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RESEARCH ARTICLE

Metabolic recovery from drowning by insect pupae

H. Arthur Woods* and Steven J. Lane

ABSTRACT

Many terrestrial insects live in environments that flood intermittently, and some life stages may spend days underwater without access to oxygen. We tested the hypothesis that terrestrial insects with underground pupae show respiratory adaptations for surviving anoxia and subsequently reestablishing normal patterns of respiration. Pupae of *Manduca sexta* were experimentally immersed in water for between 0 and 13 days. All pupae survived up to 5 days of immersion regardless of whether the water was aerated or anoxic. By contrast, fifth-instar larvae survived a maximum of 4 h of immersion. There were no effects of immersion during the pupal period on adult size and morphology. After immersion, pupae initially emitted large pulses of CO_2. After a subsequent trough in CO_2 emission, spiracular activity resumed and average levels of CO_2 emission were then elevated for approximately 1 day in the group immersed for 1 day and for at least 2 days in the 3- and 5-day immersion treatments. Although patterns of CO_2 emission were diverse, most pupae went through a period during which they emitted CO_2 in a cyclic pattern with periods of 0.78–2.2 min. These high-frequency cycles are not predicted by the recent models of Förster and Hetz (2010) and Grieshaber and Terblanche (2015), and we suggest several potential ways to reconcile the models with our observations. During immersion, pupae accumulated lactate, which then declined to low levels over 12–48 h. Pupae in the 3- and 5-day immersion groups still had elevated rates of CO_2 emission after 48 h, suggesting that they continued to spend energy on reestablishing homeostasis even after lactate had returned to low levels. Despite their status as terrestrial insects, pupae of *M. sexta* can withstand long periods of immersion and anoxia and can reestablish homeostasis subsequently.

KEY WORDS: Immersion, Anoxia, Reperfusion, Oxygen, Lactate, Carbon dioxide, Cyclic ventilation, Discontinuous gas cycles, Spiracle

INTRODUCTION

Although insects typically are thought of as 'terrestrial' or 'aquatic', these labels do not capture the biology of some species. Aquatic species, for example, usually have one or more terrestrial stages, most frequently the adult. Other species are terrestrial but spend at least some time in water, either to lay eggs or to feed and grow, or because their ecology makes them prone to intermittent flooding (Adis and Messner, 1997; Hoback and Stanley, 2001). Immersion likely exerts strong selection for the evolution of behaviors, morphologies and physiologies that help insects survive contact with water and its associated risks of hypoxia or anoxia (Hoback and Stanley, 2001).

One common flood-prone habitat is soil (Cavallaro and Hoback, 2014; Hoback and Stanley, 2001), which can become saturated by snowmelt or rainfall (Hoback et al., 1998) or by overflowing streams and rivers. Many insects spend part or all of their lives in soil (Hoback et al., 1998; Hunter, 2001; Lilly, 1956; Tuttle, 2007; Uvarov, 1977; White and Strehl, 1978). For soil insects, flooding may transport pathogens to the insect or encourage preexisting infections to proliferate. It may alter soil structure so that the insect is trapped (Sprague and Woods, 2015). And it may alter the exchange of respiratory gases. Compared with dry soils, water-logged soils provide less oxygen (Baumgärtl et al., 1994; Hoback and Stanley, 2001), both because water holds much less oxygen per unit volume than does air and because the oxygen it contains moves more slowly by diffusion (Aachib et al., 2004; Denny, 1993; Verberk et al., 2011; Woods, 1999). In addition, soils can support large populations of microbes, whose respiration may depress oxygen locally (Singh and Gupta, 1977).

Anoxia can be highly stressful, and even in anoxia-tolerant individuals it may drive large-scale changes in metabolism and development (Harrison et al., 2006; Hoback and Stanley, 2001; Hochachka, 1997; Yocum and Denlinger, 1994). Negative effects stem mainly from declining production of ATP, which can starve the pumps that maintain ion gradients (Storey and Storey, 1990). Of the resulting changes in ion distributions, calcium entry into cells appears to be the most dangerous (Choi, 2005; Thompson et al., 2006; Van Voorhies, 2009). Other major cellular effects include changes in levels of NADH, ADP, AMP and pH (Weyel and Wegener, 1996). To slow down or prevent these changes, many hypoxia- and anoxia-tolerant species sustain low levels of ATP production by switching over to anaerobic pathways. In insects, common end products from anaerobic metabolism are lactate, succinate and alanine (Hoback and Stanley, 2001). Anaerobic end products can also accumulate in normoxia if demand for oxygen outstrips its supply, as occurs transiently in jumping insects (Kirkton et al., 2005).

Recovery from anoxia poses an additional set of physiological problems (Lighton and Schilman, 2007; Wegener, 1993). The first problem is to reestablish energy and ion homeostasis. This includes reactivating mitochondria to produce ATP aerobically and then using it to power the pumps that generate trans-membrane gradients and the metabolic pathways that rid the body of accumulated organic end products. The second problem is to minimize oxidative damage arising from reperfusion of the tracheal system. In many animals, including insects and humans, reperfusion after ischemia can be more dangerous than ischemia per se, because reperfusion generates reactive oxygen species (ROS) (Boardman et al., 2012; Collard and Gelman, 2001; Doelling et al., 2014; Joanisse and Storey, 1996; Lighton and Schilman, 2007). ROS attack organic components of cells, including proteins, DNA and lipids, and can disrupt cellular functions or kill cells outright. In insects, reperfusion after anoxia can occur rapidly because oxygen is delivered not by a circulatory

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system, but directly, in the air phase, by the tracheal system (Wegener, 1993). Such a respiratory system should make insects prone to oxidative damage, and that damage could alter patterns of respiratory gas exchange (Lighton and Schilman, 2007), disrupt development, modify adult morphologies, reduce performance or lead to death.

Using the moth *Manduca sexta* (Linnaeus 1763), we tested the hypothesis that life stages exposed to immersion (see Fig. 1), even rarely, evolve mechanisms for surviving the respiratory challenges it poses, and for reestablishing respiratory homeostasis once air becomes available again (Hoback and Stanley, 2001). We examined mortality as a function of immersion time in both larvae and pupae, whether pupae accumulate lactate and, once pupae have access to air again, how rapidly lactate levels decline and the process by which patterns of gas exchange are reestablished. Immersion resulted in profound changes in patterns of CO_2 emission in post-immersion pupae, and we discuss the relationships between our results and the spiracle-control models proposed by Förster and Hetz (2010) and Grieshaber and Terblanche (2015). Finally, we examined whether there are long-term effects of immersion on the pupal–adult ecdysis and on adult morphologies.

MATERIALS AND METHODS

Animals

Manduca sexta has four terrestrial life stages, and it pupates underground. When a larva has finished feeding and growing, it descends at night from its host plant, searches for a suitable pupation site, and digs several centimeters into the ground (Madden and Chamberlin, 1945; Sprague and Woods, 2015), where it excavates a space. Over the subsequent days, the larva (becoming a prepupa) constructs a soil chamber, the main function of which is to provide space for larval–pupal and pupal–adult ecdyses (Sprague and Woods, 2015). For the pupa, an underground lifestyle raises the risk of flooding. The pupal chamber does not exclude water and therefore does not act as a physical gill (see Kolesnikov et al., 2012; Seymour and Matthews, 2013; J. C. Sprague and H.A.W., unpublished observations), nor do pupae trap air films on the cuticle as do many semi-aquatic insects (Hutchinson, 1981; Pedersen and Colmer, 2012). In nature, the risk of flooding may be high for pupae because larval host plants often occur in disturbed areas, such as along stream beds. In the grasslands of southeastern Arizona (USA), we have seen wandering larvae descend from their host plants into dry arroyos, the banks of which they were unable to climb, eventually pupating in the arroyo sands. During summer monsoon rains, the arroyos can sustain strong flows and hold persistent pools of standing water (Fig. 1).

Fig. 1. Flooded habitat of *Manduca sexta*, 25 July 2013, along Animas Creek in far southwestern New Mexico, USA. The Chiricahua Mountains (Arizona) are in the background. The flowering plants in the foreground are silverleaf nightshade (*Solanum elaeagnifolium*), which is an occasional host plant of *M. sexta*. Less visible in the background are several of *M. sexta*'s primary host plant in this area, *Datura wrightii*. When larvae have finished feeding and growing, they descend from their host plant and search on the ground for appropriate pupation sites. Often along dry creeks, larvae become trapped in the bottom and pupate there. Intermittent rains during the summer monsoon season can then flood those habitats.

We used individuals from a laboratory line (Carolina Biological), which were reared individually on artificial diet (Bell and Joachim, 1976). Larvae were reared in an incubator at 26.7±0.5°C and under a 15 h:9 h light:dark photoperiod to prevent pupal diapause (though not entirely successfully; see Results). At the onset of wandering, larvae were placed into 2.54 cm diameter holes drilled in wooden blocks, where they pupated. Larvae were held in blocks for 10 days, after which they were assigned randomly to immersion treatment groups. Prepupae shed their larval cuticles after 3–5 days and the pupal cuticle was hardened by day 6 or 7. *Manduca sexta* for the larval immersion assay were reared in a similar way, but were taken out of the incubator as mid-fifth instars. They weighed an average (±s.e.m.) of 5.57±0.33 g.

Survival of immersion by pupae and larvae

Pupae (5.67±0.07 g) were put into one of two immersion treatments or a control treatment. In immersion treatments, 230 ml of tap water was placed into 250 ml plastic cups (Karat, Chino, CA, USA) with lids, and the cups were maintained at 22.1±0.1°C in constant darkness. Pupae were immersed individually using 15 cm zip ties gently placed against them, which prevented them from floating to the surface without compressing the cuticle or otherwise restricting movement.

We used two different immersion treatments to account for the possibility that pupae obtain oxygen directly across the cuticle, or across an air–water interface at the spiracles. In an anoxic treatment, oxygen was purged by bubbling nitrogen gas into each cup at ∼30 ml min^{-1} (regulated using a Sable Systems MFC-4, Sable Systems International, Las Vegas, NV, USA). Pupae in this treatment had no access to oxygen by any means (we confirmed that the water was anoxic using a calibrated Clark-style electrode, Unisense). In an aerated treatment group, air was bubbled into each cup to give air-saturated levels of dissolved oxygen. In a control group, pupae were placed into plastic cups with no water, held down with zip ties, and then placed in a similar environment to the two immersion treatments for the duration of the experiment.

From each treatment group, we sampled pupae at 1, 3, 5, 7, 9 and 13 days post-immersion and assessed whether they were alive. A pupa was determined to have survived if it eventually eclosed as an adult. After immersion, pupae were held in 12.7×7.9×24.9 cm waxed paper bags (Brown Paper Goods Company, Waukegan, IL, USA) and kept at room temperature (approximately 21°C). Starting 2 weeks after the end of immersion, relative humidity was raised in each paper bag by placing a 20×30 cm piece of water-soaked paper towel in it. The towel was rewetted daily until eclosion. Humidity was monitored in the paper bags using a HOBO data logger (Onset Computer Corp., Bourne, MA, USA) and averaged 69.4%. Saturated paper towels were placed into the bags of control pupae 2 weeks after the start of the experiment. Bags were checked once per day. Upon eclosion, moths were killed by freezing for 2 h and body mass, body length, thorax width and wing length were measured. Body mass was measured using a PB303 balance

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(±0.001 g; Mettler-Toledo, Columbus, OH, USA) and body length, thorax width and wing length were obtained with digital calipers (±0.01 mm; Mitutoyo Corp., Aurora, IL, USA). We also estimated the pupal period for each individual, by recording the number of days between wandering and eclosion.

In a separate experiment, we examined how well larvae survived immersion, as a kind of positive control using a life stage that likely has little evolutionary history with immersion. Different life stages within particular species often have different sensitivities to low oxygen (Hoback and Stanley, 2001). Twenty-eight *M. sexta* were reared to the fifth instar and then were placed individually into 28 ml screw-top glass vials filled with water (Hoback et al., 1998). Larvae were returned to air at 1, 2, 4, 8, 12, 16, 20 and 24 h post immersion, left to dry at room temperature, and were scored as alive if they successfully pupated.

Metabolic recovery from immersion

Using flow-through respirometry (Lighton, 2008), we measured rates of CO_2 emission by pupae at room temperature (21–23°C) from the moment they were removed from immersion through the subsequent 24–48 h. Levels of CO_2 were measured by an LI-7000 CO_2/H_2O Analyzer (LI-COR, Lincoln, NE, USA) plumbed in differential mode: air from a cylinder of CO_2-free compressed air was sent (300 ml min^{-1}) first through the reference cell, then looped externally through a metabolic chamber (glass, 110 ml volume), then back through the measurement side of the analyzer. Flow rates were controlled by an MFC-4 (Sable Systems). The analyzer was calibrated frequently with CO_2-free air (zero) and 2000 ppm CO_2 in nitrogen (span). Data were sampled at 1 Hz, and analog outputs from the analyzer were sent to an A/D converter (UI-2, Sable Systems) and then to a computer running Expedata data logging software (v1.4.8, Sable Systems). For most pupae, we measured baseline values of CO_2 for several minutes at the beginning and at the end of the run, and these were used to correct the analyzer for drift (which generally was very slight).

Pupae 7–11 days post-wandering were immersed in a water-filled plastic cup for 0, 1, 3 or 5 days. Because there was no difference in survival rates of pupae in the two immersion treatments (Table 1), neither air nor nitrogen was bubbled into the water. At the end of the immersion period, pupae were removed from water, blotted briefly and placed into the respirometry system. Day 0 pupae were taken directly out of the wood blocks and put into the metabolic chamber; these pupae were the metabolic controls. Each 0- and 1-day immersion pupa was sampled for 24–48 h, while 3- and 5-day immersion pupae were sampled for 48 h (to account for longer recovery times after immersion). Six pupae (three male, three female) were measured for each immersion time period, one at a time (no multiplexing), for a total of over 1000 h of metabolic data collection.

Accumulation and clearance of lactate

Lactate concentrations were measured using methods similar to those described by Kirkton et al. (2005). After immersion (see below), pupae were weighed, sexed and frozen at −80°C, then cut in half length-wise to reduce the total mass of tissue going into the assay. Pupal halves were placed in a cold 1:10 dilution of $HClO_4$ in a 40 ml ground-glass homogenizer on ice and homogenized using a Teflon plunger powered by an 18 V cordless drill (Makita, La Mirada, CA, USA). A subsample (1 ml) of the mixture was centrifuged at 15,850 ***g*** for 5 min. Supernatants (750 µl) were removed and diluted with 169 µl of KOH-MOPS and centrifuged again for 1 min. Additional KOH-MOPS was added until pH was approximately neutral, as judged by pH strips (Hydrion Insta-Check pH Paper 6.0-8.0, Micro Essential Laboratory, Brooklyn, NY, USA).

Along with 20 µl of the above lactate solution, 100 µl 2× Tris-Hydrazine buffer with 5 mmol l^{-1} oxidized nicotinamide adenine dinucleotide and 3.75% lactate dehydrogenase (LDH) were mixed together in individual wells of a 96-well plate with black sides and a clear bottom (Greiner Bio-One, Monroe, NC, USA). Immediately after adding the LDH, the mixture was shaken for 30 s, and then the lactate solution was analyzed fluorometrically using a SpectraMax M3 spectrometer (Molecular Devices, Sunnyvale, CA, USA) with the following settings: kinetic test, 45 min, 20 s intervals, bottom read with the fluorometer excitation wavelength set to 355 nm and the emission wavelength set to 460 nm. The data were recorded by a computer running SoftMax Pro acquisition software (Molecular Devices). Raw readings were converted to lactate concentrations using a standard curve constructed from dilutions of a 2 mmol l^{-1} lactate solution. Standard curves were run on every plate.

Pupae 10–12 days post-wandering were immersed in water-filled plastic cups for 0, 1, 3 or 5 days as described above. To measure the amount of lactate produced during immersion, pupae were removed from the water at the above intervals, blotted briefly and then immediately frozen at −80°C. To measure whether levels of lactate declined after immersion, some individuals were frozen at 12, 24 and 48 h after being removed from water. Day 0, or lactate control, pupae were sampled twice, once immediately after being taken out of the wood blocks and after 48 h. Five pupae were measured for each immersion period and subsequent recovery period, for a total of 70 individuals.

Table 1. Summary of survival and death by pupal *Manduca sexta* immersed in water

	1 day	3 days	5 days	7 days	9 days	11 days	13 days
Aerated							
Alive	3	4	4	0	0	0	0
Dead	0	0	0	4	4	4	4
Anoxic							
Alive	3	4	4	0	0	0	0
Dead	0	0	0	4	4	4	4

Table 2. ANOVA results for factors affecting pupal period, thorax width, body length, wing length, body mass and lactate concentration in *M. sexta*

	Variable (d.f.)	*F*	*P*
Pupal period (days)	Treatment (1)	42.361	1.90e–06
	Time (2)	8.698	1.77e–03
	Treatment×Time (2)	5.567	0.012
Thorax width (mm)	Treatment (2)	0.032	0.968
	Time (2)	0.885	0.427
	Treatment×Time (4)	1.631	0.202
Body length (mm)	Treatment (2)	1.633	0.218
	Time (2)	1.113	0.346
	Treatment×Time (4)	0.718	0.589
Wing length (mm)	Treatment (2)	0.920	0.413
	Time (2)	0.303	0.742
	Treatment×Time (4)	0.820	0.527
Body mass (g)	Treatment (2)	0.455	0.640
	Time (2)	0.578	0.570
	Treatment×Time (4)	0.234	0.916
Lactate concentration (mmol l^{-1})	Days immersed (3)	63.69	<2e–16
	Hours of emersion (3)	72.76	<2e–16
	Days immersed×Hours of emersion (4)	18.95	1.07e–12

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Statistical analyses

All data were processed and analyzed using scripts written in R v. 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria). Raw metabolic data were read into R using the package SableBase (T. D. Förster, Sable Systems) and then stitched together, corrected for baseline drift and converted into molar rates of CO_2 emission.

We used a two-way ANOVA to test the effects of immersion time and treatment group on adult thorax width, body length, wing length and body mass. The normality of the data was confirmed with an Anderson–Darling test. We also used two-way ANOVA to test the effects of immersion time and treatment group on pupal period, but here we compared pupal period in the control versus immersion. As we found no difference between the treatment groups for survival or adult morphologies, this inclusion was justified. To test for differences in pupal period, *post hoc* analyses were conducted using Tukey's test. Fluctuations in the traces of 1–2 h segments of CO_2 emission were analyzed using autocorrelation analysis, from which significant peaks were extracted as estimates of cycling frequency. To analyze the lactate data, we fit a two-way ANOVA to the three experimental groups, with days of immersion and hours of emersion as factors.

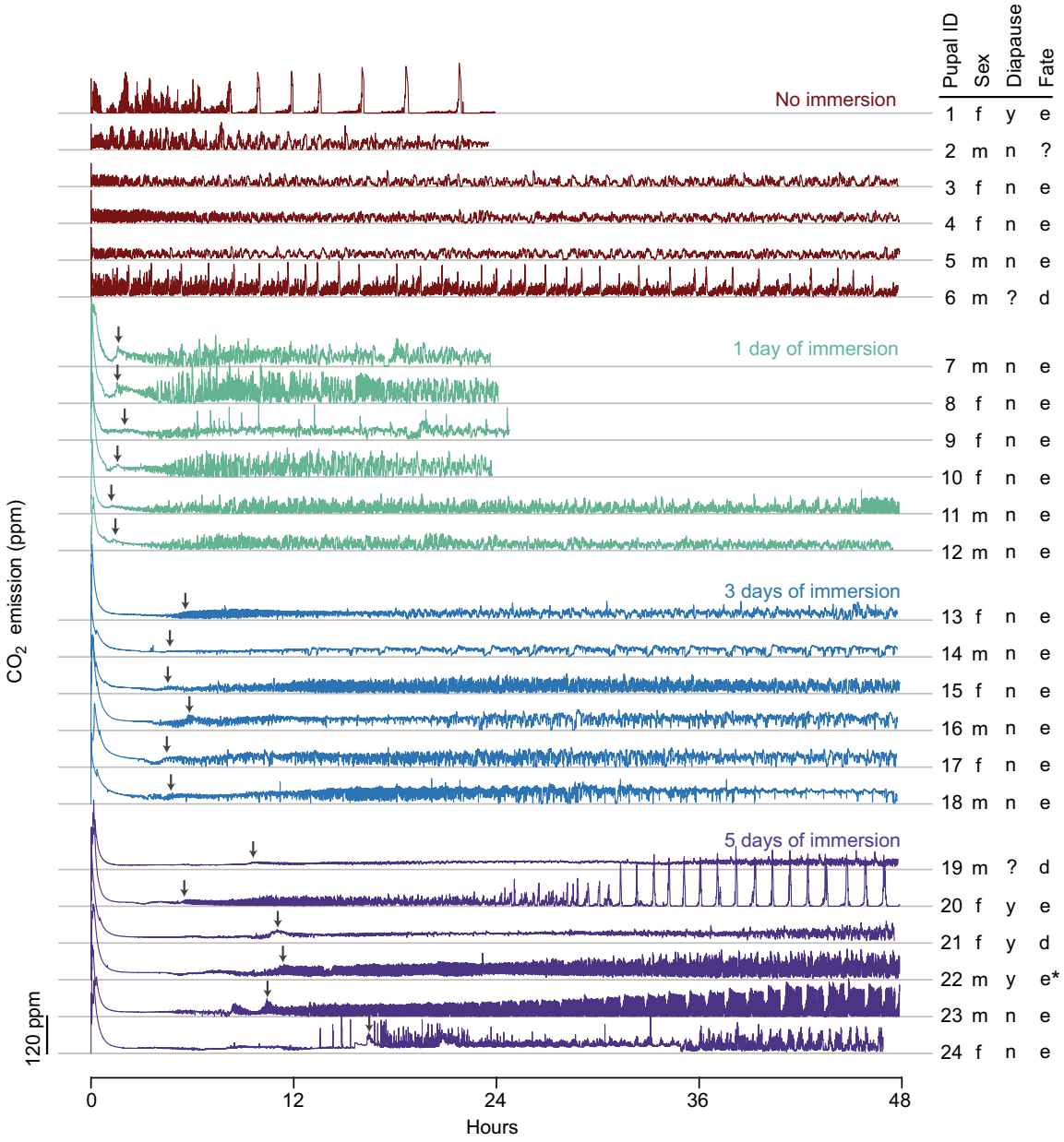

Fig. 2. Traces of CO_2 emission for *M. sexta* pupae (*N*=24) in the immersion recovery experiment. Emission by most pupae was recorded for 48 h, but a subset (*N*=6) was recorded for just 24 h. The original data were sampled at 1 Hz, but to render this figure, they were subsampled to 0.1 Hz. Gray horizontal lines represent 0 ppm for each pupa, and the gray lines are 120 ppm apart. Vertical arrows represent, for each immersion pupa, the time at which we estimate it starts to resume normal respiratory function, based on the joint occurrence of increased spiracular activity and a relatively sudden rise in average level of CO_2 emission after a sterotypic lull. The right-hand columns contain additional descriptive information: sex (f=female, m=male); diapause status (y=yes, went into diapause, n=no, did not); and fate (e=successfully eclosed as an adult, d=died during pupation, e*=died during eclosion). Question marks represent missing data.

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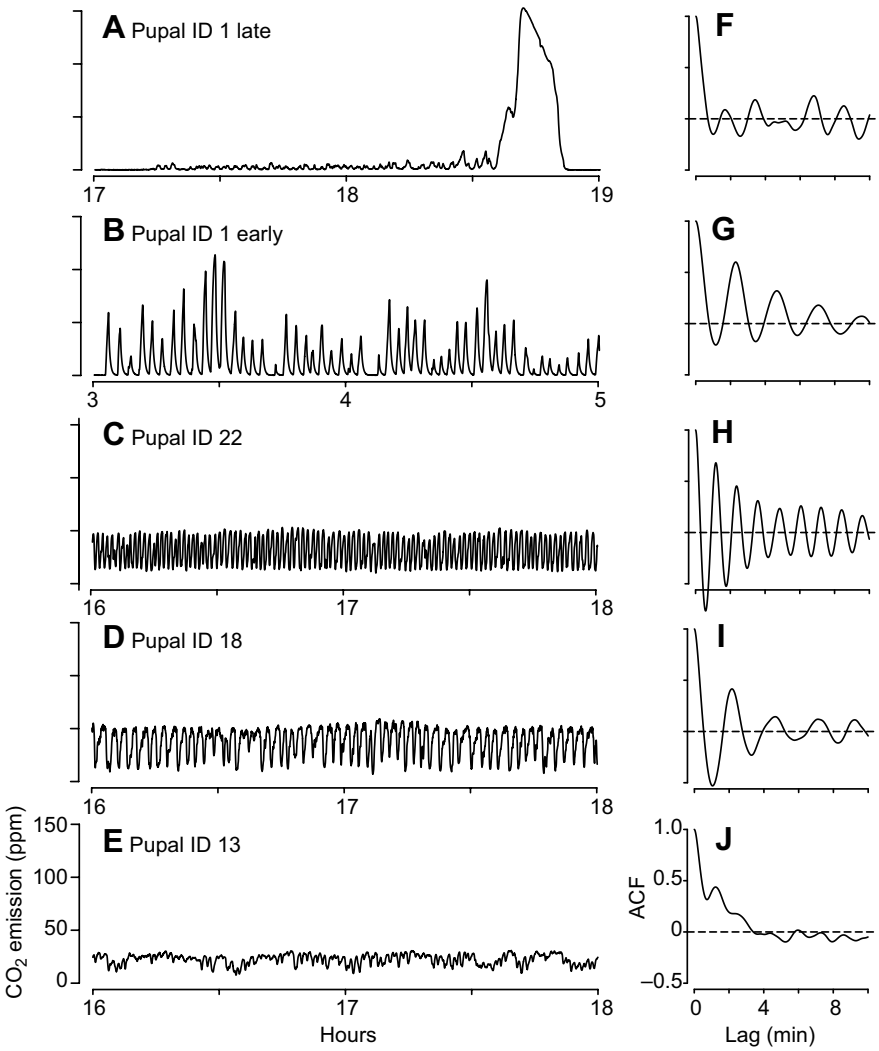

Fig. 3. Diversity of cyclic patterns of CO_2 emission and associated autocorrelation functions in *M. sexta*. (A) A control pupa (ID 1) that showed discontinuous gas-exchange cycles (DGC) starting approximately 5 h after being placed in the respirometry chamber (see Fig. 2). The associated autocorrelation function applies only to the flutter phase, between 17.3 and 18.3 h. (B) Pupa 1 in hours 3–5, during which it showed cyclic ventilation. (C) Pupa 22 from the 5-day immersion group, showing highly regular cycles of CO_2 emission. (D) Pupa 18 from the 3-day immersion group, showing regular cycles of CO_2 emission in which emissions are mostly high but are punctuated by 'anti-bursts' of low CO_2 emission. (E) Pupa 13 from the 3-day immersion group, showing an extreme version of the pattern from D. Associated autocorrelation functions are shown in F–J.

Data have been deposited into the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.v7fd5; Woods and Lane, 2016).

RESULTS

Survival of immersion by pupae and larvae

At 21°C, all pupae survived up to 5 days of immersion, but none survived 7 days or more; there was no difference between gas treatment groups (Table 1). Control pupae all survived (N=80). Because there was no variation within time points, these results could not be modeled by logistic regression; the best estimate of the LT_{50} (median lethal time) is simply the midpoint between survival and death, which is 6 days. By comparison, fifth-instar larvae of *M. sexta* survived immersions of up to 4 h but no longer (N=28; data not shown). Several larvae immersed for longer initially reestablished body turgor and resumed moving, but then died within 24 h.

Duration of the pupal period and adult morphologies as a function of immersion time

Pupal duration was affected by immersion time and treatment, and there was an interaction between them (Table 2). Immersed pupae took 6 days longer to eclose than those not immersed (Fig. S1). Within the immersion treatments, those immersed for 1 day did not eclose any later than those in the control group, but pupae immersed for 3 or 5 days took almost 7 days longer to eclose compared with the controls. There was, however, no difference in pupal period between those immersed for 3 or 5 days (Fig. S1).

There was no effect of immersion time or treatment group (anoxic or aerated) on any of the measured adult morphologies: body mass, body length, thorax width or wing length (Table 2, Fig. S2).

Metabolic recovery from immersion

Pupae in the control group (no immersion, N=6) showed more high-frequency fluctuations in CO_2 emission at the beginning of their metabolic traces than at the end (Fig. 2). These results suggested that even brief handling (<2 min) was enough to disturb them for many hours. We analyzed this effect in more detail (Fig. S3), showing that for five of six pupae, disturbed patterns of respiration persisted for 5–10 h after handling. After 10 h, patterns of emission became more consistent: four of the pupae showed slow, irregular shifts between very low and intermediate levels of CO_2 emission. One pupa (ID 1) clearly also exhibited discontinuous gas-exchange cycles (DGC), and this pupa turned out to have gone into diapause, despite our having set the larval light cycle with day lengths putatively long enough to prevent this. Diapause was diagnosed from very long times to eclosion. Most pupae (non-diapausing) eclosed approximately 1 month after wandering (mean 35 days, range 33–66 days). The four pupae (Fig. 2) identified as diapausing eclosed after 120–207 days. One other pupa (ID 6) continued to show high-

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frequency fluctuations, with superimposed DGC-like spikes, for the entire 48 h of measurement.

All pupae in the experimental groups showed similar patterns of metabolic recovery, with differences arising from the timing of events (Fig. 2). All pupae initially emitted large spikes of CO_2, which peaked within 5–10 min and then fell rapidly. The absolute peak level of emissions did not vary strongly as a function of immersion time (Fig. 2, Fig. S4). During this time, changes in CO_2 emission were smoothly graded. In eight of 12 pupae in the 3- and 5-day immersion groups there was an additional pulse of CO_2 emitted between 20 and 30 min post-emersion (arrows in Fig. S4).

Regular cycles of CO_2 emission commenced around 2 h in the 1-day immersion group, and the amplitude of cycling increased rapidly, reaching high levels by 4–6 h post emersion (Fig. 2). In pupae immersed for 3 days, cycling began 3–4 h post-emersion, and the amplitude of cycling increased slowly through ∼12 h; in general, cycling amplitude in the 3-day group never reached the level seen in the 1-day group. In pupae immersed for 5 days, cycling began 4–10 h post-emersion, and, for some pupae, cycling amplitude continued to increase throughout the 2-day measurement period. Besides the onset of cycling, there was, for most pupae, a distinct decrease and then increase in metabolic rate either associated directly with cycling or occurring several hours afterward (arrows in Fig. 2).

Most pupae in both control and experimental groups showed distinct high-frequency cycling of CO_2 emission for several hours during recovery, and some from the longer immersion groups showed the pattern for the entire trace after cycling commenced. Cycles occurred on two time scales. Seventeen of 24 pupae showed high-frequency cycling, with peaks of CO_2 emission having a mean period of 1.56 min (range 0.78–2.17 min; Fig. 3A–E), and 13 of 24 showed lower-frequency cycling, with a mean period of 12.4 min (range 6.5–31 min; data not shown). The total sample size is greater than 24 because some pupae showed both cycling frequencies simultaneously. Periods were found by examining the timing of major peaks in the autocorrelation function with lags of 0–10 min (Fig. 3F–J) or longer. We saw at least three discernible variants of high-frequency cycling. Some pupae, especially those in the control group, emitted CO_2 at low levels with regular, brief pulses (Fig. 3B). Others had rates that cycled symmetrically between high and low without going all the way to zero (Fig. 3C); these were primarily from the 5-day immersion group. And a third group had continuous, high levels of emission, with brief drops (Fig. 3D,E); these were primarily from the 3-day immersion group. Two of the pupae (Fig. 2, pupal IDs 1 and 20) showed clear patterns of DGC. Only the first pupa, however, had distinct fluttering preceding the long open phase (Fig. 3A), and the fluttering period in this individual was 1.67 min. There thus appears to be a common high-frequency oscillation in rates of CO_2 emission with a period of 1–2 min.

Even after the onset of cycling, metabolic traces differed among treatment groups. Patterns from the 1-day group were largely stationary starting approximately 10 h after emersion. However, those traces still did not resemble those of the control group: the 1-day pupae had more high-frequency fluctuations for the rest of their measurement periods. Pupae in the 3-day group mostly had very high-frequency fluctuations between 12 and 24 h post-emersion, with longer frequencies dominating after 24 h. Pupae from the 5-day group mostly had very high-frequency fluctuations through their 48-h measurement periods, and their traces were the least stationary.

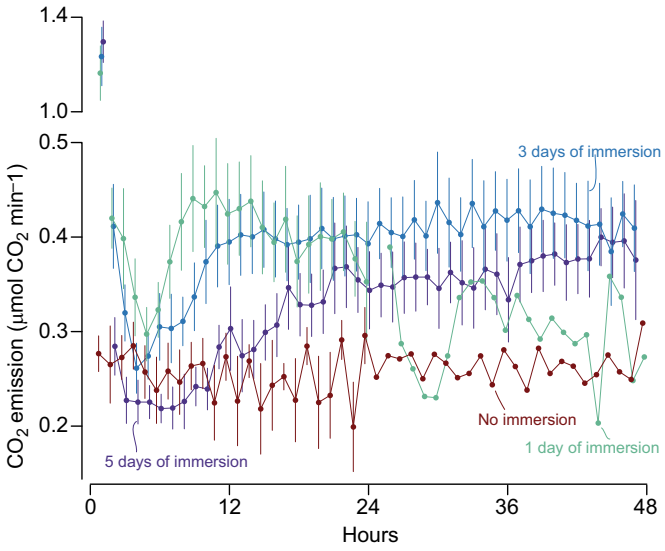

Fig. 4. Hourly averages of CO_2 emission by *M. sexta* pupae. Bars are standard errors (*N*=6 per point). Points are offset slightly in the *x* direction to reduce overlap. There are no error bars after 24 h for control pupae (brown) or those that were immersed for 1 day (light green) because there were fewer than six pupae at those time points (four and two, respectively).

Finally, there were consistent differences among groups in average metabolic rate across time after emersion (Fig. 4). Pupae from the no-immersion group had low metabolic rates (0.2–0.3 µmol CO_2 min^{-1}). Average emission rates for immersion groups depended on length of immersion. After the first hour (which had very high average rates of CO_2 emission associated with the initial spike; see Fig. 2), pupae showed a 50–75% increase in metabolic rate, with the peak reached at different times: 1-day pupae reached that peak by 10–12 h post-emersion, 3-day pupae showed it from 18 to 48 h, and 5-day pupae gradually rose to that level by the end of the 48-h trace. Presumably, pupae from the 3- and 5-day immersion groups would have exhibited declining rates of CO_2 emission after 2 days had we continued our measurements.

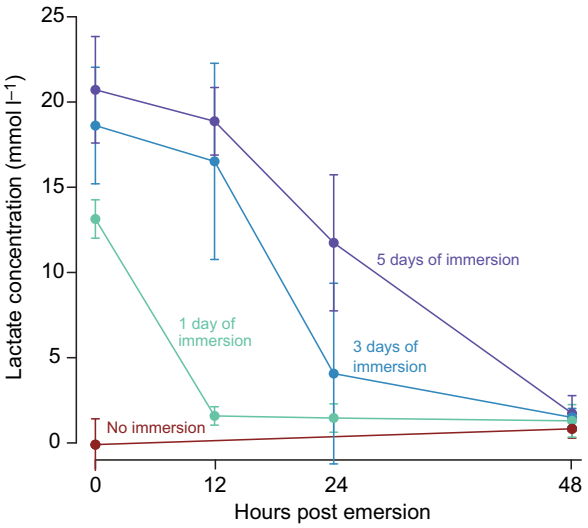

Fig. 5. Lactate concentrations in *M. sexta* pupae after 1, 3 or 5 days of immersion in water at room temperature, and in control pupae that were not immersed. Five pupae were assayed in each treatment at each time point (*N*=70 total). Bars represent ±s.e.m.

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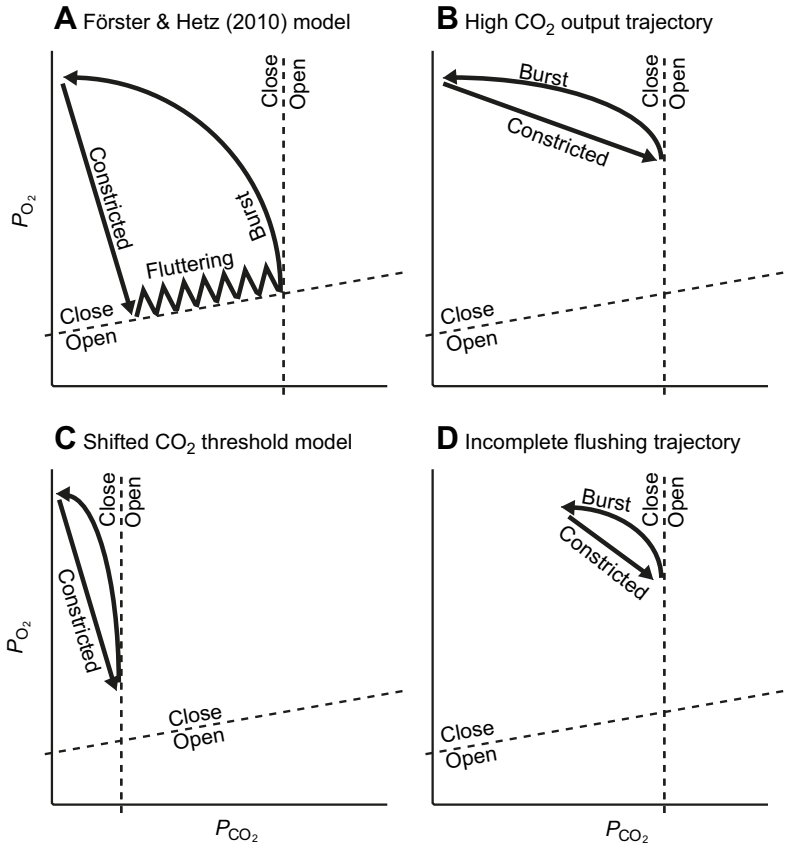

Fig. 6. Phase-space models of spiracular control. (A) The model of discontinuous gas-exchange cycles (DGC) proposed by Förster and Hetz (2010), which invokes two thresholds for spiracle opening, one at low tracheal partial pressure of O_2 (P_{O_2}) and another at high partial pressure of CO_2 (P_{CO_2}). When the spiracles are closed (constricted), tracheal P_{O_2} falls and P_{CO_2} rises until P_{O_2} eventually falls below the O_2 threshold. At that point, the spiracles start to flutter, which allows in small amounts of O_2 while not allowing out equal amounts of CO_2. P_{CO_2} thus rises and eventually exceeds the CO_2 threshold, at which point the spiracles open wide (burst) for a longer time, allowing the accumulated internal CO_2 to be emitted. (B) Alternative trajectory in which pupae recovering from immersion have accumulated large stores of CO_2 (Harrison et al., 1995), such that the rate at which CO_2 appears in the tracheal system is much higher than the rate at which O_2 disappears. If so, the slope in phase space will be shallow when the spiracles are closed, such that it strikes the P_{CO_2} threshold before the tracheal P_{O_2} falls low enough to strike the P_{O_2} threshold. A related possibility is that post-immersion pupae had less capacity to store CO_2 in the hemolymph, e.g. if tissues and hemolymph were acidified from other sources, such as lactic acid (Fig. 5), which would drive the CO_2↔bicarbonate equilibrium toward CO_2. Such an effect also could arise if immersion or anoxia reduces levels of nonbicarbonate buffers in the hemolymph (Harrison, 2001). Like the first scenario above, these processes would cause CO_2 to appear in the tracheal system at high rates, leading also to shallow slopes in phase space. The counterargument is that even control pupae showed cycling after they were handled, and they would not have accumulated CO_2. In addition, the pupae that continued to show cycling for much of the 2-day measurement period (3- and 5-day immersion groups) were unlikely to have retained high levels of internal CO_2 for such a long time. (C) A second alternative model in which the dynamics of tracheal P_{O_2} and P_{CO_2} are normal but the CO_2 threshold is lowered, perhaps by stressors of any kind or by lactic acid (Wigglesworth, 1935). (D) Another alternative in phase space, in which the CO_2 unloading from the tissues and hemolymph is so rapid, and the cycles so short, that P_{CO_2} remains high in the tracheal system throughout the entire cycle. Distinguishing among these alternatives will require additional experiments.

Accumulation and clearance of lactate

Initial levels of lactate were lowest in control pupae and progressively higher in pupae that had been immersed for longer durations (Fig. 5, Table 2). Lactate levels fell rapidly in pupae with just 1 day of immersion, approaching zero at the next sampled time point (12 h). Levels fell more slowly in pupae immersed for 3 or 5 days, but all groups reached levels indistinguishable from those of the controls by 48 h. Given that the data appeared somewhat sigmoidal, we attempted to fit a three-parameter sigmoid equation to individual immersion groups (1, 3 and 5 days); however, the non-linear fitting routine (nls function in R) failed to converge in some cases. As an alternative, we fit a two-way ANOVA to the three experimental groups, with days of immersion and hours of emersion as factors. The fit is summarized in Table 2 and it confirms the patterns observed in the figure: both factors and their interaction were highly significant.

DISCUSSION

Survival of immersion

Although *M. sexta* is ostensibly terrestrial, pupae but not larvae are able to withstand immersion in water for 5 days. This pupal physiology probably reflects the evolutionary outcome of infrequent but dangerous events in which heavy rains saturate soils locally or cause streams and rivers to flood and produce persistent standing water (see Fig. 1). In our experiments, O_2 levels in the surrounding water did not affect survival rates, indicating that pupae did not absorb O_2 across their cuticles or spiracles or exchange gases via cryptic cuticular air films (Pedersen and Colmer, 2012). Rather, immersed pupae accumulated high levels of lactate, indicating that they relied on anaerobic metabolism; we have no information on other possible anaerobic end products. Although we did not explore its effects directly, temperature probably plays an important role in setting survival times (Hoback et al., 1998; Hutchinson, 1981;

Kolesnikov et al., 2012; Topp and Ring, 1988). Pupae were immersed at 22°C, which is a typical nighttime soil temperature at 8 cm depth at our main field site in southeastern Arizona (Sprague and Woods, 2015). By contrast, pupae in diapause over the winter have lower metabolic demand (Hahn and Denlinger, 2007) and experience lower temperatures, which likely extends survival times (Kolesnikov et al., 2012; Topp and Ring, 1988).

Across life stages, differential tolerance of anoxia is common (Cavallaro and Hoback, 2014; Hoback and Stanley, 2001), and *M. sexta* is no exception. In our experiments, pupae survived longer (up to 5 days) than did larvae (no longer than 4 h). Besides being more prone to flooding than larvae, pupae also had substantially lower mass-specific metabolic rates, which should promote anoxia tolerance (Hoback and Stanley, 2001). In our study (see also Odell, 1998), non-immersed pupae had rates of CO_2 emission in the range of 3.0–3.4 µmol g^{-1} h^{-1} (see Fig. 3), which were an order of magnitude lower than those of fifth-instar larvae (30–50 µmol g^{-1} h^{-1}; Greenlee and Harrison, 2005). In several cases, emersed larvae regained turgor and the ability to move, but then died within 24 h, suggesting that they sustained significant damage as a result of prolonged anoxia or reperfusion (Lighton and Schilman, 2007). Though not studied here, eggs and adults appear to have intermediate tolerance to anoxia (Wegener and Moratzky, 1995; Woods and Hill, 2004). Thus, larvae are the most sensitive to anoxia, pupae the least sensitive, and eggs and adults somewhere in between. This pattern contrasts with the scheme proposed by Hoback and Stanley (2001), in which eggs and adults are thought to be the most sensitive. We propose that the life stage most likely to experience immersion will be the least sensitive to hypoxia and anoxia.

Compared with other species, survival times of pupal *M. sexta* in immersion (anoxia) are impressive but not unprecedented. Larvae of the tiger beetle *Cicindela togata*, which frequently are flooded in nature, survived over 4 days in pure N_2, whereas larvae of a related species (*Amblycheila cylindriformis*), which are not flooded, survived less than 1.5 days (Hoback et al., 1998, 2000). Pupae and pharate adults of the bruchid beetle *Callosobruchus subinnotatus*, which live inside bambarra (*Vigna subterranea*) seeds and may be exposed naturally to hypoxia, survived more than 4 days in atmospheres containing only 1% O_2 (Mbata et al., 2000). Some alpine insects held at cold temperatures can survive anoxia for more than 100 days (Conradi-Larsen and Sømme, 1973; Meidell, 1983).

Using immersion rather than pure N_2 complicates the interpretation because immersed insects sometimes obtain O_2 from the surrounding water. Indeed, we suspect that immersed pupae opened their spiracles from the accumulation of internal CO_2 or changes in body pH (see below), and that water was prevented from entering the tracheal system by the small diameters of the openings and highly hydrophobic cuticle. If so, there would have been a small total area (the summed area of all spiracles) of air–water interface across which gases could in principle move. However, our experiments suggest that such an exchange was negligible, as pupal *M. sexta* did not survive immersion differentially between aerated and N_2-purged water. Other species may use physical mechanisms to obtain oxygen. For example, adults of several species of floodplain ground beetles (Carabidae) can survive 10 days of immersion in cold water using air trapped under their elytra as a physical gill (Kolesnikov et al., 2012; see also Adis and Messner, 1997). We suggest that anaerobic processes are useful over short periods of time (days) but that survival for longer times requires either very cold temperatures or mechanisms for extracting oxygen from water.

Metabolic recovery from immersion

Pupae showed stereotyped patterns of recovery during the first few hours (Fig. 2, Fig. S4) after emersion: a large initial spike of CO_2 emission without superimposed spikes indicative of spiracular opening and closing, probably because pupae were anaesthetized by accumulated CO_2 or low pH (Badre et al., 2005); a secondary, smaller peak in some immersed individuals (see arrows in Fig. S4), the cause of which is unknown but may reflect reconnection of a significant body compartment to the tracheal system (perhaps blocked by liquid prior to this point) or sudden reactivation of aerobic metabolism in some portion of the pupa (for comparison, see fig. 1 in Lighton and Schilman, 2007); followed by depressed rates of CO_2 emission and, finally, elevated rates of CO_2 emission (Fig. 4). The relative timing of these events was stretched out for pupae immersed longer. Pupae immersed 1 day recovered normal levels of lactate (near zero) and patterns of CO_2 emission by approximately 1 day later, whereas those immersed 3 or 5 days had low levels of lactate by the end of 2 days, but even then still had elevated rates of CO_2 emissions and altered patterns of respiratory exchange.

These data indicate that: (1) immersed pupae use anaerobic respiration; (2) early in recovery, pupae either lack control over spiracles or are forced by high internal levels of CO_2 or acidification of the hemolymph to open them continuously (Badre et al., 2005; Colinet and Renault, 2012; Förster and Hetz, 2010; Harrison, 2001; Hoyle, 1960; Matthews and White, 2011; Nicolas and Sillans, 1989); (3) recovery from immersion is an extended process, requiring at least several days and possibly longer, depending on duration of immersion; and (4) just removing the accumulated lactate is not enough to effect complete recovery. The long period of metabolic recovery is reminiscent of the long-term changes observed in some insects after CO_2 anesthesia (Colinet and Renault, 2012).

Patterns of respiratory exchange

Immersion had diverse effects on patterns of CO_2 emission. The most striking pattern was the high-frequency cycles of continuous CO_2 emission shown by the majority of post-immersion pupae (12 of 18). We use the term ‘cycling’ to mean something different from the ‘cyclic ventilation’ described by Contreras and Bradley (Contreras and Bradley, 2009, 2010; Contreras et al., 2014): in their descriptions, cyclic ventilation involves brief but complete closure of the spiracles with regular peaks of emission between – essentially shortened DGC with very short inter-burst intervals and no fluttering phase. If the spiracles do not close all the way, they call this a ‘continuous’ respiratory pattern. However, even their continuous patterns can show regular peaks of emission (see fig. 1 in Contreras et al., 2014). Here we use the term cycling to describe more broadly any rhythmic emission that is not DGC.

During high-frequency cycling, pupae emitted peaks of CO_2 separated by 0.78–2.2 min. Five of six control pupae also showed similar periods (Fig. 3, Figs S3, S4) soon after being put in the respirometry system. Control pupae were handled briefly (< a few minutes), suggesting that pupae transition into this pattern in response to multiple stressors. The least disturbed patterns were shown by the control pupae after 12 h (Fig. S4, upper right panel). Their emissions showed no rhythmicity and fluctuated in a kind of random walk. This may indicate asynchronous action of pairs of spiracles in response to local conditions in each segment (T. D. Förster, Spiracular control in moth pupae, PhD thesis, Humboldt University of Berlin, Germany, 2010; Förster and Hetz, 2010), such that only occasionally did all spiracles close simultaneously.

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What underlying mechanisms drive cyclic emission of CO_2? The likeliest candidates are (1) cyclic opening and closing of one or more spiracles or (2) a combination of spiracle cycling and pressure-driven ventilation of the tracheal system. Spiracles are under dual control by local, intra-segmental levels of O_2 and CO_2. Low O_2 in the ganglion signals the spiracle to open (Burkett and Schneiderman, 1974; Förster and Hetz, 2010). By contrast, high CO_2 affects the spiracle muscle directly (Case, 1956), either by interfering with the transmission of action potentials at the neuromuscular junction (Badre et al., 2005; Hoyle, 1960), or by diffusing into muscle cells and disturbing local pH (Förster and Hetz, 2010; Grieshaber and Terblanche, 2015; Heinrich and Bradley, 2014). Wigglesworth (1935) showed that fleas perfused with high levels of lactic acid exhibited high-frequency (5–15 oscillations per minute) fluctuations in some spiracles. This suggests that high concentrations of lactic acid accumulated by pupae of *M. sexta* (Fig. 5) influenced spiracle cycling.

How many spiracles oscillated in pupae? Although control is local, local conditions are coupled by longitudinal transport of gases and hemolymph. Such systems of coupled oscillators can give complex patterns of control depending on the sensitivity of individual segments to O_2 and CO_2 and the strength of coupling among segments. A model of this process suggested that, with increasing coupling strength, fewer spiracles should cycle between open and closed (T. D. Förster, Spiracular control in moth pupae, PhD thesis, Humboldt University of Berlin, Germany, 2010). Interestingly, Sláma (1988) may have observed this outcome in diapausing pupae of Lepidoptera, including *M. sexta*. Using an anemometric technique to record convective flows across multiple spiracles simultaneously, he showed that a diapausing pupa of *M. sexta* used only a single spiracle for all gas exchange. Moreover, the inter-burst period was approximately 2 min, similar to the periods we observed.

These observations suggest a sequence of spiracular states in post-emersion pupae. In the first hours, high internal CO_2 and/or low pH forced all spiracles open, during which time CO_2 washed out at high rates. Eventually, CO_2 levels fell enough, or pH rose enough, that spiracles began to close locally. And ultimately, all but one or a few spiracles closed, and those last spiracles then began to cycle in response to local O_2 and CO_2 levels in their segments.

A second mechanism that can drive cyclic emission of CO_2 is convective ventilation. Pupae of many species ventilate their tracheae using pulsations of pressure in the hemocoel, originating as abdominal contractions (Sláma, 1999). Changes in hemolymph pressure drive convective flow into and out of the spiracles (Greenlee et al., 2013; Harrison et al., 2013; Matthews and White, 2011; Sláma, 1988, 1999; Westneat et al., 2003), and contractions and spiracular openings can be coordinated to give unidirectional flow (Sláma, 1999). Pupae in our experiments may have used such a strategy, although confirming this would require additional work.

Challenges to models of spiracle control

A longstanding problem in insect respiratory biology has been to explain the mechanisms that underlie DGC (Burkett and Schneiderman, 1967; Förster and Hetz, 2010; Grieshaber and Terblanche, 2015; Hetz and Bradley, 2005; Lighton, 1996). DGC has three phases: a closed phase, in which the spiracles are sealed shut and no gases exchanged; a flutter phase, in which the spiracles open briefly, letting in small amounts of O_2 and possibly letting out CO_2 (Hetz and Bradley, 2005; Wobschall and Hetz, 2004); and an open phase, in which the spiracles open for many minutes, during which time O_2 levels in the tracheal system rapidly approach ambient and large amounts of CO_2 are emitted. Two pupae in our study showed DGC (Fig. 2) and others showed possible DGC superimposed on other patterns. Two models of DGC have recently been proposed – the first by Förster and Hetz (T. D. Förster, Spiracular control in moth pupae, PhD thesis, Humboldt University of Berlin, Germany, 2010; Förster and Hetz, 2010), and the second, derived from the first, by Grieshaber and Terblanche (2015). These models have two goals: to reproduce DGCs having characteristics that match observed DGCs reasonably well, and to reproduce the observed effects of changes in temperature, metabolic rate and external gas concentrations on DGC.

The Förster and Hetz model proposes that DGCs emerge from interactions between thresholds for O_2 and for CO_2 (see also Chown and Holter, 2000). When spiracles close, tracheal partial pressure of O_2 (P_{O_2}) is high and partial pressure of CO_2 (P_{CO_2}) is low. Because the system is sealed, the insect's metabolism draws down P_{O_2} and raises P_{CO_2}. Even though aerobic metabolism produces essentially one molecule of CO_2 per O_2 consumed, tracheal P_{O_2} falls faster than P_{CO_2} rises because much of the CO_2 dissolves into tissues and hemolymph. Once tracheal P_{O_2} falls to its threshold, the spiracles start to flutter, opening briefly to let in O_2. Fluttering frequency may be altered to regulate P_{O_2} to a relatively constant, low level in the tracheal system (Contreras and Bradley, 2009; Förster and Hetz, 2010; Hetz and Bradley, 2005). Fluttering can also let out CO_2, depending on whether there is suction ventilation across the spiracles (Kestler, 1985; Sláma, 1988; Wobschall and Hetz, 2004). Either way, CO_2 continues to accumulate, at some point reaching the CO_2 threshold and triggering the spiracles to open for an extended duration. In its original form (Förster and Hetz, 2010), this model may adequately describe the DGCs observed in two of the pupae in our experiments. However, the model cannot explain the high-frequency cycling observed in most other pupae. The default set of parameters examined gave inter-burst periods of approximately 10 min. In a sensitivity analysis, essentially all reasonable sets of parameter values gave cycle times of 10 min or longer. The remaining panels in Fig. 6 graphically summarize potential additional modifications to thresholds and trajectories in phase space that may account for high-frequency cycling.

In an extension of Förster and Hetz's model, Grieshaber and Terblanche (2015) examined additional details of the two-threshold approach, focusing on CO_2/pH triggers for spiracular opening and closing. The model was parameterized using data from Orthoptera. The authors examined four trigger (threshold) scenarios (TS1–TS4) that invoke single or double triggers and different physiologically realistic mechanisms. A sensitivity analysis revealed a suite of potential patterns of opening and closing, including full DGC (TS2–TS4) with the right timing of each phase. With respect to the high-frequency fluctuations we observed, their most interesting trigger system was TS1. It failed to produce DGC under all combinations of parameters examined, suggesting that it does not capture some essential aspect. Nevertheless, it was the only system that produced short open phases, some of them <1 min long with intervening closed phases lasting >10 min. Perhaps with additional rapid efflux of stored CO_2 from tissues into the tracheal system, such a model could produce cycles with periods of ∼1 min.

These models significantly advance our understanding of spiracular behavior. Neither model, however, produced cycles short enough to account for our *M. sexta* data, and the version of Grieshaber and Terblanche's model that comes closest otherwise fails to generate DGC. We therefore still await a future model that captures the full range of observed patterns.

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No long-term effects of pupal anoxia on adult morphology

The danger from immersion can arise from anoxia per se, or from oxidative damage from the ROS produced during reperfusion of the tracheal system (Joanisse and Storey, 1996; Lighton and Schilman, 2007). Either could explain the death of pupae immersed for 7 days or more. However, adults eclosing from pupae that had been immersed 5 days or less had no trouble escaping from the pupal cuticle and expanding their wings, and they showed no other external abnormalities (Fig. S2). There could have been internal or biochemical abnormalities that we missed. These results suggest that surviving pupae suffered no other long-term effects from anoxia or reperfusion.

Conclusions

Pupae of *M. sexta* survived up to 5 days of immersion in water by using anaerobic respiration. During recovery, they emitted large quantities of CO_2, showed diverse patterns of cyclic emission of CO_2 and rapidly catabolized accumulated lactate. Immersed pupae did not metamorphose into abnormal adults, but those subjected to longer periods of immersion showed long-term changes in patterns of CO_2 emission during recovery. Among life stages, pupae were the most tolerant of immersion, probably reflecting their evolutionary history of occasional immersion. These findings suggest that pupae of *M. sexta* would make a good model system for further studies linking immersion, anoxia tolerance and the mechanisms underlying patterns of gas exchange in insects. Lastly, the high-frequency cycles of CO_2 emission pose an interesting challenge to current models of spiracular control. Future models should examine spiracular behavior under the conditions of high CO_2 production (from dissolved gases and bicarbonate) coupled with relatively low O_2 consumption.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

H.A.W. and S.J.L. designed the experiments, and S.J.L. carried them out. H.A.W. and S.J.L. analyzed the results, and H.A.W. wrote the manuscript.

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Data availability

Data are available from the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.v7fd5).

Supplementary information

Supplementary information available online at
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