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Cavitation in the embryonic tracheal system of *Manduca sexta*

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SUMMARY

Insect tracheae form during embryonic development and initially contain liquid, which impedes transport of oxygen and carbon dioxide. Only later do tracheae fill with gas and come to support high rates of gas flux. This liquid-to-gas transition is poorly understood. Using eggs of the sphingid moth *Manduca sexta*, we show that longitudinal tracheae in embryos fill with gas in less than 5 s, without invasion of external air, by a process of cavitation. Cavitation requires that tracheal liquids be under tension, and we propose two complementary processes for generating it. One likely, classical mechanism is tracheolar fluid absorption, first proposed by Wigglesworth. Our data support this mechanism in *Manduca*: after cavitation, liquids are progressively drawn out of finer tracheal branches. The second, previously unknown, mechanism is evaporative water loss across the eggshell, which leads both to declining egg volume and to a larger negative pressure potential of water. The pressure potential helps to drive rapid expansion of small bubbles nucleated near spiracles. Once bubbles are large enough to have displaced liquid across the diameter of a trachea, negative capillary pressure reinforces subsequent expansion of the bubble. Together with predictions from modern cavitation theory, our observations substantiate Wigglesworth's contention that gas filling is promoted by increasing hydrophobicity associated with tanning of the spiracles and major tracheal branches.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/212/20/3296/DC1

Key words: cavitation, development, embryo, gas exchange, tracheal system.

INTRODUCTION

The insect respiratory organ, the tracheal system, carries out one of the more unusual modes of gas exchange: it connects ambient gases essentially to every cell or group of cells in the organism *via* branching, air-filled tubes (Wigglesworth, 1983). Because respiratory gases diffuse much faster in air than in water, and because air flows more readily, air-filled tracheae can exchange gases at high mass-specific rates (Bartholomew and Casey, 1978). Early in development, however, the tracheal lumen is filled with liquid, which is replaced with gas late in the egg stage or after hatching (Sikes and Wigglesworth, 1931; Wigglesworth, 1953). Although the molecular and cellular events underlying tracheal morphogenesis are increasingly well known (Manning and Krasnow, 1993; Ghabrial et al., 2003; Devine et al., 2005), the process of gas filling – i.e. of transitioning into a functional state – is not. Here we examine this process in embryos of the moth *Manduca sexta* L. (Sphingidae), in which gas appears suddenly throughout the entire tracheal system and often also appears outside the embryo in the yolk (Dorn et al., 1987; Broadie et al., 1991; Ziese and Dorn, 2003).

The origin of the gas falls into a class of problems recognized by early workers (Keilin, 1924; Sikes and Wigglesworth, 1931): air appears from within the system rather than infilling from an external source. Endogenous filling has been observed in embryos and larvae with closed tracheal systems (Odonata) and in embryos with open tracheal systems that are immersed in egg fluids at the time of gas filling (Lepidoptera, Diptera) (Wigglesworth, 1953). Wigglesworth (Wigglesworth, 1953) proposed a model for filling, consisting of two parts: (i) a mechanism for placing tracheal liquids under tension, namely absorption of fluid by tracheolar cells; and (ii) a mechanism favoring the initial formation of small gas bubbles, namely increasing hydrophobicity of tracheal walls associated with tanning or secretion of waxes. After rupture, gas invades finer branches of the system as the liquids are drawn progressively into tracheal and tracheolar tips, as described by the water potential gradient model of Kestler (Kestler, 1985).

There is little doubt that tracheolar cells absorb fluid during insect activity (Wigglesworth, 1983), but it is uncertain whether they generate substantial, or even slight, negative pressure potentials in embryos; at a minimum, it is worth considering alternatives. We noticed a possible alternative during preliminary observations of developing *Manduca* eggs. In *M. sexta*, embryos show rapid and complete gas filling of the tracheal system about 3/4 of the way (day 3) through development, often with gas bubbles appearing in extra-embryonic fluid (apparently by extrusion from the spiracles). Earlier, usually by day 2 of development, a section of chorion opposite the glue plate invaginates to form a spoon-shaped depression. This observation led us to propose a novel mechanism for producing negative pressure potentials in egg liquids. In particular, the chemical potential of water across the eggshell drives water loss to the environment. Depending on eggshell mechanical properties, the energy contained in the water lost is transformed into either declining egg volume or declining (negative) pressure inside the egg. Because the eggshell appears to have an intermediate compliance, the pressure potential of water becomes progressively more negative. We hypothesize that this negative potential drives the growth of small bubbles of gas nucleated in the tracheal system.

We tested this idea by observing the pattern and timing of liquid-to-gas transitions and by experimentally reducing the magnitude of the pressure potential inside eggs. The rapidity of tracheal air filling (a few seconds) suggested that air originates by cavitation rather than secretion. Further, the timing, location and extent of filling suggested that cavitation occurs almost simultaneously near most

of the spiracles, with liquid subsequently drawn into finer tracheal tips. In eggs with experimentally reduced pressure potentials, embryonic tracheal systems still filled with gas but no longer extruded bubbles into the extra-embryonic fluid. Collectively, these findings indicate that the negative pressure potential derived from water loss across the eggshell contributes to tracheal air filling. However, transpiration must complement some other negative pressure-generating mechanism. Our observations suggest that the mechanism is tracheolar fluid absorption, which Wigglesworth proposed more than 50 years ago. We integrate these data into modern cavitation theory (Brennen, 1995; Franc and Michel, 2004) to develop an updated, quantitative version of Wigglesworth's original gas filling model.

MATERIALS AND METHODS

Moths and egg collection

Eggs were collected from a laboratory colony at the University of Montana derived several years ago from Lynn Riddiford's colony at the University of Washington. Adult moths were kept in black Plexiglas flight cages (1 m×1 m×1.3 m) with the light cycle reversed (16 h light:8 h dark, lights off at 09:00 h). Adults were provided with 20% honey (in water) *ad libitum* in artificial white flowers. Eggs were laid from 09:00–11:00 h on tobacco plants introduced into cages. Eggs were then removed by hand from leaves and held in small plastic cups, with pinholes in the lids for gas exchange, at 27°C until used. Eggs of *M. sexta* undergo a typical lepidopteran development (Dorn et al., 1987; Broadie et al., 1991) and hatch 3–8 days after oviposition, depending on temperature (3.5–4 days at 27°C) (Woods and Bonnecaze, 2006).

Visualization of air filling in embryonic tracheal systems

Patterns of tracheal air filling were visualized in two ways. The first was an automated device for taking time-lapse videos of multiple eggs. Eggs were fixed with clear, double-sided tape to a black Plexiglas stage with small holes (diameter ~0.5 mm, which is less than egg diameter) covered by clear Plexiglas, so that the holes transmitted light but prevented gas movement. The lid of the stage was made of clear Plexiglas and contained multiple lanes for imposing different gas mixtures and humidities on subsets of the eggs. The entire Plexiglas stage was fitted onto a robotic positioner (sub-micrometer repeatability in 3-dimensions; Bunton, Mt Airy, MD, USA) controlled with custom-written software. In operation, the stage was positioned so that each egg was illuminated briefly by a small fiber optic light source (Schott North America, Inc., Auburn, NY, USA) transmitting light through the hole in the bottom of the stage. Eggs were photographed through a stereomicroscope (Nikon SMZ-1500 with a DS-5M digital camera). Eggs were visualized in sequence, each one photographed every 20–30 min throughout development. In separate runs, a few eggs set up on an immobile stage were photographed every 4.5 s across the liquid-to-air transition. All time-lapse equipment and eggs were housed in a 27°C temperature-controlled cabinet.

The time-lapse method provided low-magnification views of embryos in intact eggs. To obtain more detailed information about gas in the tracheal system, we also removed embryos from the eggs and visualized them under a compound microscope (Leica DM3000) at different magnifications (×40–400). Through a stereomicroscope, embryos were dissected from their shells under room-temperature Grace's insect medium (G9771, Sigma-Aldrich, St Louis, MO, USA), moved to fresh droplets of medium on a glass slide, squashed under a glass coverslip, and visualized under both bright- and dark-field illumination. Embryos from three time periods were visualized – in the few hours preceding tracheal air filling, in the 10–20 min after air filling, and within a few hours after air filling (N=4–6 per group).

Effects of relative humidity on transpiration, eggshell deformation and tracheal air filling

To manipulate the rate and extent of water loss, we imposed different ambient humidities on eggs across the entire developmental period (constant illumination, 27°C). Dry air (<5% relative humidity, RH) was produced by pumping air through a column of indicating Drierite, and higher humidities (55, 85 and 99% RH) were obtained by bubbling air streams through temperature-controlled flasks of tap water.

To measure eggshell deformation during development in different humidities (<5, 55, 99% RH; N=8–11 per group), we fixed eggs from a single batch of eggs laid over a 2 h period to the automated stage for time-lapse videomicroscopy. So that deformation would be visible, each egg was positioned with its flattened axis parallel to the field of view. Eggs were photographed every 15 min. Images were extracted from the photo series starting 6 h after oviposition and every 8.25 h subsequently until hatching. Long and short egg diameters were measured digitally using ImageJ software (v. 1.40g), with the measurement tool calibrated from a photograph of a stage micrometer taken at the same height and magnification as the egg photographs.

In a separate experiment, we assessed the effects of four different humidities (<5, 55, 85, 99% RH; N=6 or 7 per group) on the timing of tracheal darkening, formation of extra-embryonic bubbles and hatching. Eggs from a 2 h oviposition period were fixed to the stage without regard to orientation and photographed every 20 min. Image series were made into movies and scored for developmental events.

Experimental introduction of air bubbles

If cavitation is driven by negative pressure potentials of water arising from transpiration across the eggshell, then eggs in which those potentials are experimentally removed should not undergo tracheal gas filling, or should show a reduced extent of gas filling. We alleviated the pressure potentials by introducing small air bubbles into eggs 4–10 h before they would normally have undergone tracheal gas filling (on day 3). Eggs were viewed at moderate magnification (×15–25), and air bubbles were introduced *via* small incisions in the eggshell made with a 30 gauge needle. Before surgery, eggs were held at 27°C and ambient RH (in our lab usually <40%). Eggshells were visibly deformed by water loss by the time of surgery. All incisions were made in the deformed region of the eggshell, and introduced air bubbles were clearly visible under the chorion. The experiment consisted of three treatments: (1) control eggs that were not cut (N=20); (2) experimental eggs in which eggshells were cut and air let in (N=14); (3) sham controls (N=10), in which incisions were made but air prevented from entering by pressing slightly on the eggshell until the wound was sealed, usually within a few minutes. Eggs were then fixed onto the automated time-lapse device and photographed until hatching.

RESULTS

Visualization of air filling in embryonic tracheal system

Early in embryonic development, the tracheal system was invisible through the eggshell (Fig. 1A,B). At ~3/4 of the way through development, however, tracheal trees in each segment began to darken, corresponding to the initial appearance of gas in the tracheal trees branching from each spiracle (Fig. 1C). At the same moment, or subsequently, large portions of the tracheal system, including main

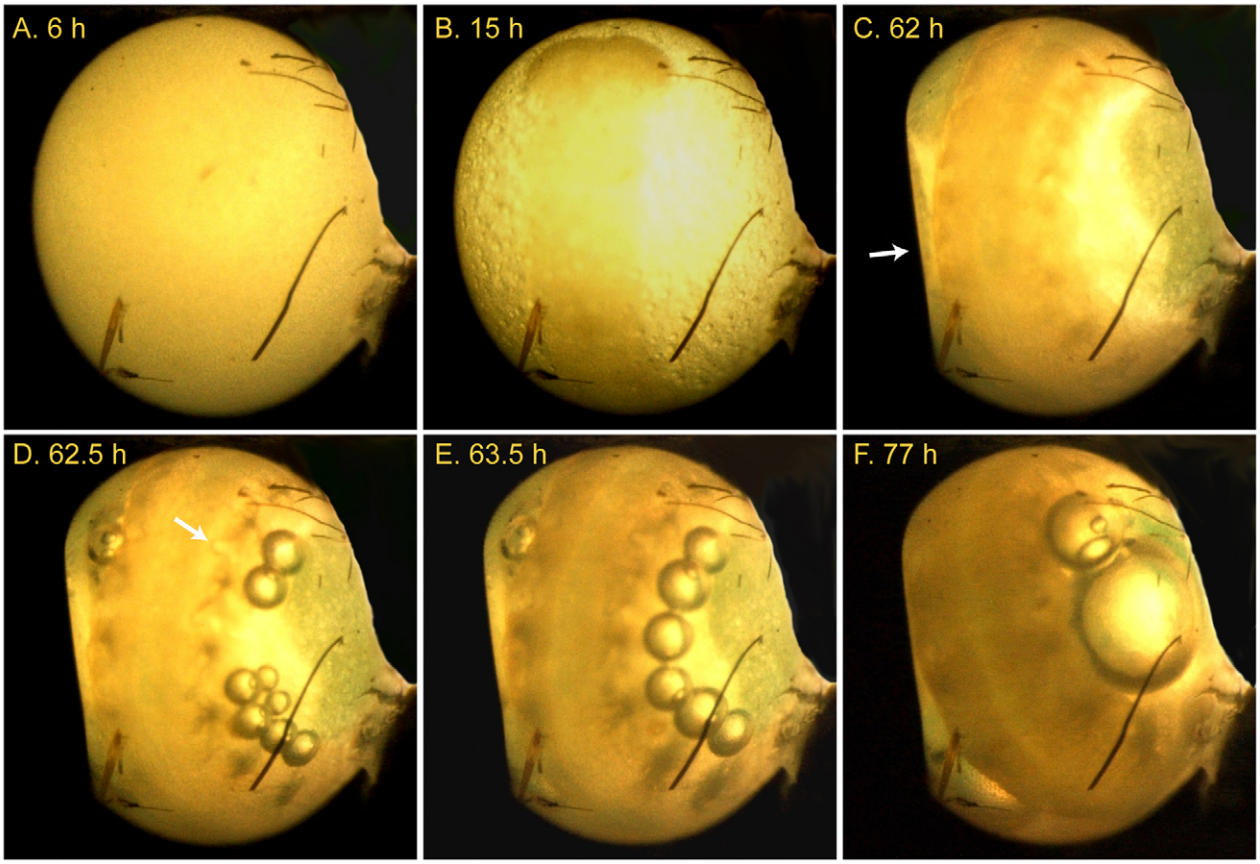

Fig. 1. Time series of development in an egg of *Manduca sexta*. The glue plate deposited by the female is oriented on the right side. Initially (A) no structures are visible through the eggshell (short axis ~1.25 mm). Subsequently, the embryo becomes visible (B) and katatrepsis and segmentation can be seen. Later, at a time dependent on how much water the egg has lost, the eggshell invaginates (white arrow in C) to form a spoon-shaped depression, which continues to grow as additional water is lost. At ~62 h of development (at 27°C; C), spiracles and attached tracheal branches begin to darken. Immediately, or soon thereafter (here 0.5 h later; D), bubbles emerge from one or more spiracles. Bubbles continue to grow and merge (E,F) until, by late in development, most extra-embryonic space in the egg is filled with air. Dark objects on the eggshell are adult scales.

longitudinal branches, became gas filled (Fig. 1D). In some cases, bubbles appeared in extra-embryonic fluids, apparently by extrusion from the spiracles (Fig. 1D), as the bubbles were always closely associated with spiracles (supplementary material Fig. S1) and, in desiccated eggs, were formed at the same moment as the tracheae were cleared of liquid (within the temporal resolution offered by our videos). Time-lapse videos taken at higher frame rates showed that longitudinal tracheae could be completely cleared of fluid in less than 5 s (Fig. 2). See online supplementary material for a time-lapse video (Movie 1) covering the period from a few hours after oviposition to hatching.

Embryos could often be dissected from their eggs with relatively little tissue damage and could be visualized rapidly. In the few hours before air filling, the tracheal system was visible but had optical properties much like those of surrounding tissues (Fig. 3A). Under higher magnification (×400), taenidial rings were visible in the branching trees under each spiracle (Fig. 3B). In a subset of eggs checked frequently we were able to pinpoint the timing of gas filling. These embryos were removed from their shells and their tracheal systems visualized within 20 min. Gas-filled sections of tracheae were much darker than fluid-filled sections. The distribution of sections indicated two characteristics of the filling process: (1) gas appeared first near the spiracles, most likely in the tracheal manifolds radiating from sub-atrial spaces (Fig. 3C), and (2) anterior sections of the tracheal system filled with gas before posterior sections (Fig. 3D). Where the gas–liquid interface was clearly visible, the liquid appeared to form a bulging meniscus (Fig. 3E), indicating that tracheal surfaces were hydrophobic. By 2 h after the start of tracheal gas filling, the entire system of large branches, including all longitudinal links between spiracles and transverse branches, was gas filled. Nonetheless, columns of liquid were sometimes trapped initially, especially in the longitudinal tracheae between gas-filled spaces (Fig. 3F). High-magnification observation of longitudinal tracheae revealed no small, branching tracheae that could be responsible for subsequently absorbing these trapped columns, although fluid may be absorbed across general tracheal walls, as suggested for *Blatella* by Bult (Bult, 1939), *Sciara* by Keister and Buck (Keister and Buck, 1949), and *Aedes* by Wigglesworth (Wigglesworth, 1953). In addition, it could be absorbed by the specialized intima associated with intersegmental nodes.

Effects of RH on transpiration, eggshell deformation and tracheal air filling

Experimental variation in ambient RH affected the rate and extent of shell deformation (Fig. 4A) in the direction of the short axis; the long axis never deformed. Clearly, lower RH gave more rapid deformation (linear mixed-effects model; significant interaction between RH and time: $F_{1,172}$=220.1, P<0.0001). Additional views

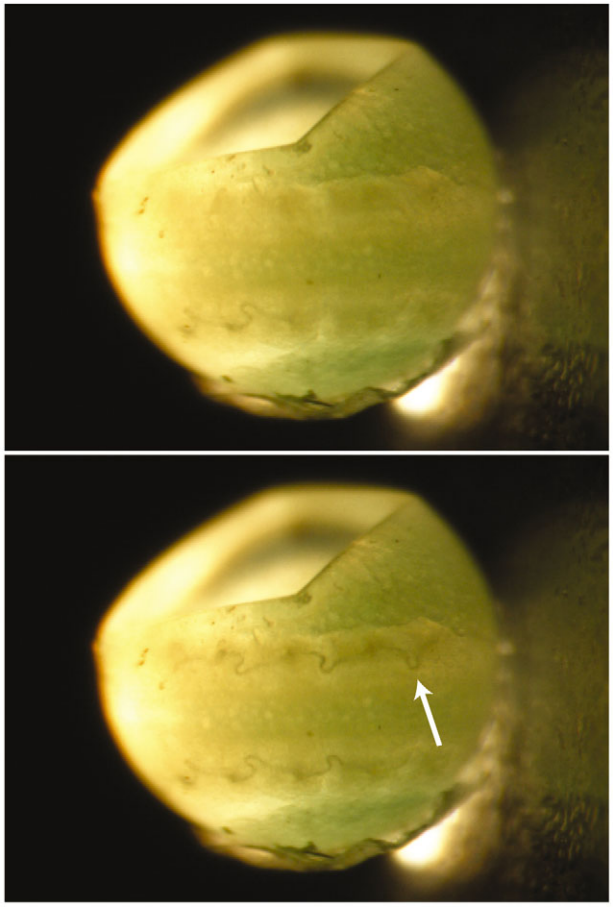

Fig. 2. Two successive frames of the same egg, held in ambient air (<40% relative humidity, RH), taken 4.5 s apart. In the upper frame, some gas filling of the lower longitudinal tracheae is visible, but the upper longitudinal tracheae still contain liquid. In the subsequent (lower) frame, the upper longitudinal tracheae are gas filled (white arrow).

of eggs in different orientations indicated that the deformation was a dimple. Eggs held in nearly saturated air (99% RH) showed no contraction of the short axis. In eggs held in lower humidities (0 and 55%), deformation stopped as soon as extra-embryonic bubbles appeared. The total volume of the deformation was calculated by treating the egg as an ellipsoid with two semiaxes, a and c, with $a>c$ (a is parallel to the glue plate; c is orthogonal). The total volume (V) displaced by eggshell deformation is twice the volume of the ellipsoidal cap defined by the sharply bent edge of the deformation (see Fig. 1C; Fig. 4):

$$V = 2\pi a^2\left(\frac{2c}{3} - x + \frac{x^3}{3c^2}\right), \quad (1)$$

where x refers to the height (from the center of the egg) of the plane that defines the base of the cap. By this calculation, eggs in dry air lost ~0.15 mm^3 (=0.15 mg water) of their volume by the time tracheal gas filling occurred.

Ambient RH had little effect on the timing of the initial appearance of gas in the tracheal system (tracheal darkening) or of hatching (Fig. 4B). However, RH strongly affected the relative timing of tracheal darkening and extra-embryonic bubble formation. At low RH (0 and 55%), tracheal darkening and bubble formation were coincident, whereas at higher RH they were progressively uncoupled.

Experimental air bubbles

None of the eggs into which air was introduced subsequently formed extra-embryonic bubbles (Table 1), but both sham-manipulated eggs (small incision made in the eggshell but no air introduced) and unmanipulated controls showed normal bubble formation (χ^2=44, d.f.=2, P<0.0001). However, all eggs in all groups showed visible darkening of the tracheal system around each spiracle, corresponding to the appearance of gas in the tracheal lumens.

DISCUSSION

In embryos of *Manduca sexta*, the tracheal system rapidly fills with gas at a point ~3/4 of the way through development. Gas originates from within the tracheal system by cavitation of tracheal liquids. Cavitation occurs in tracheal manifolds (sub-atrial spaces), and air subsequently invades finer branches of the system. Cavitation requires that liquids be under tension (negative pressure potentials); our results suggest that the tension is generated in part by water loss across a compliant eggshell and in part by liquid absorption into tracheolar cells.

Origin of negative pressure potentials of water in eggs of *M. sexta*

Eggshells of many insects, including *M. sexta*, exhibit spoon-shaped deformations that increase in size during development (Sikes and Wigglesworth, 1931). The deformations reflect water lost across the eggshell: (i) eggshells deformed more in dry than in moist air (Fig. 4), and (ii) in dry air the volume of the deformation was ~0.15 mm^3 (corresponding to 0.15 mg water), which agrees well with experimentally measured water losses from eggs of *M. sexta* (Woods and Singer, 2001). In energetic terms, the chemical potential of water (μ_w) inside an egg declines as water is lost across the eggshell, and the free energy released can be invested either in volume change (dV) or in pressure change (dP). If eggshell compliance (=dV/dP) is high, most of the free energy is invested in dV; if compliance is low, most of the free energy is invested in dP. Our experiments suggest that compliance is intermediate to high – i.e. the eggshell deforms as it loses water, so it clearly is not rigid; similarly, its compliance is not extremely high, as air was sucked in through incisions made in the eggshell. Thus, free energy released by water loss leads to both declining volume (dV) and increasingly negative pressures (dP). The resultant negative pressure potential can then be used to drive cavitation (triggers for cavitation are discussed below).

Table 1. Outcome of experiment to alleviate internal tension by introducing air bubbles before tracheal gas filling normally occurs

Treatment	*N*	No. with tracheal darkening	No. with extra-embryonic bubbles	No. hatching
Air introduced *via* incision	14	14	0	14
Sham incision	10	10	10	10
Unmanipulated	20	20	20	16

Eggs of *Manduca sexta* were held at 27°C in dry air for 2 days, after which a small incision was made in the eggshell.

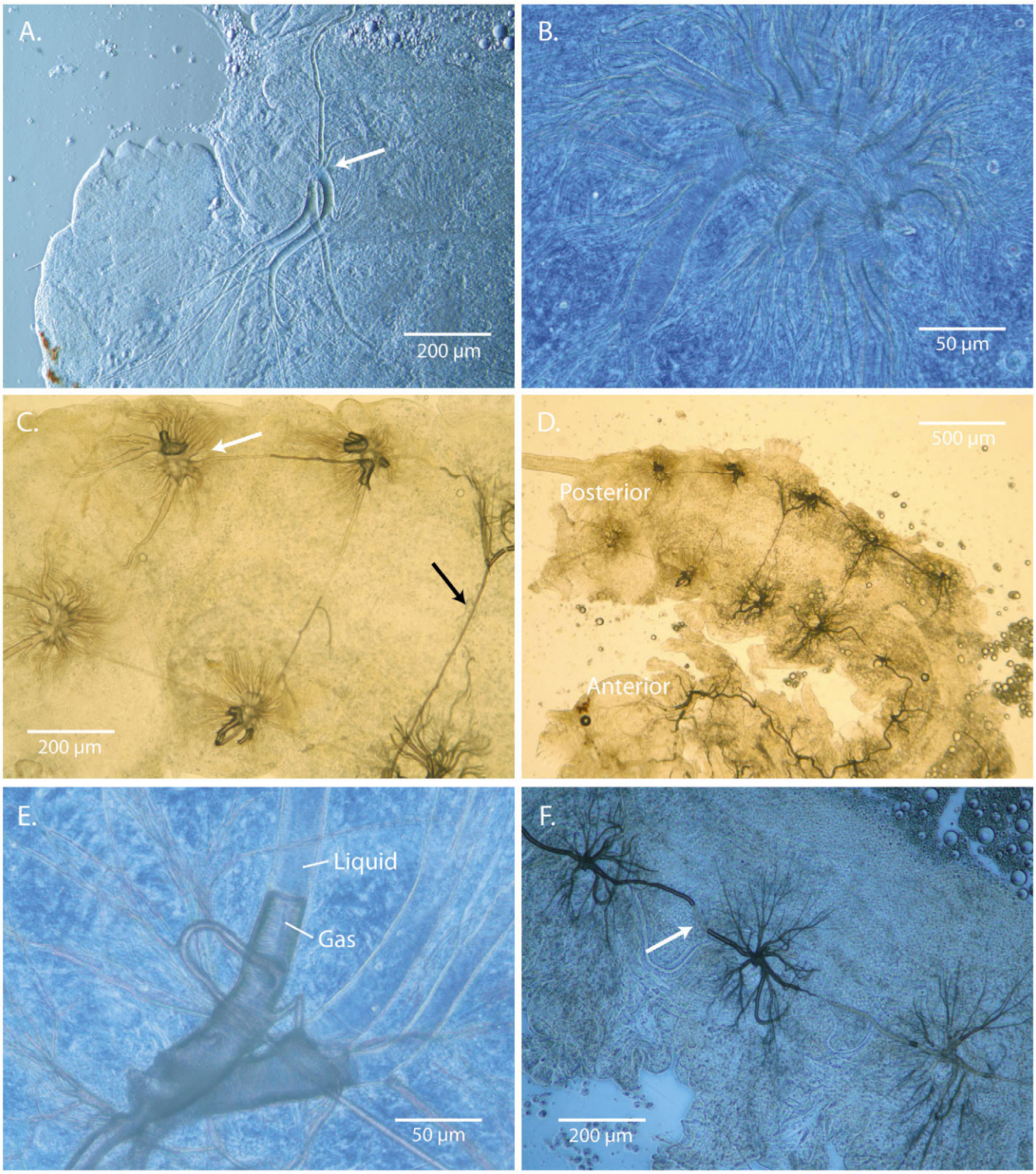

Fig. 3. Tracheal system of *Manduca* embryos before and after gas filling. (A) Thoracic spiracle and major tracheal branches sprouting from it (white arrow points to the spiracle). Tracheal branches are visible but liquid filled. Red spots in lower left are tanned mandibles. (B) In higher magnification views of a single spiracle and its tracheal branches, taenidia on the tracheal walls are clearly visible. (C,D) Embryonic tracheal system within 20 min of the onset of gas filling. (C) Gas-filled spaces appear dark. Nucleation occurs in the main tracheal branches near each spiracle (white arrow), and gas rapidly comes to occupy most of the longitudinal and transverse tracheae. The black arrow points to a transverse trachea that is completely gas filled; the next most posterior transverse trachea (to the left) is only partially filled. (D) In lower magnification views of several embryos, there clearly was an anterior–posterior gradient in the degree of tracheal gas filling. The thoracic spiracles and anterior abdominal spiracles appeared to undergo gas nucleation earlier, and the longitudinal and transverse tracheae from them were more completely gas filled. However, by 2 h after the onset of gas filling, all major tracheal branches were gas filled (not shown). (E) Gas–liquid interface inside a major tracheal branch. The positive curvature of the liquid indicates that the tracheal walls are hydrophobic; the contact angle is difficult to measure precisely, but appears to range between 100 and 110 deg. (F) In some embryos, columns of liquid were trapped in longitudinal tracheae between gas-filled spaces (white arrow). How these columns are later cleared is unknown.

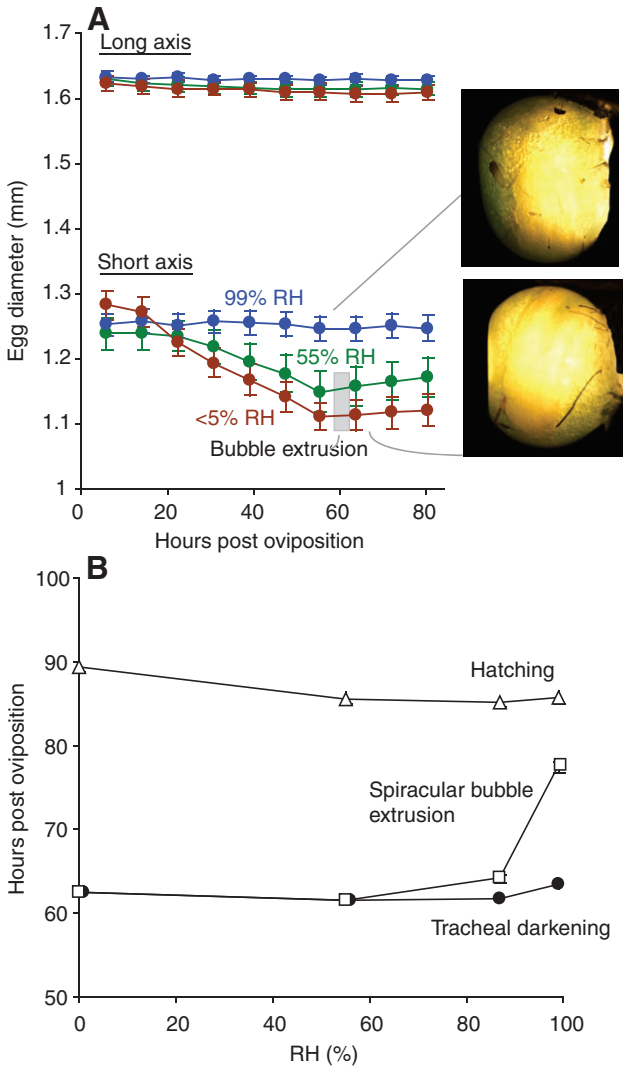

Fig. 4. (A) Compared with eggs in high humidity, those in low humidity showed deformation along the short axis but not long axis (N=8–11 per group). No additional deformation occurred after bubble extrusion from the spiracles. (B) Ambient RH had little effect on initial tracheal darkening (gas filling) or hatching time (N=6 or 7 per group). However, high humidity significantly delayed the appearance of bubbles from the spiracles.

The appearance of air in the tracheal system has several consequences. First, the negative pressure potential disappears, its energy used to rupture tracheal fluids, expand the air bubble, and in some rebound of the eggshell deformation (Fig. 4A). Second, post-cavitation water loss across the eggshell no longer leads to declining egg volume (Fig. 4A), as internal air bubbles uncouple water losses from eggshell deformation. Further, incomplete rebound of the eggshell after cavitation suggests that its deformation is partly plastic: the eggshell resists declining internal pressures, but there is some permanent deformation such that the eggshell does not return to its initial conformation even when those pressures are relieved. Biomechanical analysis of eggshells would be useful in quantifying this effect.

We also experimentally relieved pressure potentials (in eggs previously exposed to dry air) by adding small bubbles of air into eggs 4–10 h before they would normally undergo natural bubble formation. In none of these eggs was there any subsequent bubble formation near (or extrusion by) the spiracles (Table 1). However, manipulated eggs did undergo tracheal air filling. This indicates that water loss results in some but not all of the negative pressure potential necessary to drive tracheal air filling. What fraction does it provide? The answer would require measurement of the negative pressure potentials, which turned out to be unexpectedly difficult. The obvious experiment – of raising the external ambient pressure of either air or water to find the pressure necessary to prevent cavitation – is flawed: high ambient barometric pressure would drive additional gas into egg liquids, and the gas would then appear in small bubbles and promote their growth (see next section), offsetting the very process we would be trying to inhibit. Eggs put under high hydrostatic pressure would have the opposite problem of rapid and severe hypoxia stemming from slow O_2 transport across water boundary layers. Finally, we tried to measure internal pressure potentials directly by pushing glass microneedles through the chorion while simultaneously lowering the barometric pressure inside the needle shaft (to measure the hypobaric pressure just necessary to prevent air entry); however, the process of insertion deformed the shell and thereby disrupted the pressures of interest. We are currently developing other methods for measuring internal pressure potentials.

Wigglesworth (Wigglesworth, 1953) proposed that negative pressure could be produced by active fluid absorption (see also Kestler, 1985). Indeed, gas appeared first in the largest tracheae and then filled finer tracheae as the liquid was drawn out of them toward the tips [as also found by Keister and Buck (Keister and Buck, 1949)]. Such a pattern is most easily explained by tracheolar fluid absorption according to the water potential model of Kestler (Kestler, 1985). Work on tracheal air filling in *Drosophila* during ecdysis suggests additional possible hormonal and molecular controls over absorption. Baker and colleagues (Baker et al., 1999) showed, using pupal *D. melanogaster*, that tracheal air filling occurred coincidentally with the release of eclosion hormone (EH), and that injection of *Manduca sexta* ecdysis-triggering hormone (MasETH) into flies induced both premature air filling and rising levels of cGMP in tracheal cells; cGMP is known to stimulate fluid transport in other tissues (Davies et al., 1995). We speculate that tracheolar fluid absorption in embryonic *M. sexta* is stimulated by cGMP, with timing controlled by embryonic release of EH or ETH.

What triggers cavitation?

Some insight is provided by an increasingly well-developed theory of cavitation (Brennen, 1995; Franc and Michel, 2004). Cavitation theory focuses on liquid tensile strength (σ), analyzing how it varies with properties of the system in which the liquid is contained. In insects, the tensile strength of tracheal liquids is given by (Brennen, 1995):

$$\sigma = 2S\sin(\theta + 90 - \alpha) / R - P_G , \quad (2)$$

where S is surface tension, θ is contact angle (the angle at which an air–water interface contacts a solid, measured through the water side), α describes the half-angle of surface irregularities idealized as cones, R is the radius of a small bubble, and P_G is the partial pressure of dissolved gases. In principle, an embryo could trigger cavitation either by raising the negative pressure potential above the tensile strength or by altering any of the five factors in Eqn 2 so that σ is depressed below a standing negative pressure potential. In practice, some mechanisms are more likely than others. Below we briefly analyze the factors and discuss their relative utility as cavitation triggers.

First, embryos may not alter σ – rather, cavitation may occur because the negative pressure potential inside the egg rises above the tensile strength. There are two possible pressure-related triggers. First, negative pressure may build up gradually as water is lost across the semi-rigid eggshell until, at the point 3/4 of the way through development, it exceeds the tensile strength of tracheal liquids. This mechanism, however, is unlikely to function as a trigger because experimentally relieving the pressure did not significantly delay tracheal air filling. Second, negative pressure may build up rapidly if the eggshell rapidly becomes non-compliant (rigid). If so, evaporative water losses would rapidly increase the negative pressure potential. This mechanism too, however, appears unlikely, as it requires sudden increases in eggshell rigidity. In our embryo dissections, eggshells appeared to be no more rigid after cavitation than before.

Second, embryos could secrete factors that reduce the surface tension of tracheal liquids. The roles, if any, that surfactants play in the insect tracheal system are poorly known (Orgieg et al., 2007). Certainly molting fluid, which fills the tracheal system during molts, contains enzymes and other molecules that could function as surfactants – but such potential functions have never been examined.

Third, embryos could make tracheal surfaces more hydrophobic (increase θ). Contact angles vary with relative hydrophilicity and hydrophobicity of the surface – hydrophobic surfaces having θ>90 deg. (Fig. 5A). Insect tracheae are lined with cuticle, which becomes more hydrophobic as the cuticle tans and waxes are secreted (Locke, 1957; Beament, 1964). Tanning biochemistry is relatively well understood (Hopkins and Kramer, 1992) and, because it has enzyme-mediated components, tanning can occur rapidly. In general, it requires the presence of 3,4-dihydroxyphenylalanine (DOPA), which is produced by the hydroxylation of tyrosine. In *M. sexta*, tyrosine hydroxylase (TH) is a key enzyme involved in producing DOPA, and both the mRNA and protein of TH appear for the first time in the egg starting on day 3 (Gorman et al., 2007), which corresponds to when mandibles and other structures begin to tan and when tracheae fill with gas (Fig. 1) (Broadie et al., 1991; Ziese and Dorn, 2003). At liquid–gas interfaces in embryos of *M. sexta*, we observed contact angles of 100–110 deg. (Fig. 3E).

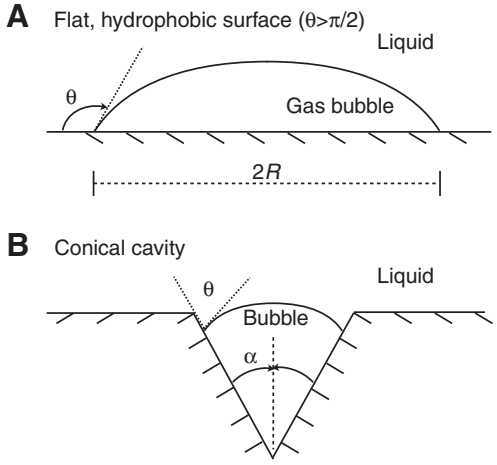

Fig. 5. (A) A small bubble of gas attached to a hydrophobic surface. The contact angle, θ, is >90 deg. (π/2). In this case, the tensile strength of the liquid is $2S\sin\theta/R$. (B) A small bubble in a conical cavity of half angle α. In the absence of dissolved gases, the tensile strength of the liquid is $2S\sin(\theta+90-\alpha)/R$. When dissolved gases are present, tensile strength is $2S\sin(\theta+90-\alpha)/R-P_G$. Panels were redrawn from Brennen (Brennen, 1995).

Fourth, insect tracheae often contain sharp surface angles (<60 deg.) in their lumens by virtue of spiral- or hoop-like rings – taenidia – in their walls (Locke, 1957). These surface irregularities may promote bubble expansion, as illustrated by the conical cavity example (Fig. 5B). In idealized cones, α is the cone's half-angle, and tensile strength falls to zero as $\theta\rightarrow\alpha$+90 deg., rather than as $\theta\rightarrow$180 deg. for a flat surface (Brennen, 1995). Embryos could decrease α by compressing tracheal walls. Indeed, Movie 1 (supplementary material) shows that embryos pulsate regularly starting about 1/3 of the way through development (before katatrepsis), although pulsation is not associated specifically with cavitation.

A final bubble-promoting factor is gas dissolved in tracheal liquids. Dissolved gases lower tensile strength by appearing in microscope bubbles (with summed partial pressures, P_G), where they act together with the vapor pressure, and any negative pressure potential, to promote bubble expansion. The partial pressures of these gases have not been measured, but estimates are possible. Because nitrogen and argon are inert, their partial pressure in the developing tracheal system should initially be ~79 kPa. Oxygen is consumed by the embryo and so is drawn down (Woods and Hill, 2004); but its level probably does not fall much below 5 kPa. Carbon dioxide exists in tracheal fluid primarily as bicarbonate but will equilibrate into the incipient bubble as gas, with a partial pressure of ~5–10 kPa. Therefore, at the beginning of development, the summed partial pressures of gases other than water vapor in any small bubble will total ~90–95 kPa (at sea level). As water is lost over development, the egg's volume declines and the partial pressure of N_2 rises. If the eggshell is relatively impermeable to N_2 (Bridges et al., 1980), the rise in P_{N_2} could be as high as ~12 kPa. However, such a rise would have modest effects on tensile strength compared with other factors; in addition, the rise is gradual and therefore probably does not act as the cavitation trigger itself.

Although we cannot definitively exclude any of the triggers discussed above, the most likely appears to be increasing hydrophobicity, as it has a well-known biochemical basis and occurs in other embryonic structures at about the same point in development. We therefore propose a three-part model for tracheal gas filling. (1) Cavitation occurs near spiracles, with timing controlled by the onset of spiracular and tracheal tanning and possibly secretion of waxes onto the tracheal epicuticle; the anterior-to-posterior wave of gas filling is controlled by an anterior-to-posterior wave of tanning [Keister and Buck observed a similar pattern of tracheal filling in molting larvae of the fly *Sciara coprophila* (Keister and Buck, 1949)]. (2) The negative pressure potential in tracheal liquids is generated by both deformation of a partially rigid eggshell and tracheolar fluid absorption; whether bubbles are extruded from the spiracles depends on whether the eggshell is sufficiently deformed. (3) Bubbles nucleate in the angular spaces between adjacent taenidial rings. This last possibility would constitute a novel function for taenidia other than the usual suggestion of mechanical support.

Air filling of the tracheal system after cavitation

The discussion above focuses on cavitation *per se*. After cavitation, however, a distinct set of mechanisms must promote gas filling of the rest of the tracheal system, with liquid moving outward through the spiracles into the periembryonic space and inward into the tracheolar cells. Inward movement may be related to the long-standing problem of understanding tracheolar fluid levels. We

propose (i) that a common factor – capillary pressure – plays a central role in expansion of the gas bubble in both directions and in the subsequent maintenance of liquid in tracheoles; and (ii) that the negative pressure potential arising from water loss across the compliant eggshell aids bubble expansion in both directions.

In a classic series of papers using the flea *Xenopsylla*, Wigglesworth (Wigglesworth, 1930; Wigglesworth, 1931; Wigglesworth, 1935) showed that tracheoles are filled with liquid whose level varies with ambient oxygen concentrations and temperature. He suggested that the level was determined by opposing forces – the osmotic pressure in other body fluids (which rises in anaerobic conditions) and the capillary pressure in the tracheolar tubes. The osmotic pressure, he proposed (Wigglesworth, 1983), originates in the tracheolar cytoplasm rather than in the hemolymph. The capillary pressure arises when water interacts with tube walls. When the walls are hydrophilic, as in tracheoles, water is pulled into the tube (capillary rise). Beament (Beament, 1965) proposed an equation for the capillary pressure (P_c):

$$P_c = 2S\cos\theta / R\,, \quad (3)$$

which is essentially Eqn 2 with cavitation-specific terms omitted.

Kestler (Kestler, 1985) (and P. Kestler, personal communication) expanded and formalized the balance in terms of overall water potential (ψ_{ow}), proposing that liquid levels reflect a balance of the pressure potential in tracheolar cells (ψ_{tlw}, which includes osmotic, matrix and other pressure potentials of water) and the capillary potential of water (ψ_{cw}). In tracheolar cells, one other potential arises from prior water loss across the chorion (which gives dimpling) and from ongoing water loss, together giving what we call the chorionic pressure potential of water (ψ_{chw}). This term can be added explicitly (P. Kestler, personal communication) to give:

$$\psi_{ow} = \psi_{tlw} + \psi_{cw} + \psi_{chw}\,. \quad (4)$$

The signs of the terms are defined with respect to the gas bubble inside the tracheal tube: a water potential is positive if it promotes bubble collapse (pulls liquid toward the gas space) and negative if it promotes expansion. Just after cavitation, both ψ_{tlw} and ψ_{chw} are negative, with ψ_{tlw} pulling only on the column of liquid between the bubble and the tracheoles (proximal liquid). Interestingly, because the chorionic pressure potential (ψ_{chw}) is distributed throughout the egg's interior, it acts both on the proximal liquid and on the distal liquid between the gas bubble and the spiracles (ψ_{chw} is negative in both cases, promoting bubble expansion; with respect to the distal air–liquid interface, ψ_{tlw} is zero). The remaining term, the capillary potential of water (ψ_{cw}), plays a key role because it changes sign at the border between tracheae and tracheoles. In tracheae, whose surfaces are hydrophobic (see Fig. 3E), ψ_{cw} is negative. ψ_{cw} therefore reinforces the actions of ψ_{tlw} and ψ_{chw} in removing proximal liquid from the system and of ψ_{chw} in removing distal liquid. If we use our measured contact angle of 105 deg. and a tracheal radius of ~20 μm, ψ_{cw} in *Manduca* embryos is about –3.7 kPa. In tracheoles, however, surfaces are hydrophilic. Therefore, when the proximal air–liquid interface reaches a tracheole, the sign of ψ_{cw} switches from negative to positive, which then opposes the action of ψ_{tlw} and retains liquid in the tracheolar tip.

Functional significance of tracheal gas filling and external bubbles

Tracheal gas filling in embryos must dramatically increase rates of internal oxygen transport, as the diffusion and capacitance coefficients of oxygen are much higher in air than in water (Kestler, 1985; Denny, 1993). However, whether gas filling occurs specifically to facilitate transport is debatable. In favor of this interpretation are our prior findings: (1) embryo metabolic rates increase 3–4 times toward the end of development (Woods et al., 2005), which draws P_{O_2} in the egg down to low levels (Woods and Hill, 2004); and (2) a mathematical model of gas flux across the different eggshell and internal layers between the environment and embryo (Woods et al., 2005) suggested that layers of embryonic tissue and yolk could be significant barriers to adequate diffusive delivery of oxygen. Air-filled trachea could thus distribute oxygen rapidly to deep tissues that may not otherwise be well supplied. Against this functional interpretation is the observation that even when tracheae contain gas, liquid outside the spiracles would prevent rapid oxygen diffusion into the tracheal system. Evaluating these possibilities will require methods for preventing tracheal gas filling; so far, we have been unable to discover one (experimental incisions prevented formation of extra-embryonic bubbles but not filling of tracheae themselves).

We first thought that gas bubbles associated with the spiracles functioned as gas gills (Rahn and Paganelli, 1968). However, subsequent observations suggested that bubbles were mobile and did not remain in contact with spiracles; therefore, total oxygen flux into the tracheal system *via* the spiracles may be quite small. Regardless, extra-embryonic bubbles still store significant quantities of O_2 (because air has a high capacitance coefficient for O_2) (Kestler, 1985), and facilitate eggshell-to-embryo transport, all the more so because embryos mix the egg contents vigorously late in development (see supplementary material Movie 1).

Conclusions

In biological systems, evaporation from enclosed spaces can readily generate negative pressure potentials, which can be used to do work. The best known example is water transport in tree trunks: water is pulled up by negative pressure potentials arising from foliar transpiration. In trees, cavitation in xylem tubes disrupts transport. In eggs of *Manduca*, the water potential gradient from egg to environment is transformed by inevitable evaporative losses (Zrubek and Woods, 2006) across the partially rigid eggshell into a pressure potential inside the egg, which in turn helps drive cavitation and air filling of the embryonic tracheal system.

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