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Biomechanics

Tracheal compression in pupae of the beetle *Zophobas morio*

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Insects that are small or exhibit low metabolic rates are considered to not require active ventilation to augment diffusive gas exchange. Some pupae with low metabolic rates exhibit abdominal pumping, a behaviour that is known to drive tracheal ventilation in the adults of many species. However, previous work on pupae suggests that abdominal pumping may serve a non-respiratory role. To study the role of abdominal pumping in pupa of the beetle *Zophobas morio*, we visualized tracheal dynamics with X-rays while simultaneously measuring haemolymph pressure, abdominal movement, and CO₂ emission. Pupae exhibited frequent tracheal compressions that were coincident with both abdominal pumping and pulsation of pressure in the haemolymph. However, more than 63% of abdominal pumping events occurred without any tracheal collapse and hence ventilation, suggesting that the major function of the abdominal pump is not respiratory. In addition, this study shows that the kinematics of abdominal pumping can be used to infer the status of the spiracles and internal behaviour of the tracheal system.

1. Introduction

Passive diffusion of gases within the tracheal system is considered to be sufficient to support aerobic metabolism in small or developing insects [1], but larger or more active insects also require convection [2]. Using synchrotron X-ray imaging, direct observation of tracheae in insects has shown that parts of the tracheal system can be rhythmically compressed, generating convective flow for ventilation [3]. However, little is known about non-adult stages, which can exhibit considerably lower metabolic rates [4]. Active ventilation is known to be produced by abdominal pumping, a dorsoventral and/or anteroposterior compression of the abdomen. However, recent studies have shown that abdominal pumping is not always correlated with external gas exchange, particularly in sub-adults [5,6]. Therefore, it is unclear whether abdominal pumping causes tracheal collapse and induces ventilation, or if abdominal pumping merely coincides with spiracle opening, while the insect respire primarily by diffusion.

Ventilation by tracheal compression has been observed in many species of adult insects [3] and in one larval form under hypoxia [7] but has never been investigated in pupae. In the pupal stage, the insect is immobile and can exhibit lower metabolic rates [4], reducing the demand for oxygen and the need for active ventilation, but pupae in fact do employ abdominal pumping [5,8]. Direct correlation between abdominal pumping and metabolic rate has been observed in adults [9], suggesting that a lower metabolic rate should indicate a reduced need for active ventilation in pupae.

Here, we examine the relationship between abdominal pumping and active ventilation in the pupal stage of the beetle *Zophobas morio*. We hypothesize that abdominal pumping produces active ventilation by pressurizing the haemolymph and inducing tracheal collapse, but only when the tracheal system is open to ambient air, meaning that at least one spiracle is open and air can flow from the animal. Conversely, if all spiracles are closed during an abdominal pump, the sealed tracheal system should not allow any tracheal collapse to

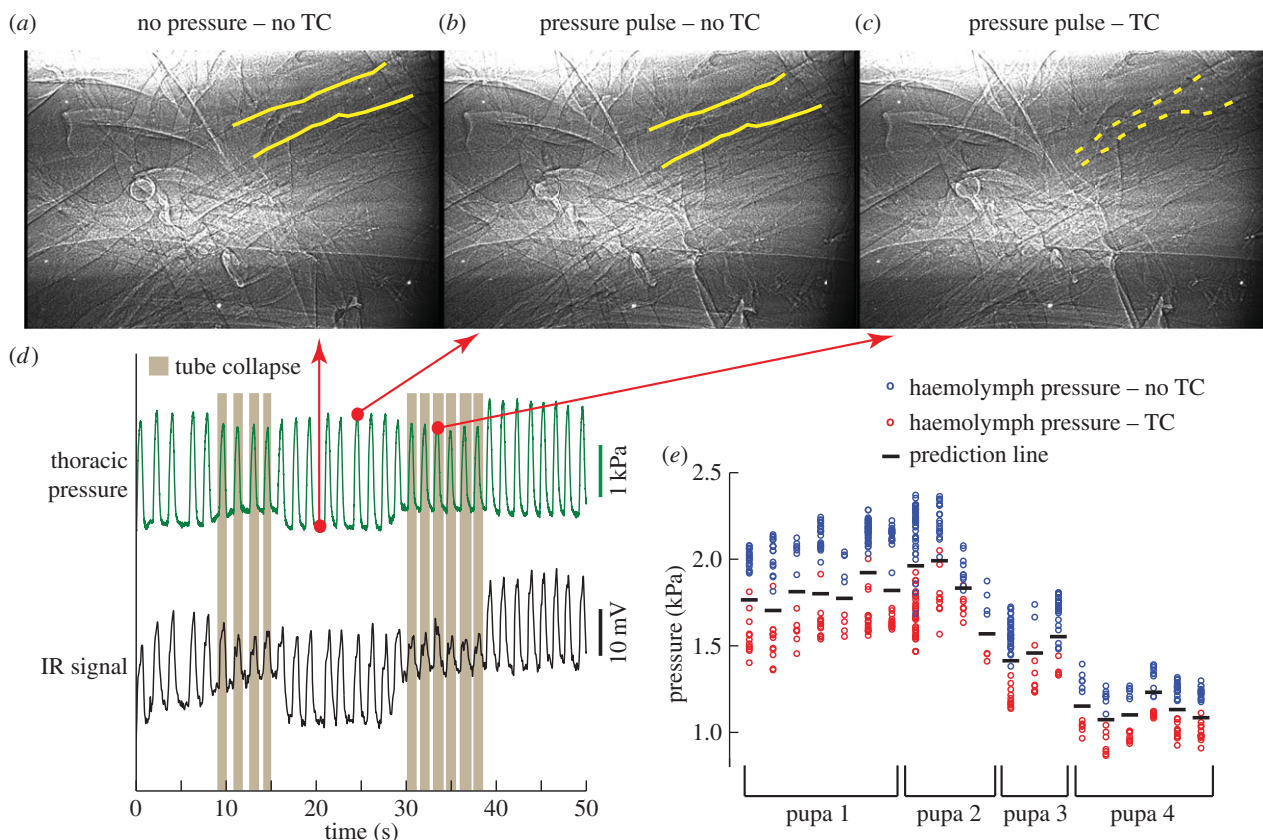


Figure 1. Correlation of abdominal pumping with tracheal compression and haemolymph pressure. X-ray video (a–c) shows that tracheal compression occurs during only some abdominal pumping/pressure cycles (d). Here, the IR signal indicates movement of the abdomen only; the magnitude does not represent absolute displacement. The results of clustering of pressure pulses can be used to predict tracheal compression (e); the points below the black bars are predicted to be associated with tube collapse. Each group represents a different temporal sequence. TC, tracheal compression. (Online version in colour.)

occur during this relatively fast event. To test this hypothesis, we visualized the tracheal system in pupae using synchrotron X-ray imaging, while also observing external abdominal movements and recording internal haemolymph pressure. In follow-up trials without X-rays, we examined the relationship between abdominal pumping, haemolymph pressure, and external gas exchange.

2. Material and methods

Pupae of the tenebrionid beetle *Z. morio* were used for experiments. Animals were bred and reared in the laboratory during the summer at room temperature ($T = 22\text{--}23^\circ\text{C}$) and weighed prior to trials ($m = 644.4 \pm 76\text{ mg}$, $N = 14$). The duration of pupation of this species is approximately 16 days during the summer and increases up to approximately 21 days in winter. Pupae were chosen for testing at ages ranging from 3 to 10 days post-pupation.

For each trial, the pupa was mounted on the ventral side of the thorax on a small platform formed of adhesive putty (Scotch adhesive putty, 3M). The animal was positioned such that the abdomen could move freely and the spiracles were not occluded (electronic supplementary material, figure S1).

Hydrostatic pressure in the haemolymph of the thorax was measured using a Fabry–Perot-type fibre-optic pressure sensor (Preclin 420, Samba Sensors; precision approx. 12 Pa). The sensor was inserted into the dorsal thorax through a drilled hole at an angle of $58\text{--}78^\circ$, a depth of 1.7–3.0 mm, and a distance of 1–2 mm from the midline of the thorax. The hole sealed rapidly owing to haemolymph coagulation.

Abdominal movements were recorded using an infrared (IR) sensor (SUNX FD-T80, Panasonic), or with a video camera (NEX-

VG10, Sony). The IR sensor was positioned facing the dorsal side of the abdomen, 2–3 cm from the animal. The camera recorded abdominal movements from a lateral view at 30 fps, and the cercus was tracked with a custom image processing code to quantify displacement (see the electronic supplementary material).

A custom-made respirometry chamber (28 ml, $25 \times 25 \times 45\text{ mm}^3$) was used to record CO_2 emission. Normoxic, CO_2 -free air (Airgas) was pushed into the chamber at a flow rate of 2.5 l min^{-1} , controlled with a flow control valve (MFC 5850E, Coastal Instruments, Inc.). The concentration of CO_2 in the outlet gas was measured with an infrared gas analyzer (LI 7000 Li-Cor; calibrated from 0 to 125 ppm, precision approx. 0.2 ppm). CO_2 data were adjusted for chamber washout using post hoc correction, and the start and end of CO_2 bursts were determined (see the electronic supplementary material).

The tracheal system was observed via synchrotron X-ray phase contrast imaging at beamline 32-ID of the Advanced Photon Source, Argonne National Laboratory. Following Socha *et al.* [10], imaging parameters were set using a sample–detector distance of 70 cm, monochromatic X-rays of 25 keV and a field of view of $3.3 \times 2.5\text{ mm}$.

(a) Trial protocols

In the first set of trials, we examined haemolymph pressure, abdominal movement (via IR) and tracheal movement with X-ray (four animals, 20–40 min each). In the second set of trials, we examined haemolymph pressure and CO_2 emission (10 animals, 2–24 h each). In three of these specimens, abdominal movement was recorded via camera for 2–8 h. All trials were conducted at room temperature ($22\text{--}23^\circ\text{C}$).

We chose a subset of the recordings for full analysis based on the presence of haemolymph pressure pulses, representing

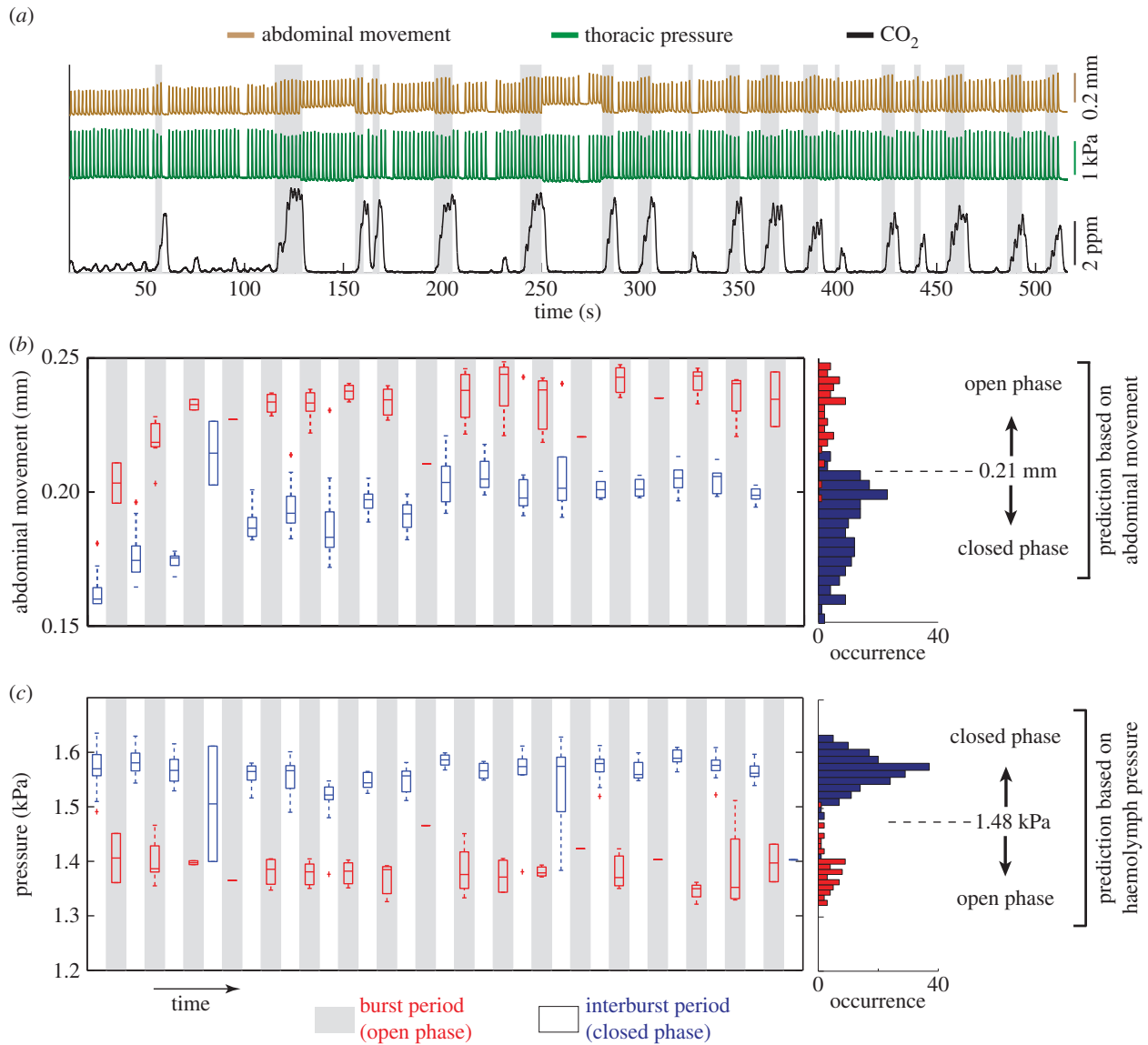


Figure 2. The magnitudes of abdominal pumping and haemolymph pressure differ during CO₂ burst (grey) and interburst (white) periods (*a–c*). Here, abdominal movements were measured from video records (see electronic supplementary material, movie S2). The status of the spiracles can be inferred by clustering the pressure pulses or abdominal movements (*b–c*). The burst and interburst periods were determined by temporal correction of the CO₂ signal (see the electronic supplementary material). (Online version in colour.)

65 min from the first trials and 250 min from the second trials. The abdominal movement and pressure signals were coded as having CO₂ bursts or no CO₂ bursts (called 'interburst'). During interburst periods, pupae emitted trace levels of CO₂, which we interpret as diffusion through the cuticle or through partially closed spiracles. CO₂ bursts were defined as CO₂ emissions with durations greater than 1 s and amplitudes greater than 20% of the average height of all CO₂ pulses.

We hypothesized that during the interburst phase, when the spiracles should be closed, the amplitude of abdominal pumping would be small and the magnitude of pressure pulses would be large in comparison to that in the open phase. To test this hypothesis, the amplitudes of pressure pulses and abdominal movements in each trial were partitioned into two clusters using the k-means clustering method [11]. The clusters were used to infer the status of spiracles (open or closed) and tracheae (relaxed or compressed) during each pressure pulse.

3. Results

X-ray imaging revealed that pupae of *Z. morio* exhibited periodic tracheal compressions. Some tracheal tubes in the thorax

deformed partially, or in some regions, collapsed completely (figure 1 and electronic supplementary material, movie S1). However, many tracheae remained intact at all times and did not exhibit obvious signs of compression. Pupae also exhibited periodic pressure pulses (figures 1 and 2) and CO₂ emission (figure 2). Every tracheal collapse event co-occurred with an abdominal pump and a pressure pulse. Similarly, every pressure pulse coincided with an abdominal pump. However, the majority (63.7%) of pressure pulse/abdominal pump events occurred without any tracheal compression. The magnitude of the haemolymph pressure pulses was 21.8% smaller when tubes collapsed compared with when they did not ($P_{\text{tube collapse}} = 1.51 \pm 0.27$ kPa, $P_{\text{no tube collapse}} = 1.93 \pm 0.36$; $p < 0.05$, $N = 562$ pulses, 10 animals). The amplitude of pressure pulses was 20.0% smaller during CO₂ burst periods than in interburst periods ($P_{\text{burst}} = 1.44 \pm 0.33$ kPa, $P_{\text{interburst}} = 1.80 \pm 0.3$ kPa; $p < 0.05$, $N = 3784$ pulses, 10 animals). Finally, the amplitude of abdominal movements was 25.3% larger during CO₂ burst periods than in interburst periods ($D_{\text{interburst}} = 246 \pm 81$ μm , $D_{\text{burst}} = 329.3 \pm 75$ μm ; $p < 0.05$, $N = 264$ and 821, respectively; electronic supplementary material, movie S2).

Clustering of pressure magnitudes in the respiratory dataset was used to predict the compression behaviour of the tracheal tubes in the X-ray trials (figure 1e). This method produced correct predictions for 524 of 544 pressure pulses that were tested (96.3% accuracy). In addition, 1085 pressure pulses and abdominal movements in three pupae were clustered to predict CO₂ emission, and thereby infer the status of spiracles (figure 2b,c). Predictions based on pressure pulses and abdominal movements demonstrated 88.9% and 92.7% accuracy, respectively.

4. Discussion

Experimental studies of pupae have found sporadic correlations between abdominal pumping and gas exchange under some conditions [5]. The rate of CO₂ emission depends on many factors, including the intra-tracheal concentration of CO₂, the opening area of spiracles, and the volume deformation of the tracheae. Previously observed associations of abdominal pumping and CO₂ emission may therefore reflect a co-occurrence of abdominal pumping and spiracular opening without any volume change in the tracheal system, a mechanical requirement for active ventilation. Using X-ray imaging, this study demonstrates conclusively that pupae compress the tracheal system during abdominal pumping, producing active ventilation.

However, abdominal pumping produces two different behaviours, which depend on the state of the spiracles. When no CO₂ is emitted, all spiracles must be closed; conversely, when CO₂ is emitted, one or more spiracles must be open. Abdominal pumping occurs during both states, but this movement only produces active ventilation during the open phase. In this phase, the abdominal pump produces a pressure rise in the haemolymph (within the range of other taxa [12,13]), and compression in the tracheal system is allowed because air can be displaced outward by bulk flow. Correspondingly, a part of the pressure rise in the haemolymph is alleviated by the compression of the tracheal system, accounting for the lower observed pressures during active ventilation. This alleviation also allows the abdomen to bend more under the action of the intersegmental muscles.

By contrast, when the spiracles are closed, the abdominal pump produces a greater rise in pressure in the haemolymph, but with no visible compression of the tracheal system. This can be explained by simple gas theory: the rise in haemolymph pressure acts on the closed tracheal system, and any

compression of the tracheae must be owing to the compression of air itself. As calculated using Boyle's law, an average pressure increase of 1.93 kPa during the closed phase should result in a 1.9% decrease in tracheal volume, which assumes that the tracheae provide no structural resistance to collapse. This small change in volume must be distributed across the tracheal system, but it cannot be discerned in our X-ray projection images owing to limits of resolution (approx. 1 µm). In this phase, the tracheal system should be more rigid, limiting movement produced by the intersegmental muscles during abdominal pumping. Such rigidity would explain the observations of decreased amplitude of the abdominal movement and increased haemolymph pressure.

These findings provide the basis for an indirect method for predicting the status of the spiracles and the state of tracheal tubes in species that employ this mechanism, by measuring either pressure or abdominal movement. Pupae do not change their behaviour rapidly, and the average magnitude of their abdominal movement and pressure stay relatively consistent in the open or closed phase. However, they do change over longer time intervals, enabling clustering of relatively short periods of time (1–10 min) to provide accurate predictions.

Abdominal pumping during the spiracular closed phase has been observed in some other pupae [5,12] and adults [6]. Most abdominal pumping events (63.7%) observed in this study occurred during this closed phase, suggesting that abdominal pumping serves other roles besides active ventilation, including internal gas mixing, reduction of water loss [6] or facilitation of haemolymph circulation [13]. The abdominal pump is a multi-functional system, and its widespread and varied usage across insects demands deeper mechanistic investigation.

Data accessibility. Data are available through Dryad (<http://dx.doi.org/10.5061/dryad.90sj5>) and in the electronic supplementary material.

Authors' contributions. H.P. and J.J.S. conceived the study; H.P., M.C.K. and J.J.S. conducted the data collection. H.P. performed the analysis. All authors contributed in writing and editing the paper.

Competing interests. We declare we have no competing interests.

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