as 'Scripts for geometry evaporation etc.'

Notes on the conversions between conductivity of water to D_{O2} values:

Step 1: Convert $G(H_2O)$ to $\Delta N/\Delta t$ (moles sec^{-1})

$$\frac{G(H2O) \times \Delta P(H2O)}{1000 \times Mw(H2O) \times 86400} = \frac{\Delta N}{\Delta t}\ (moles\ sec^{-1})$$

M(H2O) = 18.015 g $moles^{-1}$

Δt = 1 day = 86400 sec

Step 2: Convert $\Delta P(H_2O)$ to $\Delta C(H_2O)$ (moles m^{-3})

Convert torr to atm => torr x (1/760) = atm

Convert °C to K => °C + 273.15 = K

Ideal gas law => pV=nRT

R = 8.206×10^{-5} (m^3 atm $mole^{-1}$ K^{-1})

$$\frac{n}{V} = \frac{\Delta P(H2O) \times \left(\frac{1}{760}\right)}{RT} = \Delta C\ (moles\ m^{-3})$$

Step 3: Calculating $D(H_2O)$ (m^2 sec^{-1})

$$D(H2O) = \frac{\Delta N \times z}{\Delta t \times A \times \Delta C}$$

A = publication value (converted to m^2) OR 0.0068 m^2

z_1 = 0.0003 m OR z_2 = 0.0004 m

Publication	G(H2O) (mg day^{-1} $torr^{-1}$)	ΔP(H2O) (torr)	Temp. (°C)	Eggshell area (cm^2)	Calc. ΔN/Δt x 10^{-7} (moles sec^{-1})	Calc. ΔC (moles m^{-3})	Calc. D(H2O) x 10^{-8} (m^2 sec^{-1})
Paganelli et al. 1975	10.67	23	24.5	66.4	1.58	1.24	z_1: 0.58 z_2: 0.77
Seymour & Visschedijk 1988	12.7	23	25	68	1.88	1.24	z_1: 0.67 z_2: 0.89
Ar et al. 1974	14.36	23.76	25	68	1.88	1.24	z_1: 0.76 z_2: 1.01

Rahn & Paganelli 1990	1.92	21	37	68	0.26	1.09	z_1: 0.11 z_2: 0.14

3.5 Determining O_2 consumption

- Files with data from estimations of the O_2 influx over incubation can be acquired from corresponding author.
- Scripts (MatLab) used for performing the calculations are available and can be acquired from the corresponding author.

Supplementary Figures

Figure S1. Oxygen-sensing setup for fertilized eggs. **A)** Drilling of a hole at the blunt end of the egg. **B)** Aiming the microsensor, using the micromanipulator. **C)** Microsensor in place, with its tip in the air cell. **D)** Incubation under a Styrofoam bell. **E)** Calibration through small holes, injecting clean air or N_2, prepared at 37°C and 100% humidity. **F)** Post-measurement inspection of embryo.

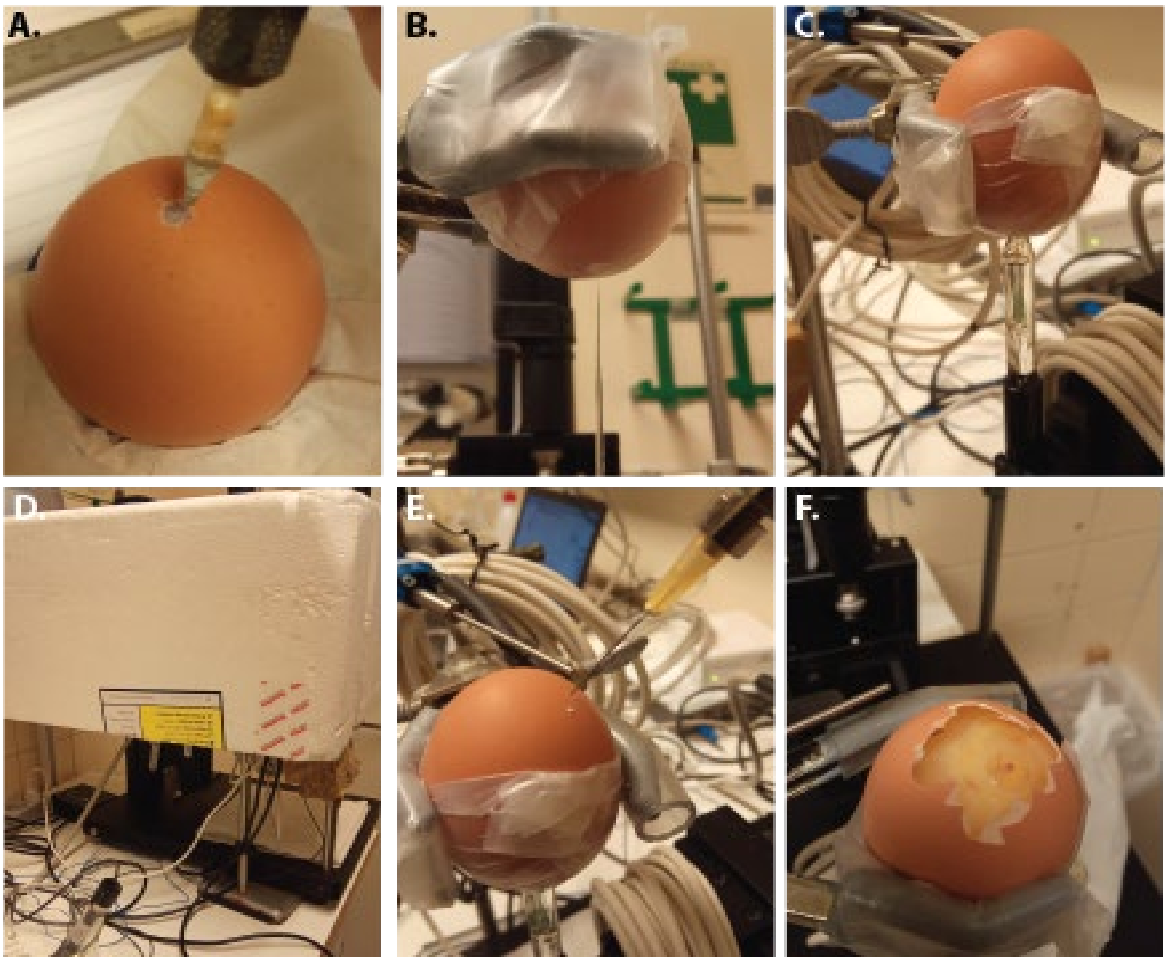


Figure S2. The incubation experiment under full access to normoxia (21%), where eggs are perforated.

Figure S3. The experimental set-up for direct determination of the oxygen transport coefficient for eggshell. A) The setup with microsensor entering from below, at the pointy end of the egg. B) The setup with a switch for air and N_2. C) For calibration, two holes were drilled through the eggshell and into the air cell. D) For calibration, air and N_2 gas were injected into the air cell while the microsensor was still in place. E) Opening to the air cell after measurements, noting air cell dimensions. F) Position of the tip of the microsensor, below the eggshell surface.

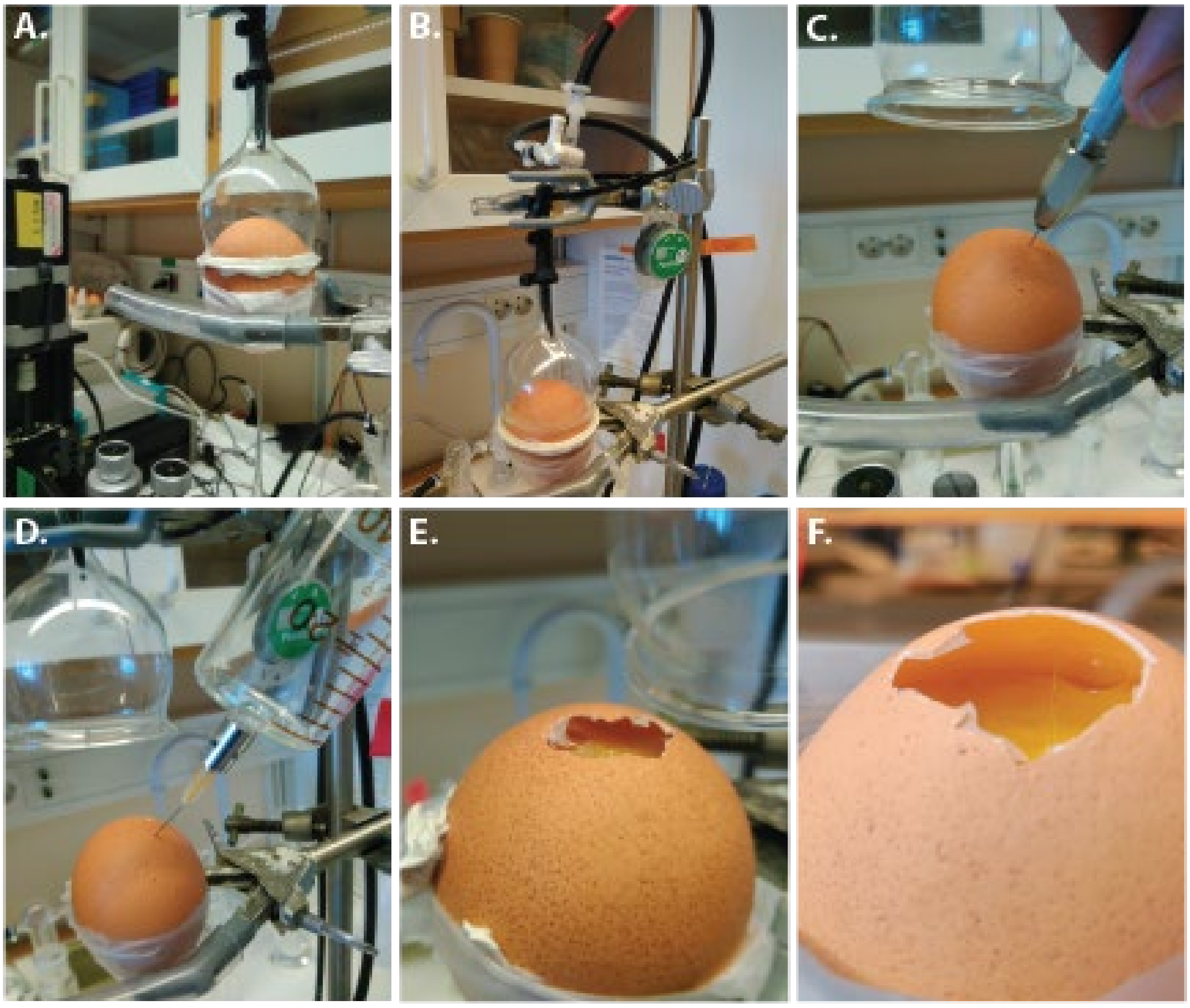


Figure S4. The experimental set-up for direct quantification of the D_{O2} of eggshell. **A)** Schematic view on the setup. **B)** The microsensor enters the egg from below and a bell jar (with N_2/air) extends below the gas cell inside the egg. Egg dimensions are noted as width (w), total height (h), and height from the widest part of the egg to the pointy end (c).

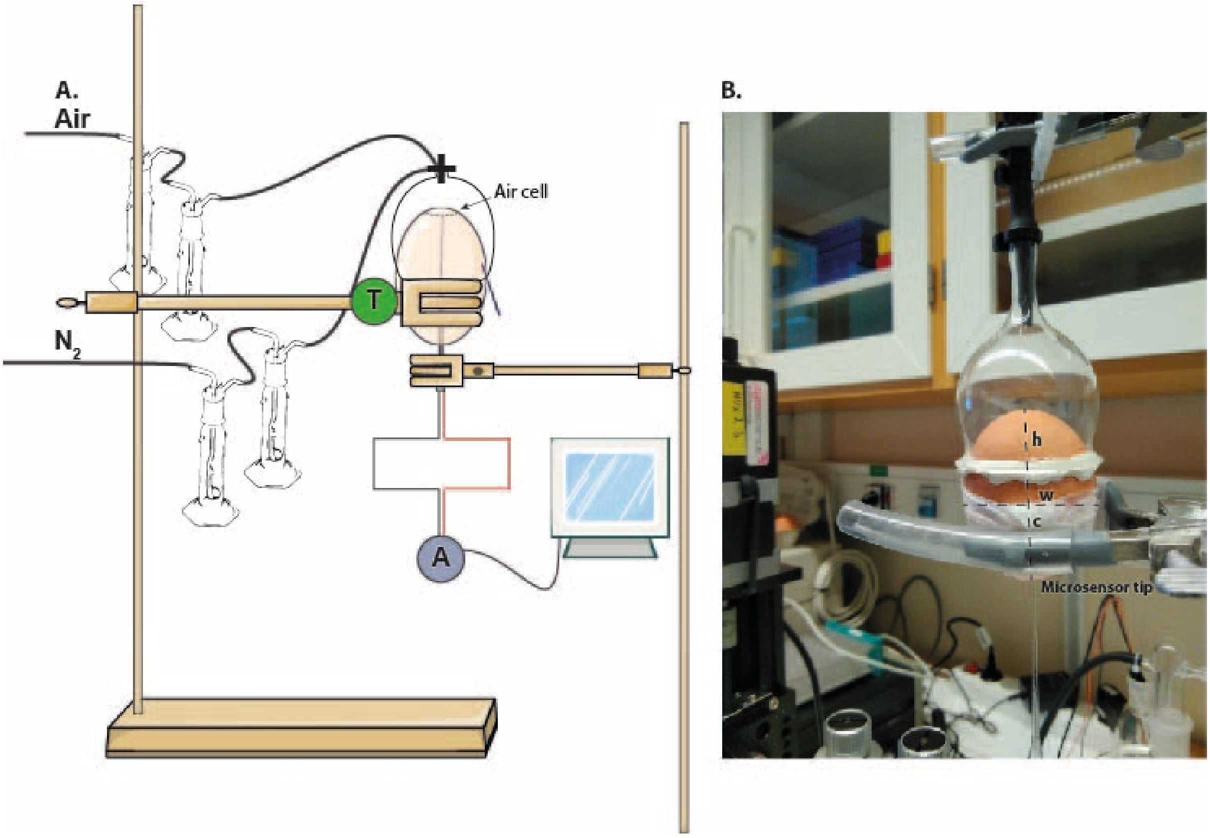


Figure S5. Oxygen concentrations in the gas cell of unfertilized eggs were in equilibrium with air before and during incubation at 37.5°C.

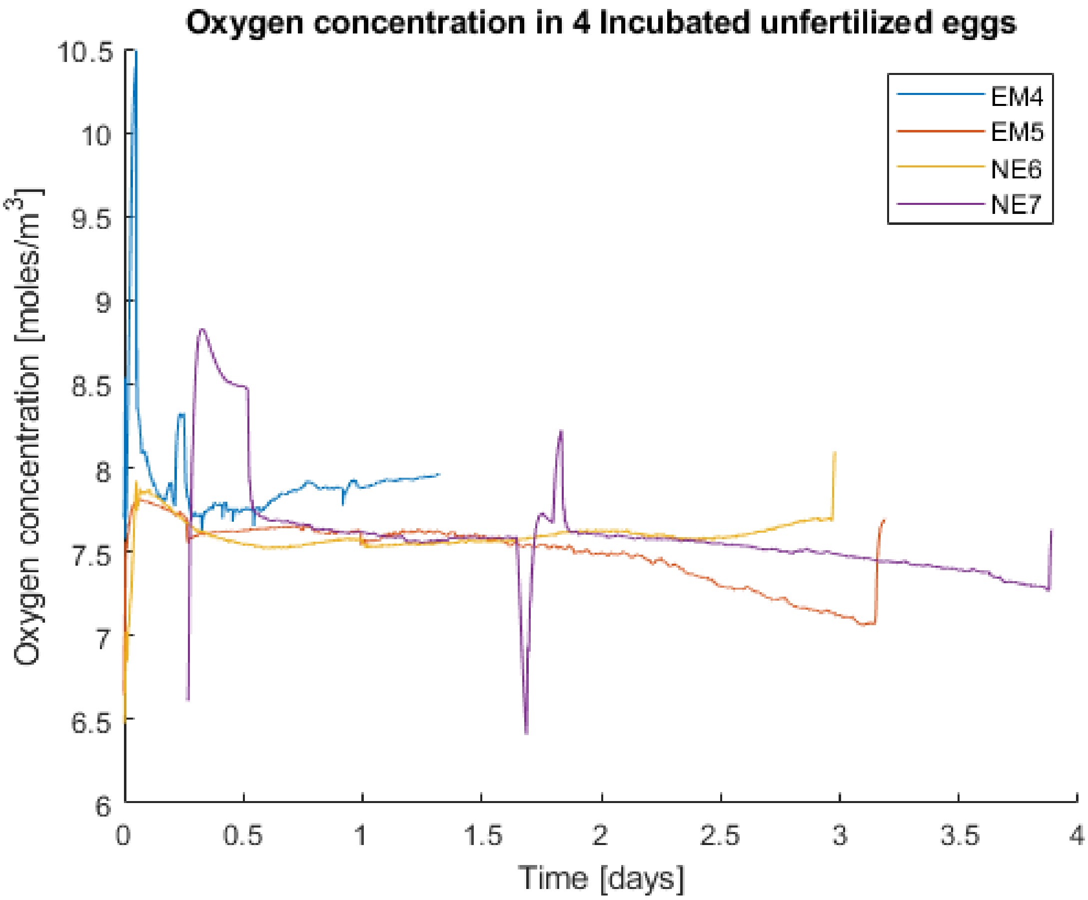


Figure S6. Images of incubation experiment under normal conditions. Examples of embryos at E3, after 3 days of incubation.

Figure S7. Images of incubation experiment under full access to oxygen. Examples of embryos at E3, after 3 days of incubation under full access to oxygen.

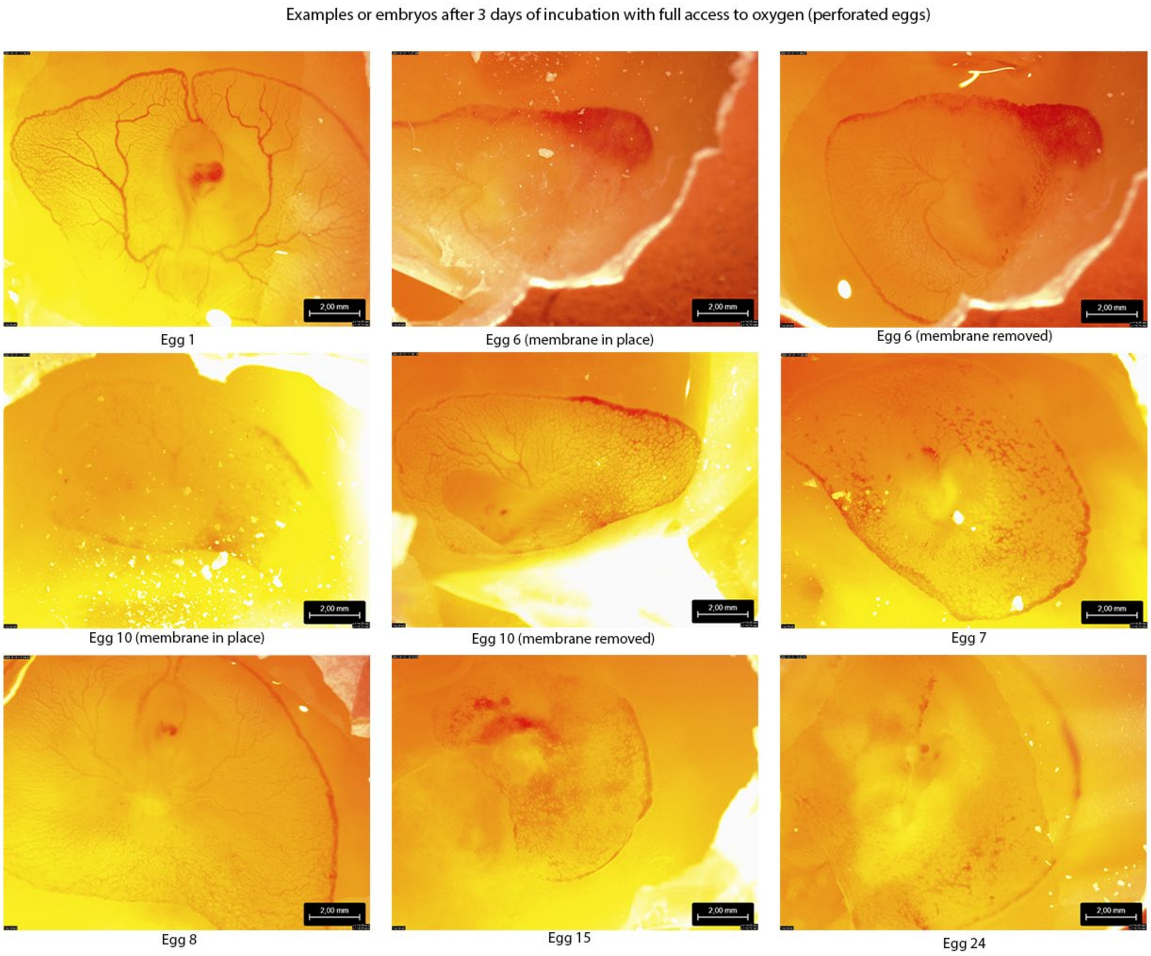
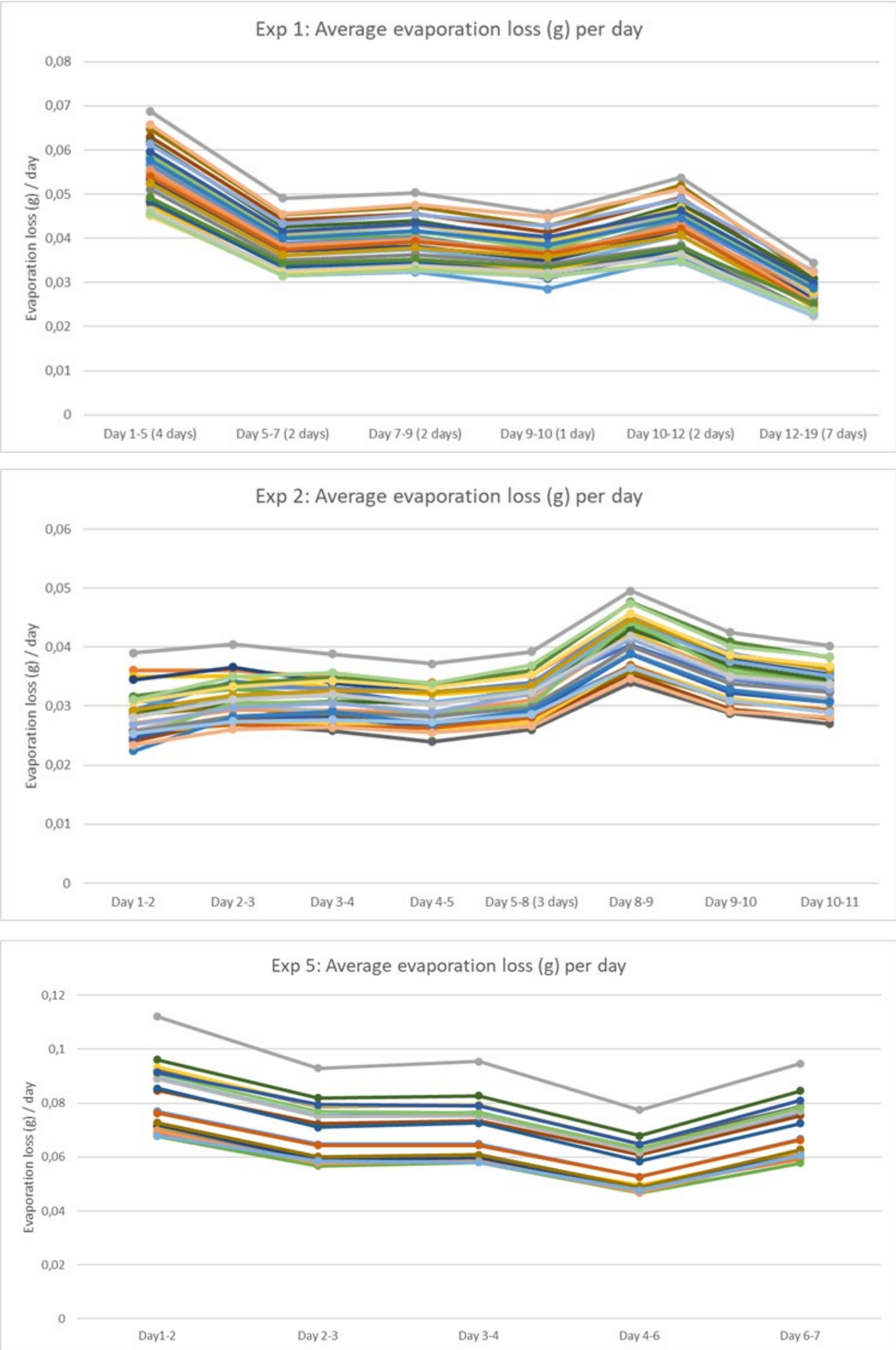
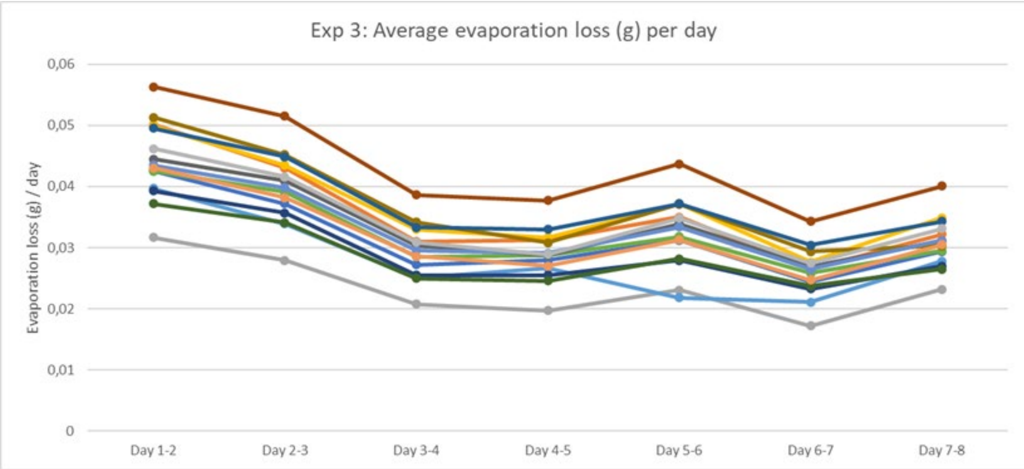
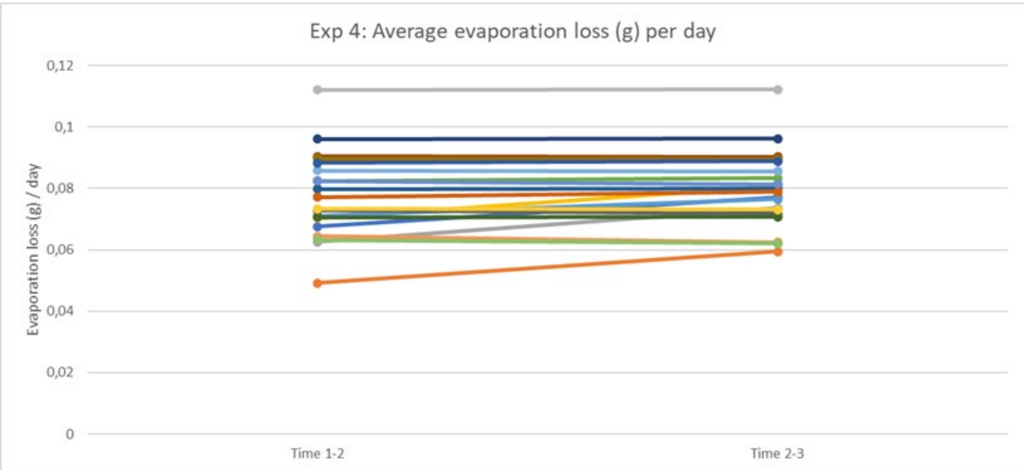


Figure S8. Weight loss in unfertilized eggs kept at 37.5°C.



(cont) **Figure S8**. Weight loss in unfertilized eggs kept at 37.5°C.





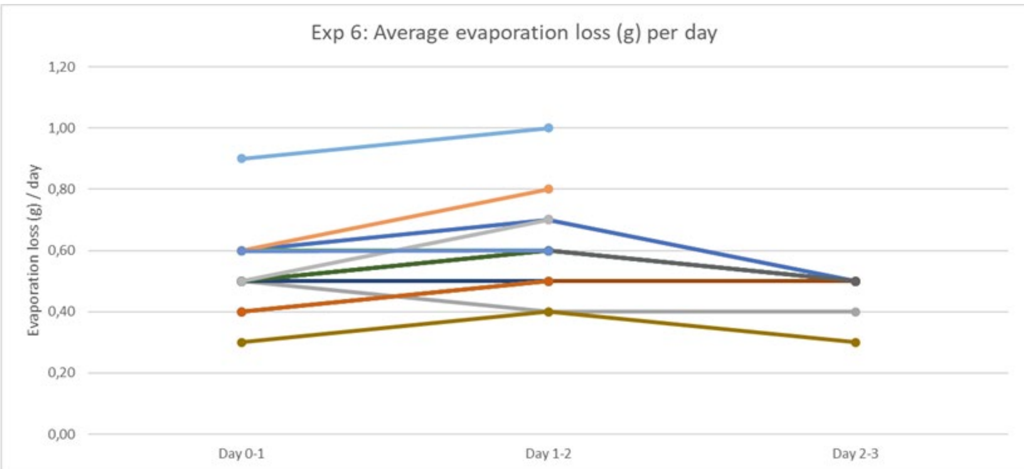


Figure S9. Manipulations of our directly determined D_{O2} values. The plot shows how our values would look different if the actual gas-cell volume was 1/3 (down arrow) or 3x (up arrow) compared to what was measured (corresponding change in gas-cell eggshell area is accounted for).

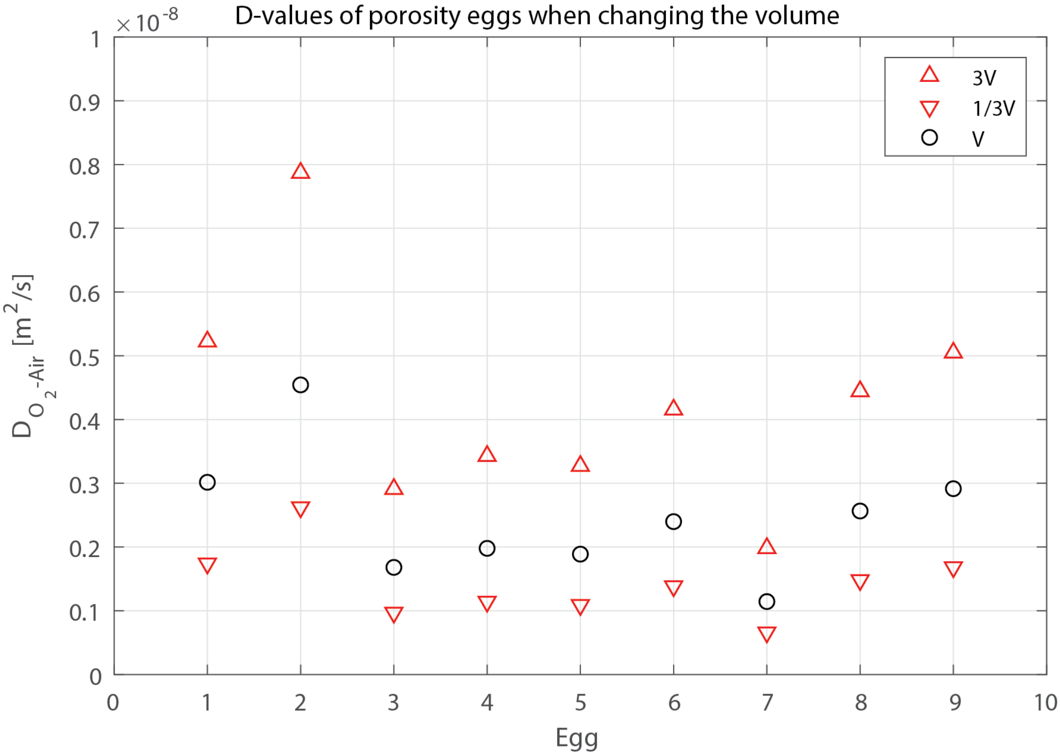


Figure S10. Influx estimates using the directly determined D_{O2} value.