The Journal of Experimental Biology 207, 2267-2276
Published by The Company of Biologists 2004
doi:10.1242/jeb.00991

Temperature-dependent oxygen limitation in insect eggs

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Accepted 18 March 2004

Summary

Most terrestrial insect embryos support metabolism with oxygen from the environment by diffusion across the eggshell. Because metabolism is more temperature sensitive than diffusion, embryos should be relatively oxygen-limited at high temperatures. We tested whether survival, development time and metabolism of eggs of a moth, *Manduca sexta*, were sensitive to experimentally imposed variation in atmospheric oxygen availability (5–50 kPa; normoxia at sea level is 21 kPa) across a range of biologically realistic temperatures. Temperature–oxygen interactions were apparent in most experiments. Hypoxia affected survival more strongly at warmer temperatures. Metabolic rates, measured as rates of CO_2 emission, were virtually insensitive to hypo- and hyperoxia at 22°C but were strongly influenced at 37°C. Radial profiles of P_{O_2} inside eggs, measured using an oxygen microelectrode, demonstrated that 3-day-old eggs had broad central volumes with P_{O_2} less than 2 kPa, and that higher temperature led to lower P_{O_2}. These data indicate that at realistically high temperatures (32–37°C) eggs of *M. sexta* were oxygen limited, even in normoxia. This result has important implications for insect population ecology and the evolution of eggshell structures, and it suggests a novel hypothesis about insect gigantism during Paleozoic hyperoxia.

Movie available on-line.

Key words: moth, *Manduca sexta*, egg, oxygen availability, temperature, metabolism, eggshell, insect gigantism, Paleozoic hyperoxia.

Introduction

Temperature and oxygen interact to affect organismal physiology and behavior. These interactions were first described in studies of fish, which showed that metabolism and circulatory function were more sensitive to dissolved oxygen at higher temperatures (Alabaster and Welcomme, 1962; Gehrke, 1988). More recent studies, primarily of marine ectotherms, suggest a mechanistic view of temperature-oxygen interactions: at high temperatures, ventilatory and circulatory systems are unable to meet metabolic demand; conversely, at low temperatures cold-inhibited mitochondria provide insufficient energy to support oxygen supply by ventilation and circulation (Frederich and Pörtner, 2000; Pörtner, 2001, 2002). Temperature-oxygen interactions also appear in behavioral studies, in which organisms exposed to hypoxia often prefer lower body temperatures (Wood, 1991; Steiner and Branco, 2002; Petersen et al., 2003).

In insects, the importance of such interactions is less clear. Two older studies demonstrated them (von Buddenbrock and von Rohr, 1923; Keister and Buck, 1961), and, more recently, Harrison and Lighton (1998) showed that flying dragonflies *Erythemis simplicicollis*, with thoracic temperatures of about 40°C, released more CO_2 in hyperoxia (30 and 50 kPa) and less CO_2 in moderate hypoxia (10 kPa). In addition, Frazier et al. (2001) showed that survival and growth of *Drosophila melanogaster* (from egg to adult) were more sensitive to oxygen availability at higher temperatures.

Here we examine temperature–oxygen interactions in eggs of a terrestrial insect, *Manduca sexta* (Lepidoptera: Sphingidae). Because of their small size and lack of mobility, terrestrial insect eggs often experience large, rapid fluctuations in temperature. Eggs of *M. sexta*, in particular, are common throughout temperate North America (see Ferguson et al., 1999), especially in the Sonoran and Mojave deserts where temperatures vary substantially from day to night. Small size would seem to minimize problems of oxygen transport. However, terrestrial eggs must also restrict water efflux and prevent attack by predators and parasitoids, and doing so requires elaborate eggshell layers (Hinton, 1981) that may also restrict oxygen influx (Tuft, 1950; Hinton, 1953; Zeh et al., 1988; Daniel and Smith, 1994). Such a set of tradeoffs appears to have driven the evolution of egg brooding in the Belostomatidae; males in the subfamily Belostomatinae allow females to lay eggs on their backs, which they then position at the air–water interface and may actively ventilate when

underwater (Smith, 1997). Although the morphology of insect eggs and their eggshells is well known (Hinton, 1981; Margaritis, 1985; eggshell of *M. sexta*: Orfanidou et al., 1992), little is known about egg metabolism and oxygen flux to embryos. The only recent physiological work, by Daniel and Smith (1994), shows that size and shape of the single aeropyle in eggs of *Callosobruchus maculatus* are correlated with metabolic rate between strains. Understanding the effect of temperature on oxygen supply and demand may provide insight both into evolutionary constraints and pressures operating on eggshell design and, more generally, into factors influencing the abundance and distribution of insects.

Our ideas about temperature–oxygen interactions are guided by two observations. First, insect embryos obtain oxygen by diffusion across their eggshells (Hinton, 1981). Second, oxygen supply by diffusion usually is less sensitive to temperature than is oxygen demand by metabolic reactions. The latter relationship reflects a more general principle that has been known for the past century: chemical reactions are, in general, more sensitive to temperature than are physical processes such as diffusion or electrical conductivity (Snyder, 1908, 1911; Belehrádek, 1935; Sidell and Hazel, 1987). The consequence is that individuals obtaining oxygen by diffusion should, all else being equal, experience oxygen limitation at high temperatures and surplus at low temperatures (von Bertalanffy, 1960; Bradford, 1990; Atkinson and Sibly, 1997; Woods, 1999). This scenario is related to Pörtner's ideas about oxygen supply and demand (Pörtner, 2001, 2002) – but invokes a different transport mechanism and predicts monotonically decreasing tissue oxygen levels with increasing temperature. Evidence for temperature-induced oxygen limitation in eggs is available primarily from studies of fish eggs (Hamor and Garside, 1976; Czerkies et al., 2001). Bradford (1990) also showed, in a large comparative study of amphibians, that egg volume among species was inversely related to incubation temperature, and he hypothesized that large eggs may be oxygen limited at high temperatures. In addition, the oxygen conductance of amphibian eggs increases during development as metabolic rate increases (Seymour and Bradford, 1987; Seymour et al., 1991) and, in some species, increases in response to environmental hypoxia (Mills et al., 2001).

Our study focuses on two primary questions: Are insect embryos limited by oxygen availability? If so, is limitation more severe at higher temperatures? We use video-microscopy to measure survival rates and development times in eggs exposed to different combinations of temperature and oxygen. We then present data on metabolic rates (measured as CO_2 emission rates) of eggs exposed to different oxygen levels and either steady or variable temperatures. Finally, using an oxygen microelectrode, we measure radial oxygen profiles in living and dead eggs at cool or warm temperatures. The results suggest that metabolism and development of eggs of *M. sexta* are oxygen limited, especially at high temperatures. Among insects the structure of *Manduca* eggs is unexceptional, implying that oxygen limitation of insect embryos may be widespread.

Materials and methods

Animals

Eggs were derived from a laboratory colony of *Manduca sexta* Johansson. All stages were exposed to a 14 h:10 h L:D photoperiod. Eggs, larvae and pupae were kept at 27°C and adults at 24°C. Adults were given 30% honey water and potted tobacco plants for oviposition. To facilitate egg collection, the adult scotophase was set to begin at 7 am.

Survival and development times

Survival and development times from oviposition to hatching were measured by video-microscopy in a 3×8 factorial experiment (three temperatures: 22, 27 and 32°C; eight O_2 partial pressures: 9, 11, 13, 15, 17, 19, 21 (normoxia), and 30 kPa, with N_2 making up the balance). Oviposition times were determined to within 1 h by introducing potted tobacco plants into the adult flight cage and removing them 1 h later. Eggs were stripped from the leaves, weighed individually (to ±1 μg) on a microbalance (Sartorius MC-5, Goettingen, Germany), and randomly assigned to positions in one of three Plexiglas blocks (11.5 cm×12.5 cm×2 cm; custom built by the Department of Chemistry's Machine Shop at the University of Texas, USA). Each block contained eight cartridges that, in turn, each contained 10 individual wells backed by Nitex mesh. Eggs were placed one to a well (80 eggs per block; 240 eggs in total). With cartridges fixed into the blocks, the wells isolated each egg and hatchling larva from other individuals but exposed all ten eggs to the experimental gas flowing beneath the Nitex mesh. The blocks were placed in incubators set to 22, 27 and 32°C.

Gases were handled with Bev-A-Line IV tubing (Cole Parmer, Vernon Hills, IL, USA). A preliminary experiment showed that eggs fared poorly in the dry air streams coming directly from gas cylinders. Therefore, upstream of the blocks, gas streams were bubbled through 100 ml distilled water in 250 ml flasks submerged in controlled-temperature baths. Bath temperatures were set so that the water-saturated gases emerging from the flasks would, once warmed to the experimental temperature, impose a uniform vapor density gradient of 10 g m^{-3} (1.3 kPa) across the eggshells. Thus, for example, the streams going into the 27°C incubator (saturating vapor density 25.8 g m^{-3}) were bubbled through water at 18.4°C (saturated vapor density 15.8 g m^{-3}). Water baths for the 22 and 32°C incubators were set at 10 and 25.5°C, respectively. Because the latter was above room temperature, which would cause condensation in the lines, it was housed inside the 32°C incubator. This design avoids confounding effects of different vapor pressure gradients across temperature treatments. Gases, flowing at 100 ml min^{-1} (checked daily with a bubble flow meter), were directed into each incubator and then into a 300 ml glass jar, allowing temperature equilibration. Outflows from the jars were connected to the Plexiglas blocks.

A digital video camera (Hitachi kP-D50, Tokyo, Japan, $\frac{1}{2}''$ CCD, 768×494 pixels) with magnifying lens (Samsung 604CN, South Korea, 6–12 mm vari-focal zoom lens) was

mounted above each Plexiglas block inside the incubator and adjusted so that all 80 eggs were visible in the frame of view. The experiment was carried out under constant illumination so that eggs were continuously visible. Images from each camera were captured at 10-min intervals using a frame grabber (Model 3153, Data Translation, Marlboro, MA, USA) controlled by image processing software (Global Lab Image/2 V3.0, Data Translation). Images were written to a hard drive, assembled into movies, and analyzed for hatching time (±10 min). Hatching was obvious; larvae emerged rapidly from their eggs and moved about their wells (see supplemental material to view one of the movies).

Carbon dioxide emission

Carbon dioxide emission was measured using flow-through respirometry. Batches of 40 3-day-old eggs (*M. sexta*) were weighed and placed into a water-jacketed stainless-steel chamber sealed by a threaded steel screw containing a built-in o-ring (custom built by the Department of Chemistry's Machine Shop, University of Texas-Austin, USA). The steel chamber was designed to interface with a gas multiplexer (TR-RM8, Sable Systems, Las Vegas, NV, USA) in which the interior plastic tubing had been replaced by brass and stainless steel. The multiplexer was also modified to accept steel tubing from the jacketed chamber. The total internal volume of the air circuit through the water jacket was ~3 ml. Chamber temperature was controlled by water from a recirculating bath (1160A, VWR, USA).

Gases were mixed from cylinders of O_2 and N_2 using calibrated mass flow controllers (Tylan FC-2900, Torrance, CA, USA and UNIT UFC-1100, Yorba Linda, CA, USA) and mixing electronics (MFC-4, Sable Systems). The stream (25 or 50 ml STPD min^{-1}) was directed past the eggs, through a small volume of indicating Drierite to remove water vapor, and into a carbon dioxide analyzer (CA-2A, Sable Systems). The analyzer was calibrated with pure N_2 and 103.4 p.p.m. CO_2 in N_2 (Praxair, Danbury, CT, USA). Data were logged using Datacan V software (V5.4, Sable systems) receiving digital signals from an A/D converter (UI2, Sable Systems), which itself received analog signals from the instruments. In addition to CO_2, we logged temperature in a separate chamber otherwise identical to the experimental chamber except that the steel screw supported a T-type thermocouple (connected to a TC-1000 thermocouple meter, Sable Systems) that extended into the chamber's air space.

We first measured CO_2 emission from batches of eggs exposed to combinations of temperature and oxygen in a 4×7 factorial experiment (four temperatures: 22, 27, 32 and 37°C; seven oxygen levels: 5, 9, 13, 17, 21, 30 and 50 kPa). Individual batches of eggs were exposed to a single combination of oxygen and temperature (N=3 batches of 40 eggs at each combination) and then discarded. Eggs were equilibrated in flowing gas to each test temperature for 8–12 min, and the CO_2 concentration (p.p.m.) was subsequently measured for 6.5 min. Mass-specific CO_2 emission was calculated from concentration, flow rate and batch mass.

In a second experiment, we measured CO_2 emission continuously while ramping the temperature from 16 to 48.5°C over 55–60 min (0.57°C min^{-1}). By virtue of their small size, eggs have so little thermal inertia that they should always be in thermal equilibrium. The high temperature exceeded the upper thermal limit tolerated by eggs, but we wanted to stimulate maximal metabolic rates. Temperatures were ramped at four O_2 levels, 11, 15, 21 and 30 kPa (N=3 batches of 40 3-day-old eggs at each level; 480 eggs total). For each ramp, average mass-specific emission rates were calculated at intervals of 1°C.

Internal oxygen partial pressure

Radial profiles of P_{O_2} inside eggs were obtained using a Clark-style O_2 microelectrode with guard cathode (model 737GC, 40 μm tip, Diamond General, Ann Arbor, MI, USA) connected to a picoammeter (Chemical Microsensor I, Diamond General). The electrode was calibrated in still water that was either air- or N_2-saturated. The water was held at constant temperature using a water-jacketed calibration cell (custom made by the Department of Chemistry's Glass Shop, University of Texas-Austin, USA) connected to a recirculating water bath. The electrode was always calibrated at the temperature at which it would be used (24 or 37°C).

Eggs of *M. sexta* were affixed individually to double-sided tape so that the same surface that had been against the leaf was against the tape. In this orientation, the developing embryo was wrapped around the margins of the egg (parallel to the working surface). Under a dissecting microscope a fine insect pin was used to bore a small hole in the chorion. Subsequently, the electrode (calibrated before and after measurements from each egg) was placed through the hole using a micromanipulator (MM33, Märzhäuser, Wetzlar, Germany). The electrode was then advanced vertically down through the hole in 100 μm increments, for a total distance of 700 or 800 μm, about 300 μm past the egg's center. In this orientation, the electrode tip was advanced through the egg's center and avoided the embryonic tissue toward the lateral margins. Attempts were made to obtain readings in embryonic tissue, but these proved unreliable due to the difficulty of seeing tip placement through the chorion. Measurements were made on living eggs that were either <12 h or 3 days old (measured from oviposition, total development time at 27°C about 4 days) at either 24 or 37°C. As controls, 2- or 3-day-old eggs were killed by freezing (in liquid N_2 or overnight in a –80°C freezer), thawed, then allowed to sit for 1–3 h at the test temperature (24°C only) before measurement of O_2 profile.

Statistics

Statistics were done using S-Plus software (V6.1, Insightful Corp., Seattle, WA, USA). Egg survival was analyzed using logistic regression (implemented in S-Plus using the generalized linear model function), which is appropriate for data with a binary response variable (e.g. alive or dead) (Armitage and Colton, 1998; Selvin, 1998). Survival data were first fitted to a logistic regression model containing different

combinations of predictor variables, and the models were then tested for significance by comparing how much additional deviance was explained by adding a term (using a chi-square test). Egg development time was analyzed by linear regression (using untransformed times) with temperature, oxygen, egg mass, and their interactions as predictors. After initially fitting a full model containing all possible interaction terms, a reduced model was constructed that discarded all nonsignificant interaction terms from the first model.

Between 16 and 38°C, egg batches (three per temperature) from the temperature ramping experiments emitted CO_2 as an approximately S-shaped function of temperature. For each batch of eggs, emission data were therefore fitted (using the nonlinear least-squares function in S-Plus) by the logistic curve:

$$\dot{M}_{O_2} = k / (1 + ae^{-b(T-16)}) , \quad (1)$$

where T is temperature (°C), K is the maximum rate of emission (analogous to 'carrying capacity'), and a and b are coefficients controlling the shape of the curve. In the equation, 16°C is subtracted from the actual experimental temperature simply in order to rescale the values to between 0 and 22°C. This linear transformation has no effect on interpreting the results of the analysis. Relationships between fitted values of K and ambient oxygen, and also between calculated emission rates at particular temperatures and ambient oxygen, were analyzed by linear least-squares regression.

Results

Survival and development times

The 240 eggs used in this experiment weighed on average 1.56±0.01 mg (mean ± S.E.M., range 1.37–1.76 mg). At P_{O_2} values ≥15 kPa, all eggs survived (Fig. 1A). At lower partial pressures, temperature and O_2 interacted to affect survival, an effect we analyzed with logistic regression. Table 1 shows significance tests of fitting models with increasing numbers of terms, added sequentially. Adding each of the three terms – oxygen, temperature, and their interaction – explained significantly more deviance. Fitted coefficients from the full model are shown in Table 2. An objection to this approach is that eggs at most P_{O_2} values survived with a probability of 1; however, analysis of a reduced dataset containing only survival data for P_{O_2} values ≤15 kPa gave qualitatively similar results (not shown).

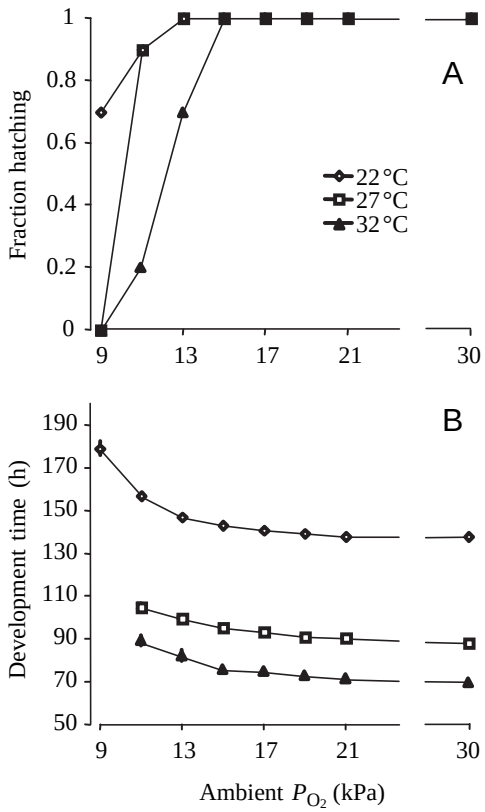

Fig. 1. Effects of temperature and ambient oxygen partial pressure P_{O_2} on development of eggs of *Manduca sexta*. Eggs were exposed to experimental conditions from within 6 h of oviposition until hatching. Ten eggs were exposed to each combination of factors (240 eggs total), and all treatments were started simultaneously. (A) Fraction hatching in each combination of temperature and oxygen. (B) Total development time from oviposition to hatching, including only those that actually hatched. In all cases, standard errors were so small that they are hidden by their respective symbol.

Total development time (h) was affected by both temperature and oxygen (Fig. 1B). At 21 kPa O_2, the Q_{10} of development rate (reciprocal of development time) was 2.3 between 22 and 27°C and 1.6 between 27 and 32°C (overall Q_{10} between 22 and 32°C was 1.9). We analyzed these data using linear regression, with temperature, oxygen and egg mass as predictors. In no preliminary analysis was egg mass significant, nor were any of the interaction terms including it. For all subsequent analyses (including those shown), egg mass itself was retained but all its interaction terms were excluded from the models. Two primary

Table 1. *Analysis of deviance table showing fits of egg survival to logistic regression models with increasing numbers of terms*

Terms	Residual d.f.	Residual deviance	Test	d.f.	Deviance	P*
Null	239	228.7				
O	238	97.6	O	1	131.1	<0.001
O+T	237	63.5	T	1	34.1	<0.001
O+T+O×T	236	58.2	O×T	1	5.3	0.022

O, Oxygen; T, Temperature.
*P values are given by Pr[χ^2(d.f.)>Deviance]. Pr, probability.

Table 2. *Fitted coefficients from the full logistic regression model shown in Table 1*

Term	Coefficient	S.E.M.
Intercept	38.06	18.49
Oxygen (O)	–2.161	1.60
Temperature (T)	–2.059	0.75
O×T	0.137	0.06

Table 3. *Summary of regression analysis of egg development time*

Term	Fitted model coefficient (± S.E.M.)	Significance test d.f.	MS	*F*	*P*
(A) Full dataset					
Intercept	373.69±20.33				
Oxygen (O)	–3.67±0.76	1	20849	221.1	<0.001
Temperature (T)	–8.95±0.56	1	165 672	1756.5	<0.001
Egg mass	–5.82±8.80	1	23.3	0.247	0.62
O×T	0.10±0.03	1	1137.9	12.06	<0.001
Residuals		190	94.3		
(B) Reduced dataset					
Intercept	329.44±17.63				
Oxygen (O)	–1.53±0.68	1	8142	124.8	<0.0001
Temperature (T)	–7.40±0.49	1	148 898	2281.9	<0.0001
Egg mass	–7.51±7.45	1	61.6	0.94	0.33
O×T	0.03±0.03	1	89.6	1.37	0.24
Residuals		183	65.3		

(A) Analysis of the full data set, including surviving eggs from the 9 kPa treatment. (B) Analysis of a reduced dataset that excludes eggs from 9 kPa.

MS, mean squares.

models were fitted. The first included development times of all eggs that hatched. Oxygen and temperature were highly significant (Table 3A), and the interaction between them, though explaining much less of the total variance, was also significant. The second model excluded eggs from the 9 kPa treatment, because only eggs from the lowest temperature treatment (22°C) withstood this degree of hypoxia. In the reduced dataset, both oxygen and temperature still were highly significant but their interaction was not (Table 3B).

Carbon dioxide emission

Linear regression revealed that temperature, O_2, and their interaction all significantly affected CO_2 emission (Fig. 2; Table 4; fitted model: emission rate = 2.89 – 0.64×oxygen level + 0.11 × temperature + 0.042 × oxygen × temperature, where oxygen level is in kPa and temperature in °C). The significant interaction term reflects that at higher temperatures, the effect of P_{O_2} was more pronounced (Fig. 2). Thus, at 37°C carbon dioxide emission rose more than fourfold between 5 and 50 kPa O_2, whereas at 22°C emission approximately doubled. In addition, hyperoxia (30 and 50 kPa) led to higher emission rates only at higher temperatures.

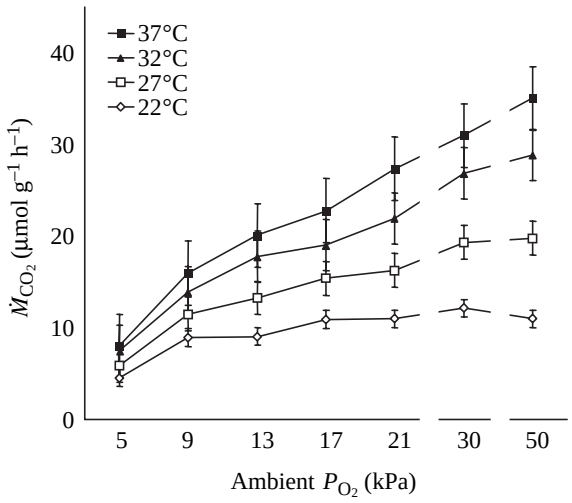

Fig. 2. Carbon dioxide emission (mean ± S.E.M.) by batches of eggs (*Manduca sexta*) at different combinations of temperature and oxygen. See text for details.

During the temperature ramps, emission rates rose sigmoidally to a peak at about 38°C (Fig. 3). Rates subsequently dipped before rising to a second peak and then falling to zero. The same qualitative pattern appeared in all traces. Fig. 4 shows emission rates presented as functions of temperature (excluding rates beyond the trough, at 48°C). Clearly, higher O_2 availability led to higher carbon dioxide production, most distinctly at the peak rates near 38°C. We analyzed this effect two ways. The simpler analysis consisted of two separate regression analyses of emission rates as a function of oxygen availability at either 16 or 38°C. P_{O_2} had no effect at 16°C ($F_{1,10}$=0.73, P=0.41), but did at 38°C [fitted

Table 4. *ANOVA summary of linear regression analysis of data shown in Fig. 2, excluding data from batches treated with 50% oxygen*

Term	d.f.	MS	*F*	*P*
Oxygen (O)	1	1673	202.1	<0.001
Temperature (T)	1	1337	252.8	<0.001
O×T	1	263	39.7	<0.001
Residuals	68	6.6		

MS, mean squares.

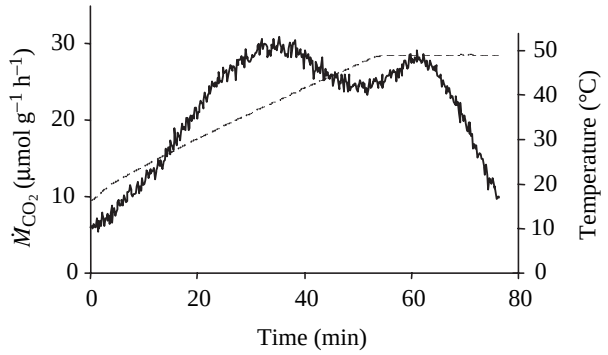

Fig. 3. Single batch of 40 eggs of *Manduca sexta* ramped from 16 to 48.5°C over 55 min (broken line is the temperature trace). Traces at all oxygen levels exhibited qualitatively similar patterns.

model: emission rate = 15.9 + 0.61 × oxygen level (kPa); $F_{1,10}$=22.5, P<0.001]. As a more complete analysis, we fitted emission traces from each batch of eggs with a logistic curve (Equation 1; model coefficients shown in Table 5). The coefficient with the most obvious biological meaning is K, which gives the asymptotic maximum emission rate. K was strongly and positively related to ambient oxygen availability (Fig. 5A). In addition, we used the fitted models to estimate emission rates at 16 and 38°C; those at 38°C were significantly related to P_{O_2} where those at 16°C were not. To examine whether fitted models described the data adequately, we analyzed residual variation from each batch of eggs around its fitted model (Fig. 5B). Although the residuals had a tendency to exhibit a U shape (data above model predictions at low and high temperatures but below inbetween), most residual values were within 1 μmol CO_2 g^{-1} h^{-1} of the model prediction, indicating a very good fit. Together, these analyses indicate that P_{O_2} had an effect at high but not low temperatures.

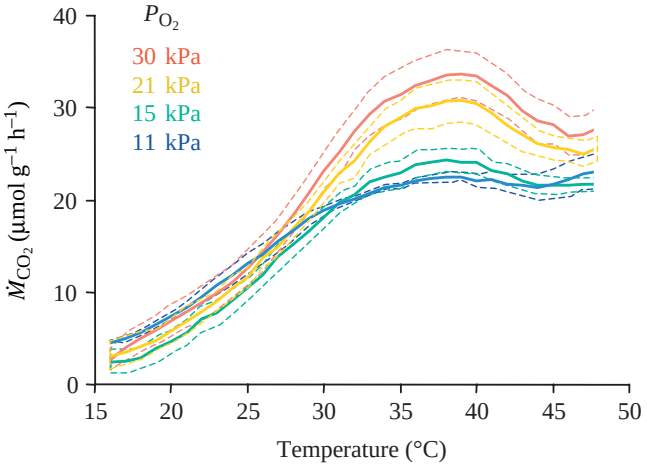

Fig. 4. CO_2 emission by batches of eggs of *Manduca sexta*, as a function of temperature. Eggs were ramped from 16 to 48.5°C over 55 min. Each color represents three batches of 40 eggs. Solid lines represent mean CO_2 emission at 1°C increments, and broken lines indicate S.E.M.

Table 5. *Summary of fitted coefficients of the logistic curve, fitted to data from the temperature ramping experiments (see Fig. 4) by nonlinear least-squares regression*

		Fitted model coefficients		
P_{O_2} (kPa)	Batch	K	a	b
30	1	37.27±1.53	9.35±0.58	0.18±0.01
	2	34.00±0.50	27.16±1.66	0.26±0.01
	3	46.88±1.74	9.05±0.50	0.18±0.01
21	1	31.68±0.89	10.64±0.54	0.19±0.01
	2	39.09±0.66	27.62±1.71	0.25±0.01
	3	36.24±0.98	6.91±0.26	0.17±0.01
15	1	27.75±0.55	7.55±0.30	0.19±0.01
	2	29.22±0.40	9.67±0.36	0.22±0.01
	3	22.28±0.29	36.56±3.78	0.33±0.01
11	1	25.91±0.78	5.16±0.25	0.17±0.01
	2	23.77±0.46	5.43±0.26	0.20±0.01
	3	22.99±0.22	4.93±0.22	0.25±0.01

Each batch consisted of 40 eggs.
For an explanation of the fitted coefficients, see Equation 1.

The location of peak emission rates was not affected by variation in P_{O_2} (Fig. 4). However, the location of the post-peak trough in emission rate (right side of graph) occurred at a distinctly lower temperature at 11 kPa O_2 than did the troughs at higher P_{O_2} values.

Oxygen content

The electrode was stable and exhibited a stirring effect of only about 2% (Fig. 6). Dead eggs showed slight radial gradients of P_{O_2} (Fig. 7). The cause of the decline is unclear but may reflect interactions between the electrode tip and yolk material or small rates of *post-mortem* O_2 consumption by persistent reactions. Living eggs showed much steeper P_{O_2} gradients than dead eggs (Fig. 7; *t*-test of live *versus* dead eggs at a distance of 500 μm, pooled across stages and temperatures: t_{27}=–9.1, P<0.001). P_{O_2} values inside freshly laid eggs (<12 h old) declined to 7–10 kPa at the center, where

Table 6. *Summary of ANOVA of radial* P_{O_2} *gradients inside living eggs (*N=*23) of* Manduca sexta

Source	df	MS	F	P
Across eggs				
Temperature (T)	1	3578.4	11.8	0.003
Age (A)	1	12617.3	41.4	<0.001
T×A	1	39.3	0.13	0.723
Residuals	19	304.5		
Within eggs				
Position in egg (P)	5	6676.4	138.3	<0.001
P×T	5	29.1	0.60	0.70
P×A	5	936.9	19.4	<0.001
P×T×A	5	219.3	4.5	<0.001
Residuals	95	48.3		

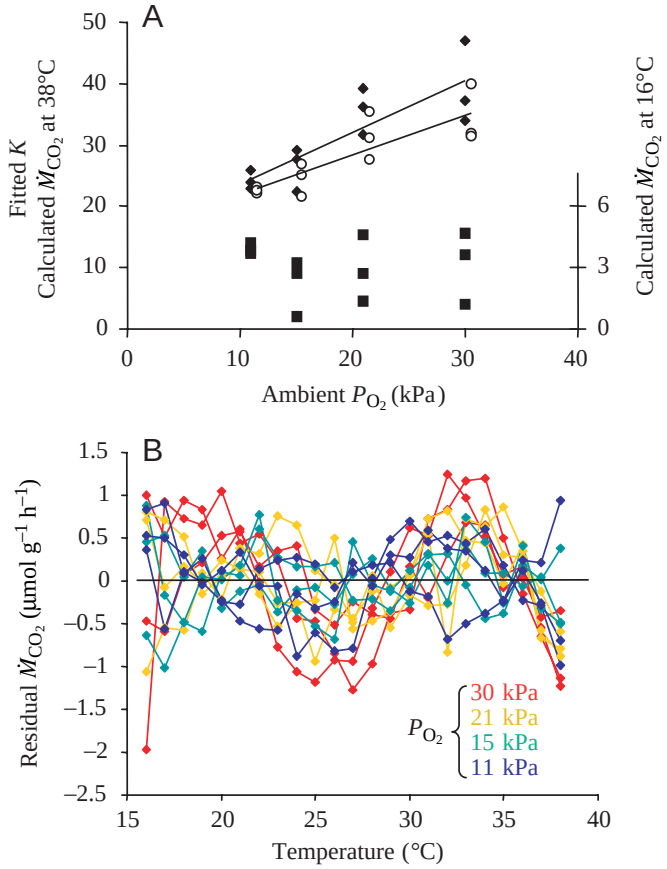

Fig. 5. Statistical analysis of data shown in Fig. 4. CO_2 emission by each batch of eggs was fitted by the logistic curve (see text for details). (A) Fitted values of the coefficient K (filled diamonds) and calculated emission rates (μg mol^{-1} h^{-1}) from the fitted model (coefficients given in Table 4) for temperatures of 16 (solid squares) and 38°C (open circles). K depends on ambient P_{O_2} by the relationship: K=15.1+0.85P_{O_2} (r^2=0.71; $F_{1,10}$=24.3, P<0.001). Calculated emission rates at 38°C depend on ambient P_{O_2} by the relationship: $\dot{M}_{CO_2}$ (38°C)=15.4+0.65P_{O_2} (r^2=0.70; $F_{1,10}$=23.6, P<0.001). Calculated emission rates at 16°C were not significantly related to ambient oxygen ($F_{1,10}$=0.05, P=0.82). (B) Residual variation about the model fitted to the emission trace from each batch of eggs.

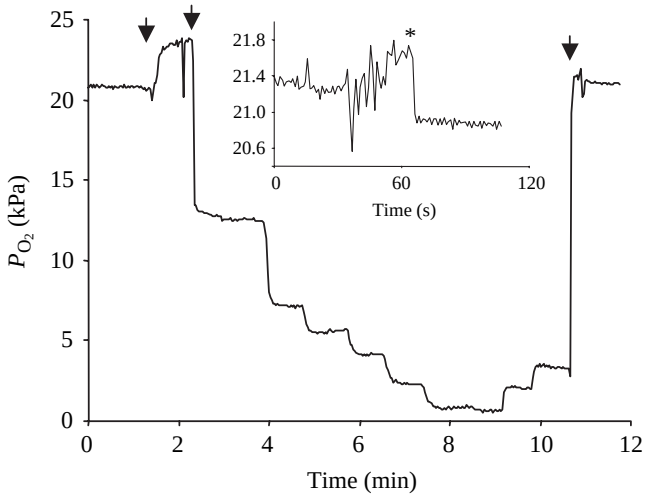

Fig. 6. Representative profile of radial oxygen partial pressure in an egg of *Manduca sexta*. The initial reading is from still water (temperature equilibrated and air saturated). The first arrow indicates electrode withdrawal into air, and the second indicates electrode penetration to just below the chorion. Each subsequent plateau represents a 100 μm increment, advancing past the central minimum P_{O_2}. The third arrow indicates where the electrode was withdrawn back into air and immediately placed back into still water. Inset: Stirring effect. The asterisk indicates when the air bubbles were turned off. All electrode calibration was done in still water after bubbles were turned off.

as P_{O_2} values inside 3-day-old eggs approached zero. At both ages, eggs subjected to 37°C appeared to contain less oxygen than those at 24°C. These effects were analyzed (positions 0–500 μm for live eggs only) in a full ANOVA model, with position, age and temperature as predictors, and taking into account the repeated measures within eggs across positions (Table 6). The analysis confirmed the significance of the main effects of age, position, and temperature. Two of the interactions (temperature × age and position × temperature) were not significant, but two others (position × age and the three-way interaction of position × temperature × age) were highly significant. The biological interpretation of these interactions is apparent from Fig. 7. The interaction between position and age reflects that P_{O_2} values are more divergent in center than in edge positions. The three-way interaction reflects that in young eggs the main temperature-driven

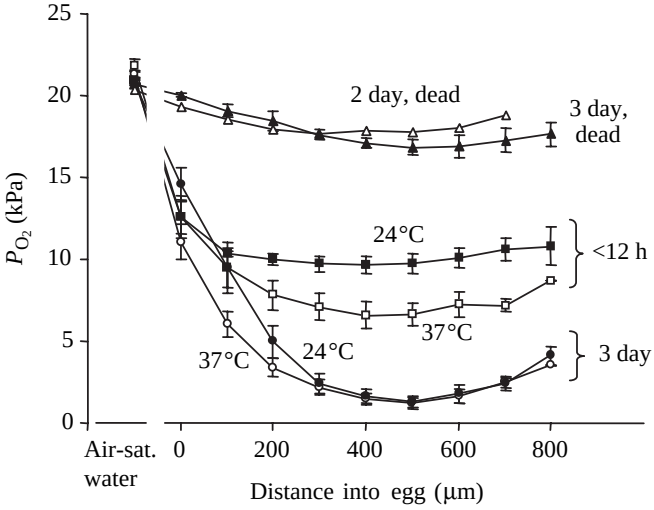

Fig. 7. Radial oxygen profiles in eggs of *Manduca sexta*. A calibrated Clark-style microelectrode was advanced through a small hole in the egg chorion (egg surface=0 μm) and then in 100 μm increments. The two bottom traces are 3-day-old eggs (at 24 and 37°C, N=7 and 9, respectively), the middle two traces are freshly laid eggs (laid in previous 12 h, N=4 and 3 at low and high temperatures, respectively), and the top two traces are 2- and 3-day-old traces of dead eggs (killed by freezing in liquid N_2 or in a –80°C freezer, N=2 and 4, respectively). Initial measurements were from still water (temperature equilibrated and air saturated). Values are means ± S.E.M. The dead 2-day-old eggs do not have error bars because N=2 eggs for this treatment.

difference appears in the central volume, whereas in old eggs it appears only in the first 200 μm.

Discussion

A common view is that insects are limited by oxygen only when ambient P_{O_2} falls to quite low values, e.g. <5 kPa (Wegener, 1993; Hoback and Stanley, 2001; other references are summarized in table 1 of Greenlee and Harrison, 2004). Our data, however, leave little doubt that eggs of a moth, *Manduca sexta*, regularly experience O_2 limitation, even in normoxia. First, development times were extended by even slight depression of ambient P_{O_2}. Second, metabolic rates of eggs (Fig. 2; measured as CO_2 emission) were depressed in hypoxia, especially at higher temperatures. Moreover, at 32 and 37°C, metabolism was stimulated by hyperoxia, implying that embryos at these temperatures were O_2-limited even in normoxia. Third, measurements of radial P_{O_2} profiles inside eggs in normoxia (21 kPa) (Fig. 7) show that 3-day eggs contain broad central areas with P_{O_2} lower than 3 kPa at both 24 and 37°C. These measurements were from non-embryonic tissue, indicating that P_{O_2} values inside embryos were very low.

Moreover, significant interactions between temperature and oxygen were apparent in most experiments. First, eggs experiencing relatively severe hypoxia were more likely to hatch when they developed at cooler temperatures (Fig. 1A). Second, metabolic rates were disproportionately affected by ambient oxygen availability at high temperatures, both at different steady-state combinations of temperature and oxygen (Fig. 2) and in experiments in which temperature was ramped gradually to very high values (Fig. 3). Third, both young and old eggs exhibited steeper radial oxygen profiles in warm (37°C) than in cool (24°C) temperatures. Together, these data strongly support the idea that oxygen limitation is more severe at high temperatures.

The sole instance in which an interaction did not emerge unequivocally was in the experiment on development time. Evaluating this dataset was complicated by the fact that development times could be measured only on surviving eggs – and at the lowest oxygen level (9 kPa) only eggs from the coolest temperature (22°C) hatched. If these eggs were included in the regression analysis of development times, the temperature–oxygen interaction term was significant; if they were excluded, it was not. Over most of the range of experimental P_{O_2} values, therefore, development times did not exhibit temperature-oxygen interactions. This result seems, at first glance, inconsistent with the metabolic and electrode data, which showed obvious interactions between the two variables. We suggest that the inconsistency stems from either of two interesting possibilities. First, the metabolic and electrode experiments exposed eggs to temperatures higher than the highest temperatures imposed in the developmental experiment (32°C); if oxygen is in increasingly short supply at high temperatures, we may simply have been more likely to see the interactions at temperatures above 32°C (though see Fig. 2). An alternative view is that temperature–oxygen interactions were stronger in the short-term experiments (metabolic and electrode experiments imposed experimental conditions for less than a few hours) than in the long-term experiments (the entire developmental period). This difference suggests that embryos receiving lengthy exposure to unusual combinations of temperature and oxygen may adjust their physiology or the gas transport characteristics of the eggshell layers.

Why, given their large surface area-to-volume ratios and short diffusion distances, do eggs of *M. sexta* exhibit O_2 limitation? Barring some exceptions (e.g. Salt, 1952; Byrne et al., 1990), eggs of most terrestrial species do not have access to water other than what is sequestered in the egg before oviposition. Water is therefore a commodity not to be wasted. Retarding water loss, however, depends on possessing a relatively impermeable eggshell, which in turn creates other potential problems. In particular, no organism has evolved a barrier capable of eliminating water loss while still conducting O_2 (Hinton, 1953), and therefore water-resistant eggshells (essentially all terrestrial eggshells, Hinton, 1981) may pay the price of reduced O_2 delivery (Smith, 1997). Adults and larvae or nymphs usually have access to water directly as free water or in their food. Although pupae usually do not have access to water, they have much lower surface area-to-volume ratios and may be protected from desiccation by underground chambers, leaves, or cocoons.

Pörtner (2001, 2002) argued that O_2 limitation at high temperatures results from the failure of ventilation and circulation to deliver adequate oxygen to tissues. In contrast, our electrode data strongly suggest that hot eggs of *M. sexta* are limited by inadequate *diffusive* supply of O_2. In particular, the largest drop in P_{O_2} was observed just below the eggshell, and most of the remaining drop occurred within 200 μm of the chorion (Fig. 7). This result indicates that the eggshell layers provided the largest resistance, but that the yolky material surrounding the embryo was also a barrier to O_2 flux. Although later stage (day 4) embryos occasionally move, possibly causing convective movement of egg liquids, we saw no indication of movement in the stages used in our electrode experiments (< 12 h and 3-day-old).

Another question is what caused declining CO_2 emission at temperatures above 38°C. One possibility is that the declines stemmed directly from low or falling internal P_{O_2} (Pörtner 2001, 2001). If so, however, the temperature at which eggs exhibited maximal metabolic rates should have been positively correlated with ambient P_{O_2}. In fact, peak locations were similar across treatments, occurring at about 38°C at all four O_2 levels (Fig. 4). Declining CO_2 emission at temperatures >38°C instead must represent direct effects of temperature on protein stability, or limitation by some other unmeasured variable. It is conceivable that O_2 affects short-term tolerance to extreme high temperatures, but this possibility awaits future tests.

Implications

The eggshell layers and arrangement of yolk and embryo are

not particularly unusual in *M. sexta* compared to other terrestrial insects (Hinton, 1981), implying that O_2 limitation may be widespread among terrestrial insect eggs. Such limitation could have interesting ecological and evolutionary consequences. First, because water-resistant eggshells probably also restrict O_2 flux (Hinton, 1953), evolutionary adjustment in eggshell structure among populations and species may be closely related to trade-offs between retarding water loss and obtaining sufficient O_2 (Schmidt-Nielsen, 1984). Second, temperature-induced hypoxia may force warm-climate females to oviposit only in relatively cool microclimates. This suggestion is particularly relevant to desert populations of *M. sexta*. During their active season in the Sonoran and Mojave deserts, daytime ambient air temperatures often exceed 40°C. Smith (1978), however, showed that leaves of some large-leafed desert perennials, including a host of *M. sexta*, *Datura metaloides*, are *cooler* than ambient air, apparently from high rates of transpiration. For example, on a day when the air temperature was 43.7°C, leaf temperatures of *D. metaloides* ranged from 26.1–36.3°C (matching well our range of experimentally imposed temperatures). This finding suggests that the ecological success of *M. sexta* in deserts – even though *M. sexta* and most other species of *Manduca* are primarily Neotropical (Rothschild and Jordan, 1903) – stems in part from the use of cool, large-leafed perennial host plants.

Our data also suggest a novel hypothesis about Paleozoic insect gigantism: hyperoxia during the late Carboniferous together with lower surface temperatures (Berner, 1994; Berner et al., 2003) may have facilitated the evolution of large eggs. Theoretical models and various indirect data indicate that atmospheric oxygen content reached up to 35% during the late Carboniferous (Berner et al., 2003). By increasing gradients driving diffusive O_2 flux, high ambient P_{O_2} may have facilitated the evolution of gigantism in numerous Paleozoic arthropods (Graham et al., 1995; Dudley, 1998), including Paleoptera, Arachnida, the extinct Arthropleurida, and others (Shear and Kukalová-Peck, 1989). Neontological tests of this hypothesis have involved manipulation of oxygen availability and measurement of adult flight performance and metabolic rates, but the results have been conflicting (summarized by Harrison and Lighton, 1998).

Selection on adult size, however, may not have been the only factor leading to insect gigantism. In general, egg and adult size are positively correlated within arthropod families (Fox and Czesak, 2000). For example, García-Barros (2000) used data from several butterfly families to show that egg size (diameter) scaled to adult size (wing length) by the relationship: egg size $\propto$ adult size$^{0.622}$. This relationship indicates that small changes in egg size are associated with large changes in adult size. Whether selection for larger egg size results in correlated shifts to larger adult size, or *vice versa*, is unclear (indeed the result could stem from selection on other correlated stages or traits). Regardless, large eggs must be able to support embryonic development with adequate oxygen flux – clearly more easily done in hyperoxic Paleozoic atmospheres. Future tests of this hypothesis should examine whether eggs of other extant insect taxonomic groups are oxygen limited and whether larger eggs (interspecifically) are more likely to show oxygen limitation. One neontological example of the latter is provided by Belostomatidae (Smith, 1997), many of which lay eggs that are very large. To obtain sufficient oxygen to support development, these eggs must be actively ventilated by a parent or deposited in air on emergent vegetation.

A special thanks to Lee Benson, Grady Rollins, Mike Ronalter, Charles Thonig and Terry Watts, who built much of the equipment used in this project. We also thank Jon Harrison, Norbert Heisler, Stefan Hetz and Wolfgang Waser for electrode training and advice, and Kendra Greenlee for access to unpublished manuscripts and comments on the manuscript. Nancy Wong maintained the *Manduca* colony. Thanks also to Chris Goforth, Robert Dudley, and two anonymous reviewers for constructive comments on the manuscript. This work was supported by the NSF (IBN-0213087 to H.A.W.) and the University of Texas at Austin.

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