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RESEARCH ARTICLE

Respiratory function of the plastron in the aquatic bug *Aphelocheirus aestivalis* (Hemiptera, Aphelocheiridae)

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ABSTRACT

The river bug *Aphelocheirus aestivalis* is a 40 mg aquatic insect that, as an adult, relies totally on an incompressible physical gill to exchange respiratory gases with the water. The gill (called a 'plastron') consists of a stationary layer of air held in place on the body surface by millions of tiny hairs that support a permanent air–water interface, so that the insect never has to renew the gas at the water's surface. The volume of air in the plastron is extremely small (0.14 mm^3), under slightly negative pressure and connected to the gas-filled tracheal system through spiracles on the cuticle. Here, we measure P_{O_2} of the water and within the plastron gas with O_2-sensing fibre optics to understand the effectiveness and limitations of the gas exchanger. The difference in P_{O_2} is highest in stagnant water and decreases with increasing convection over the surface. Respiration of bugs in water-filled vials varies between 33 and 296 pmol O_2 s^{-1}, depending on swimming activity. The effective thickness of the boundary layer around the plastron was calculated from respiration rate, P_{O_2} difference and plastron surface area, according to the Fick diffusion equation and verified by direct measurements with the fibre-optic probes. In stagnant water, the boundary layer is approximately 500 μm thick, which nevertheless can satisfy the demands of resting bugs, even if the P_{O_2} of the free water decreases to half that of air saturation. Active bugs require thinner boundary layers (~100 μm), which are achieved by living in moving water or by swimming.

KEY WORDS: Aquatic insect, Metabolic rate, Optode, Oxygen, Respiration, Spiracle, Tracheal system

INTRODUCTION

Insects rely primarily on their tracheal system to exchange respiratory gases with the atmosphere (Wigglesworth, 1931). The system consists of air-filled tubes that open to the air through spiracles on the surface of the thorax and abdomen, branch throughout the body, and eventually reach the cells with blind-ended tracheoles. Oxygen (O_2) and carbon dioxide (CO_2) move through the smaller tubes largely by diffusion down gradients in partial pressure (P_{O_2}, P_{CO_2}), although the outer tracheae are connected and can be ventilated by movements of the body (Harrison et al., 2013). Many aquatic insects still use the gas-filled tracheal system, but interface it with snorkels, tracheal gills or air bubbles on their bodies that act not only as an O_2 store, but also as a 'physical gill' capable of exchanging O_2 and CO_2 with the water (Seymour and Matthews, 2013). Physical gills are classified into two types: 'compressible' or 'incompressible', depending on whether the air–water interface is unsupported or supported, respectively (Flynn and Bush, 2008). Compressible gas gills decrease in volume during a dive, because: (1) O_2 is removed by respiration; (2) CO_2 does not replace the O_2 removal as it easily dissolves in water; and (3) nitrogen (N_2) dissolves in the water because its partial pressure (P_{N_2}) increases over the P_{N_2} of the surrounding water because of the decrease in P_{O_2} in the gill (Ege, 1915; Rahn and Paganelli, 1968). The limited lifetime of compressible gas gills forces the insects to renew the bubble periodically at the surface. Incompressible gas gills, defined as 'plastrons' by Thorpe and Crisp do not collapse, because the air–water interface is supported by hydrophobic cuticular hairs that push against it (Marx and Messner, 2012; Thorpe and Crisp, 1947a). Oxygen can diffuse from the water into the plastron gas space and from there through spiracles into the tracheal system of the insect. The physical gill is permanent, and the insects never have to surface if the water is sufficiently oxygenated.

Plastron respiration has been considered sporadically for about a century (Balmert et al., 2011; Ege, 1915; Flynn and Bush, 2008; Hinton, 1976; Messner and Adis, 1995; Thorpe, 1950). A uniform picture of the anatomy, physiology and physics of this interesting system emerges, indicating that the hydrophobic surface of the plastron matches the hydrostatic pressure with surface tension of the curved menisci on the plastron. Dissolved O_2 taken up by the insect reduces the P_{O_2} in the plastron gas, but P_{N_2} does not increase as it does in collapsible gas gills. Instead, plastron P_{N_2} remains in equilibrium with the water and the total pressure in the plastron gas becomes less than the combined atmospheric and hydrostatic pressures outside the plastron. The decrease in total pressure is roughly equivalent to the decrease in P_{O_2}.

Conclusions about plastron function have been largely theoretical, although good measurements of plastron structure of the river bug *Aphelocheirus aestivalis* (Hinton, 1976) and rates of O_2 consumption have been measured for several species of plastron bugs (Kölsch and Krause, 2011; Thorpe and Crisp, 1949; Verberk and Bilton, 2015). According to a recent analysis (Seymour and Matthews, 2013), respiration rates of resting bugs range from 27 to 55% of the prediction of resting metabolic rates based on an interspecific allometric analysis of 391 species of insects in general (Chown et al., 2007). Such low values might be related to diffusion limitation through the layer of water adjacent to the plastron. This is the 'boundary layer', which is calculated according to the Fick general diffusion equation as the thickness of an imaginary layer of completely stagnant water next to the exchange surface. Thus the measured rates of O_2 consumption could be satisfied with effective boundary layers of approximately 100 to 800 μm thick in air-equilibrated water (Seymour and Matthews, 2013). It is expected that the effective thickness would decrease if the insect swam through the water or lived in moving water. However, there are no

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Received 14 May 2015; Accepted 6 July 2015

Journal of Experimental Biology

Journal of Experimental Biology (2015) 218, 2840-2846 doi:10.1242/jeb.125328

List of symbols

A	surface area of the air–water interface plastron
G_{O_2}	O_2 conductance of the gas exchange surface
K_{O_2}	Krogh's coefficient of O_2 diffusion in water
L	thickness of boundary layer
$\dot{M}_{O_2}$	rate of O_2 consumption
P_{CO_2}	partial pressure of CO_2
P_{O_2}	partial pressure of O_2
P_{N_2}	partial pressure of N_2
Q_{10}	temperature coefficient over 10°C range
β	oxygen capacitance of water

direct measurements of boundary layer thickness in any aquatic insect. It is important to know the boundary layer thickness because several models of gas exchange of aquatic insects assume that it is very thin (Chaui-Berlinck and Bicudo, 1994; Chaui-Berlinck et al., 2001; Rahn and Paganelli, 1968).

Actual measurements of P_{O_2} in the boundary layer or within plastron gas have not been possible, because the volume of the gas is miniscule. In particular, anatomical data from the river bug *A. aestivalis* indicate that the plastron hairs are only 3 µm long and the entire gas space between the hairs of the plastron is only about 0.14 mm^3 (calculated in this study from data in Hinton, 1976). Despite this problem of size, we were able to measure the P_{O_2} across the boundary layer and inside the plastron gas of *A. aestivalis*. The technique involves a modification of O_2-sensing fibre optics (optodes) that have been used to measure P_{O_2} in collapsible gas gills of other diving insects with collapsible gas gills (Matthews and Seymour, 2006, 2008, 2010). We also investigate the interaction between swimming activity, respiration rate and boundary layer thickness over the surface of the plastron.

RESULTS

Plastron morphometrics

The mean body mass of 15 adult *A. aestivalis* was 43.5±3.2 mg (mean±95% confidence interval, CI). There was no significant difference between 8 males and 7 females (*t*-test; P=0.39). Mean body length was 9.40±0.19 mm. The surface area of the silhouette from above, without the head and appendages, was 47.5±1.8 mm^2. Males had a slightly, but significantly, smaller silhouette (45.7 mm^2) than females (49.5 mm^2) (P=0.016). The bugs were highly dorsoventrally flattened (Fig. 1), so assuming that they were perfectly flat, the total surface area of the plastron was taken as twice the area of the headless silhouette, or 95 mm^2. Hinton (1976) measured the structure of the plastron of *A. aestivalis*, concluding that the hairs were 3 µm high, 0.4 µm in diameter and their centres spaced 0.5 µm apart. These data indicate that the air fraction is 0.5. Our measured surface area of 95 mm^2 multiplied by the thickness of 0.003 mm^2 gave a total plastron volume of 0.28 mm^3 and an air space of 0.14 mm^3.

We do not know the total volume of the tracheal plus plastron system accurately, but can estimate the maximum volume by assuming that the density of the air-less body is 1.078 g ml^{-1}, which is the measured density of the aquatic bug *Anisops deanei* (Matthews and Seymour, 2008). Because *A. aestivalis* was observed to be negatively buoyant, the total air space is somewhat less than 3.39 mm^3 or 8.4% of body volume.

P_{O_2} of plastron gas

When the bubble on the tip of the optode sheath contacted the plastron, there was an immediate change in P_{O_2} that progressed toward a relatively stable value (Fig. 2). The speed of this change was related to how fast the bubble was absorbed, with equilibration occurring generally between 1.5 and 4 min. Although it was thought that placing a bubble of nitrogen instead of air on the optode tip would speed up equilibration, it was not always evident in the traces. The most successful traces occurred when equilibration coincided with complete absorption of the bubble and the sheath was contacting the plastron. Sometimes the lowest P_{O_2} occurred before the bubble was completely absorbed, in which case we waited until it was absorbed and then recorded the value. Data were discarded if equilibration was incomplete when the bubble disappeared totally.

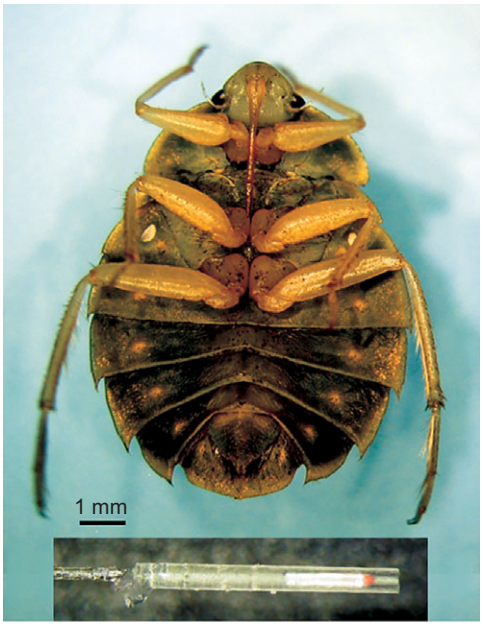

Fig. 1. Ventral side of *Aphelocheirus aestivalis* showing the broad surface covered with the plastron. The light spots on the sternites are the spiracles (except for the two bright spots on the second abdominal sternites, which are presumptive sense organs (Thorpe and Crisp, 1947a). The inset shows the fibre-optic 'optode' encased in its polyethylene sleeve, open to the right and sealed with petroleum jelly to the left. The O_2-sensitive dye is the pink dot on the end of the optode. The 1 mm scale relates to both the insect and the optode.

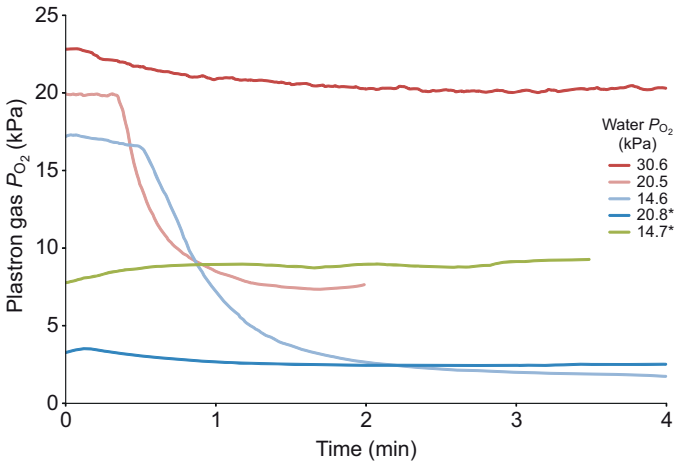

Fig. 2. Examples of P_{O_2} traces from selected runs at different levels of aquatic P_{O_2}. Equilibration occurred after approximately 2 min. The top three initial traces involved optodes tipped with bubbles of air and the bottom two traces (*) initially had bubbles augmented with N_2.

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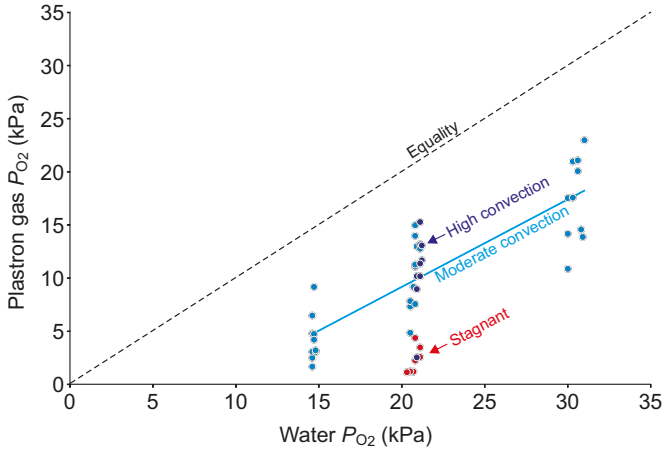

Fig. 3. P_{O_2} at equilibrium in the plastron gas of *Aphelocheirus aestivalis* in relation to the P_{O_2} of the free aquarium water around the tethered insect. The points represent individuals under three qualitative regimes of aquarium convection caused by bubbling from a gas diffuser. Water P_{O_2} was varied only under moderate convection.

In normoxic water (~20.7 kPa), the difference in equilibrium P_{O_2} between the water and the plastron was 10.6±2.5 kPa (mean±CI) in 8 tethered bugs with high convection, 10.2±1.7 kPa in 12 bugs with moderate convection and 18.7±0.9 kPa in 8 bugs in stagnant water (Fig. 3). There was no significant difference between high and moderate convection (P=0.38), but plastron P_{O_2} in stagnant water was lower than both other treatments (P<0.0001; t-tests). The effect of ambient P_{O_2} was tested only with moderate convection. The slope of the linear regression for data for 32 points for aquatic P_{O_2} between 14 and 32 kPa was slightly less than that for equality ($P_{O_2,plastron}$=0.82 $P_{O_2,water}$−7.3; r^2=0.75).

Boundary layer thickness

Boundary layer thickness was evaluated from P_{O_2} transects above the plastron surface in a miniflume by observing where the P_{O_2} began to decrease from ambient levels in the free water (Fig. 4). This began at approximately 500 µm away from the ventral sternite surface in stagnant water, 200 µm in water flowing through the flume at 92 ml min^{-1} and 100 µm in water flowing at 256 ml min^{-1}. The velocity of the water over the surface of the bugs is not known precisely, but the mean velocity across the cross section of the empty flume was approximately 2.5 mm s^{-1} and 5.8 mm s^{-1}, respectively. Transects of the boundary layer on the dorsal side tergites in stagnant water were similar to those on the ventral surface, beginning to decrease at approximately 600 µm away and dropping to 6.3±0.8 kPa (mean±CI) at the surface.

Respiration rates

Respiration rates of 14 *A. aestivalis* in the present study were standardized to 20°C from data taken between 20 and 23°C assuming a Q_{10} of 2.3 (Fig. 5). We could not control the activity of the insects in the respirometer, but the lowest rates were from mainly stationary animals and the highest rates were from bugs that were swimming almost constantly. There was no significant difference between the overall means of the present study and Thorpe and Crisp, but their data appear overall slightly higher (Thorpe and Crisp, 1947b). Our lowest rate was 0.66 nmol s^{-1} g^{-1} (wet mass; =32.6 pmol s^{-1}) and the highest 6.73 nmol s^{-1} g^{-1} (=296 pmol s^{-1}), giving a maximum factorial metabolic scope of approximately 9.1. By comparison, Thorpe and Crisp's data were 1.67 and 9.92 nmol s^{-1} g^{-1}, respectively, giving a scope of 5.9. A recent study of metabolic rate in inactive *A. aestivalis* at 5–15°C extrapolates to 2.62 nmol s^{-1} g^{-1} (=100 pmol s^{-1}) at 20°C (Verberk and Bilton, 2015).

DISCUSSION

The data gathered in this study allow us to evaluate plastron gas exchange in *Aphelocheirus aestivalis* according to Fick's general diffusion equation: $\dot{M}_{O_2}=G_{O_2}(P_{O_2\,out}-P_{O_2\,in})$, where $\dot{M}_{O_2}$ is the rate of O_2 uptake of the whole animal (pmol s^{-1}), G_{O_2} is the O_2 conductance of the gas exchange surface (pmol s^{-1} kPa^{-1}) and ($P_{O_2\,out}-P_{O_2\,in}$) is the difference in P_{O_2} (kPa) between the free water and the plastron gas. $G_{O_2}=K_{O_2}(A/L)$, where K_{O_2} is Krogh's coefficient of O_2 diffusion in water (constant at 0.277 pmol s^{-1} kPa^{-1} cm^{-1} at 20°C) (Seymour, 1994), A is the surface area of the air–water interface plastron (constant at 0.95 cm^2) and L is the thickness of the boundary layer (cm). The exchange surface area is less than the surface area of the plastron because the interface is supported by hairs that take up some of the area. This is estimated from direct measurements of the plastron morphology for *A. aestivalis* (Hinton, 1976). Each square mm of the surface has about 4,000,000 hairs that rise perpendicularly from the

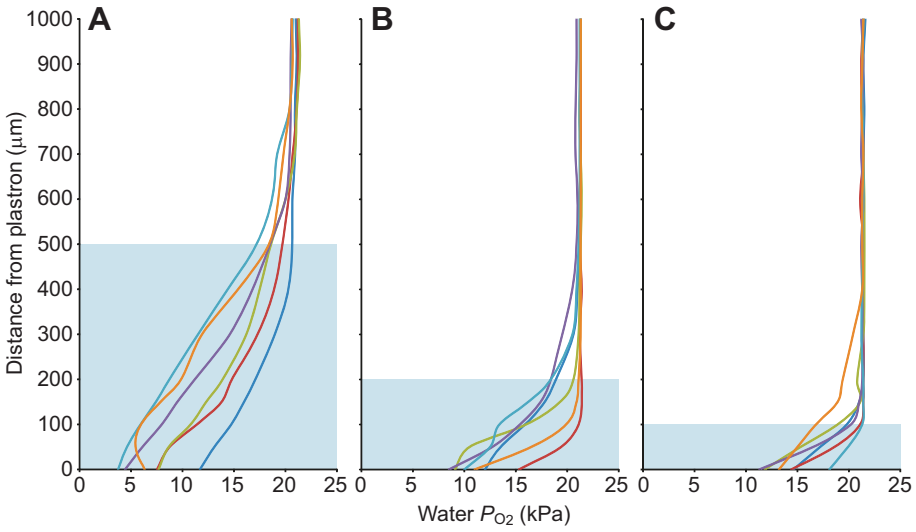

Fig. 4. Transects of P_{O_2} measured close to the surface of the ventral plastron of *Aphelocheirus aestivalis* in a small flume with water flowing at three speeds. (A) Stagnant water. (B) Water flowing at 92 ml min^{-1}. (C) Water flowing at 256 ml min^{-1}. Estimated boundary layer thickness is indicated to the closest 100 µm by boxes that enclose approximately linear decreases in P_{O_2}.

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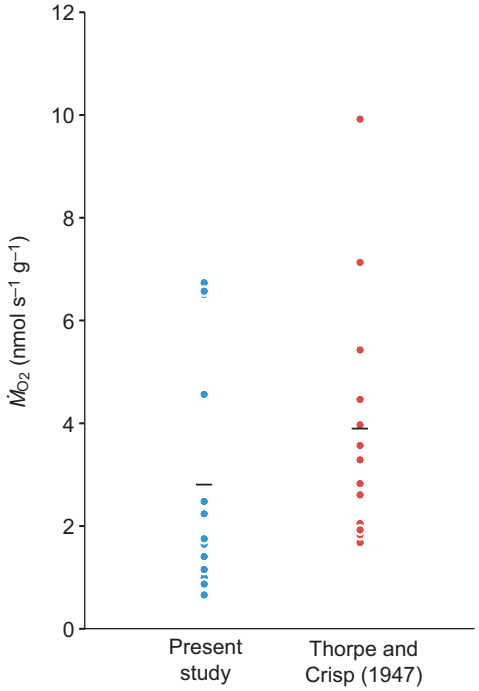

Fig. 5. Respiration rates of *Aphelocheirus aestivalis* in closed respirometer vials at 20–23°C, but adjusted to 20°C for comparison with data for this species collected at 20°C by Thorpe and Crisp (1947b). The variability in the data relate to different levels of activity during individual measurements. Means are horizontal bars.

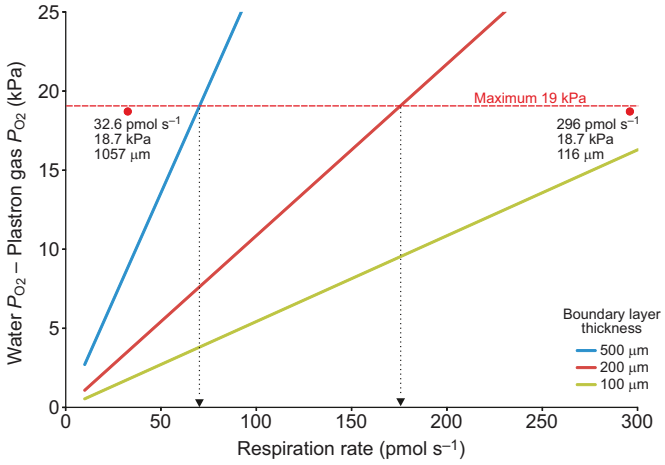

Fig. 6. Theoretical relationships between total respiration rate and the difference in P_{O_2} between the free water and the plastron gas in *Aphelocheirus aestivalis*, according to Fick's general diffusion equation. Plastron surface area is taken as 0.95 cm^2 and the fraction of air–water interface on the plastron is 0.7. The lines assume constant boundary layer thicknesses of 500, 200 and 100 μm, corresponding to those approximate distances measured in optode transects (Fig. 3). The lowest and highest measured respiration rates are indicated as points, providing the calculated boundary layer thickness, assuming a constant P_{O_2} difference of 18.7 kPa. The maximum P_{O_2} difference without reducing respiration rate is assumed to be 19 kPa.

cuticle and make a right angle turn at the top. The hairs taper, such that the average width of the bent tip on the surface of the plastron is 0.15 μm. Assuming that the hairs form a grid with 2000 rows of hairs in each square mm, the area covered by hairs is 0.15×1000×2000=300,000 μm^2 (width of hair×length of row×number of rows). This is 30% coverage of hairs, leaving 70% of area between the hairs, or equivalent to 0.66 cm^2 for the whole insect. Different packing and overlap of hairs or effects of meniscus shape might change this fraction a little, but this seems to be a reasonable approximation. Independently, the free area for exchange in adult *A. aestivalis* was estimated to be 0.60 cm^2 (Thorpe and Crisp, 1947b).

The relationships between $\dot{M}_{O_2}$, P_{O_2} difference and boundary layer thickness indicate that a higher $\dot{M}_{O_2}$ requires a proportionally higher P_{O_2} difference or an inversely proportional boundary layer thickness (Fig. 6). However, there is a maximum P_{O_2} difference that apparently can occur without reducing metabolic rate. Although we have not measured the critical P_{O_2} below which $\dot{M}_{O_2}$ decreases in this species, it is 2 kPa in aquatic waterboatmen *Agraptocorixa eurynome* (Matthews and Seymour, 2010), which is equivalent to a P_{O_2} difference of approximately 19 kPa. This seems reasonable because *Aphelocheirus* is reported to survive P_{O_2} around 5% of saturation (~1 kPa) in running water of 15°C (Marten et al., 1994). The mean P_{O_2} difference in *A. aestivalis* is close to this limit: 18.7 kPa in stagnant water. Taking the endpoints of the range of $\dot{M}_{O_2}$ measured in this study, namely 32.6 and 296 pmol s^{-1}, and the P_{O_2} difference of 18.7 kPa, the boundary layer thickness would be 1057 and 116 μm, respectively (these points appear on Fig. 6). It is evident from this analysis that a boundary layer 500 μm thick, as measured in optode transects in stagnant water (Fig. 4) can support $\dot{M}_{O_2}$ up to approximately 70 pmol s^{-1} before the P_{O_2} difference reaches 19 kPa. Resting $\dot{M}_{O_2}$ is about half of 70 pmol s^{-1} (Fig. 4), so resting metabolic rates should be achievable in stagnant water if the P_{O_2} is above 10 kPa.

Active metabolic rates require convection to reduce the boundary layer thickness. The effect of convection on effective boundary layer above plastrons has been analysed theoretically, but not experimentally (Flynn and Bush, 2008). Our data indicate that with a thickness of 200 μm, $\dot{M}_{O_2}$ could rise to approximately 175 pmol s^{-1}, and with 100 μm, it could rise above 300 pmol s^{-1} (Fig. 6), which is about the maximum rate we ever measured (=6.82 nmol s^{-1} g^{-1}) (Fig. 5). Boundary layers of approximately these thicknesses occurred in the flume measurements when the flow rate was between 92 and 256 ml min^{-1} (Fig. 4). Unfortunately we do not know the velocity distribution of the water within the hydraulic boundary layer; however, the mean velocity of the water in the flume without the insect was about 2.5 mm s^{-1} and 5.8 mm s^{-1}, respectively. The bugs can swim faster than this, so it is apparent that simply moving through the water reduces the effective boundary layer enough to prevent diffusion limitation of respiration at the highest levels we measured. An increased mobility of *Aphelocheirus* was reported in aquaria when the water current was interrupted (Larsén, 1931).

The thicknesses of the boundary layers that we measured and calculated are considerably larger than previously indicated in the literature. Thorpe and Crisp modelled the oxygen diffusion cascade through four layers of the plastron–tracheal system: (1) the external boundary layer; (2) vertically and laterally in the plastron gas; (3) the tracheal system to the ends of the tracheoles; and (4) through the tissues (Thorpe and Crisp, 1947b). They assumed a resting O_2 consumption rate of 6 mm^3 h^{-1}, which is equivalent to 74 pmol s^{-1}. For this rate, they calculated a P_{O_2} drop across several layers of the oxygen cascade: external boundary layer next to the plastron (0.7 kPa), across the plastron to spiracles (0.1 kPa), spiracles to tracheoles (0.3 kPa), through tissues to mitochondria (2.0 kPa), adding to a total of 3.1 kPa for whole oxygen cascade. The obvious problem with their calculations is the gross underestimation of the

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boundary layer thickness. Our experiments in stagnant water show that a $\dot{M}_{O_2}$ of 74 pmol s^{-1} would create a boundary layer of 466 µm and a P_{O_2} difference of 18.7 kPa (Fig. 6), rather than 0.7 kPa. If we ignore Thorpe and Crisp's calculated decrease of 0.7 kPa across the boundary layer, the decrease in the plastron–tracheal and system–tissue layers totals 2.4 kPa. This almost exactly matches the mean plastron P_{O_2} (2.0 kPa) that we measured in air-equilibrated stagnant water (Fig. 3). We conclude that the calculations of the oxygen cascade within the plastron–tracheal and system–tissue layers calculated by Thorpe and Crisp are reasonable, but the boundary layer is much too thin. Higher metabolic rates due to activity would be expected to increase the P_{O_2} difference between the plastron and the tracheoles, but would also increase plastron P_{O_2} by reducing the effective boundary layer thickness.

There is a technical question of how the gas in the optode sheath and applied bubble affected the measurements of plastron P_{O_2}. The volume of air in the optode sheath was calculated from the internal dimensions of the sheath less the volume of the optode as 0.236 mm^3. The bubbles were approximately the same outside diameter of the sheath and therefore nominally 0.113 mm^3. The total volume of air associated with the optode was therefore approximately 0.349 mm^3. By comparison, the total volume of the tracheal and plastron gas is calculated above to be about 10 times larger, at 3.39 mm^3. Assuming that the total pressure in the plastron is 80 kPa and equal to that in the tracheal system, and normal barometric pressure is 101.3 kPa, then the potential air volume associated with the pressure difference is up to 0.713 mm^3 (3.39×21.3/101.3), which is about six times larger than a 0.113 mm^3 bubble. Therefore the absorption of several optode bubbles can be explained by expansion of the sub-atmospheric plastron-tracheal system when the bubble made contact with the plastron gas.

Three additional arguments indicate that the effect of the optode gas was minor. First, the application of the bubble required about 2 min to reach equilibrium (Fig. 2). During this time, the respiration rate of a resting bug consumes about 0.19 mm^3 of oxygen, which is more than the estimated maximum of 0.14 mm^3 of oxygen in the entire optode–plastron–tracheal system when the optode was applied. (This is calculated assuming that the P_{O_2} in the 3.39 mm^3 plastron–tracheal system is 2 kPa, yielding 0.07 mm^3 of O_2 plus 0.349 mm^3 of gas in the bubble and optode sheath at a P_{O_2} of 21 kPa, yielding another 0.07 mm^3 of O_2.) Second, the traces appeared to plateau when the rate of consumption equalled that entering the plastron from the water (Fig. 2), so equilibrium was reached at the same level as occurred before the optode bubble was applied. Third, by the time equilibrium was reached, the size of the bubble had disappeared and the sheath contacted the plastron. In a separate set of experiments, we measured the O_2 conductance of naked bubbles on the end of the optode (our unpublished data). Conductance was high on large bubbles, but decreased to practically zero when the sheath contacted a gas-impermeable surface. This indicates that the P_{O_2} represented that in the plastron and was not significantly influenced by aquatic P_{O_2}.

Ecological implications

Diving insects that use a plastron are under severe constraints because of the limitations of gas exchange inherent in this mechanism. There are several lines of evidence for this idea. First, true plastron insects are generally small, because scaling principles show that metabolic rate generally increases faster than surface area as insects increase in size, reaching a limit set by surface area (Seymour and Matthews, 2013). This applies to species that never have to surface to replenish the bubble, including some beetles (Madsen, 2008, 2012). *A. aestivalis* and some other species of *Aphelocheirus* may be the largest plastron insects at approximately 40 mg body mass (Xie and Liu, 2014). Second, resting metabolic rates of true plastron insects are in the region of one-quarter to one-half of the rates predicted from most insects of the same body size and temperature (Seymour and Matthews, 2013). Third, plastron insects tend to occur in well-oxygenated, flowing water (Hutchinson, 1981), which reduces the effective boundary layer thickness and its resistance to diffusion. In particular, *A. aestivalis* live typically in moving streams (Basu et al., 2013; Hoffmann, 2008; Miguélez and Valladares, 2010). In stagnant water, the measured boundary layer thickness in *A. aestivalis* is about 500 µm, which is thin enough to satisfy a bug with respiratory demands twice the level of the minimum that we measured. Therefore, resting bugs could withstand P_{O_2} levels in stagnant water at approximately half of air-saturation. Even if the insects find themselves in more severely O_2-depleted stagnant water, they can survive by moving, either to reduce the boundary layer, to enter water near the surface with higher P_{O_2} or to obtain O_2 from the surface of aquatic vegetation (Pedersen and Colmer, 2012). If O_2 becomes very low, *A. aestivalis* moves near the surface and can even voluntarily emerge from the water (Thorpe, 1950). Finally, the critical thermal maximum of *A. aestivalis* decreases in aquatic hypoxia (P_{O_2}=5 kPa), whereas that of a comparable air-breathing bug *Ilyocorus cimicoides* does not (Verberk and Bilton, 2013, 2015).

MATERIALS AND METHODS

Animals

Aphelocheirus aestivalis (Fabricius 1794) Hemiptera were captured in small rivers in the northern part of Germany (Warnow River as well as in the northern run off of the Schweriner See). The summer water temperature was 22 to 23°C and oxygen saturation was between 70 and 90%. Free water flow rate was approximately 0.1 to 0.4 m s^{-1}. The bottom of the rivers was covered with gravel and small stones and was similar to the biotopes of *Aphelocheirus* described elsewhere (Larsén, 1931; Ussing, 1910). The population density was very high and up to 20 adult individuals could be caught on a patch of 30×20 cm. They were maintained in small holding aquaria (~50 litres), which were aerated and filtered. They were fed living red blood worms (*Chironomus* sp.) and small gammarid crustaceans (*Hyalella* sp. and *Gammarus* sp.), which form part of their natural diet (Lemb and Maier, 1996).

Bugs were removed from the holding aquarium, blotted lightly with facial tissue and weighed to within 0.1 mg on a balance (model 1201 Sartorius GmbH; Göttingen, Germany). They were photographed from above, against a grid and the length and area of the silhouette was determined with ImageJ (Version 1.47, Wayne Rasband, NIH, USA). Legs of the bugs were removed from the photographs to produce a body outline. Each photograph was converted into a binary image where particle analysis was used to determine the surface area of the bugs' silhouettes. Proportion of surface area attributed to the head was removed as the plastron does not occur there.

The bugs were inverted and attached to a bent wire stand with a tiny drop of cyanomethacrylate adhesive on the thorax between the two wing buds. A cloth mesh, about 12 mm square with six openings per centimetre, was used to restrain the legs by cutting four to five strands in a central slit and pulling the mesh over the abdomen toward the head. The anterior end of the mesh was secured above the head on two wires attached to the stand. The wire stands were placed on an inverted beaker in a small aquarium (30L×20W×16H cm), so that the bugs were approximately 1 cm below the water surface. The aquarium had a sintered glass gas diffuser on one side that stirred the water and equilibrated it with selected gas mixtures. Pure oxygen and nitrogen were mixed with mass flow controllers [models 1179A (200 ml min^{-1}) and 1259B (1000 ml min^{-1}), MKS Instruments, Andover, MA, USA] and a custom-made control unit. The surface of the water was covered with a polystyrene raft that cut off most of the exposure to air except

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for an 8-cm-diameter opening above the insects. It was possible to control the gas flow through the diffuser to create different convective environments around the insect described qualitatively as 'stagnant' when the diffuser was stopped after equilibration, 'moderate convection' with a gas flow rate of 460 ml min^{-1}, and 'high convection' at 920 ml min^{-1}. The system was allowed to equilibrate for 30 min between P_{O_2} treatments, 10 min between convection treatments and 10 min between boundary layer measurements. P_{O_2} was measured continuously in the water with one optode, while P_{O_2} in the plastron gas was measured with another optode.

P_{O_2} measurements

The oxygen-sensing, glass fibre-optic optodes were fast-response, tapered tipped fibres 140 µm in diameter and 20 mm of exposed length (sensor model B2, meter model TX-3 with thermal compensation, PreSens Precision Sensing GmbH, Regensburg, Germany). The optode output was logged at 1 s intervals by Presens software in the Microsoft Windows environment. The optodes were mounted in a Pasteur pipette held in a 3D micromanipulator above the aquarium. They were calibrated with the manufacturer's software by equilibrating them in a separate chamber with pure nitrogen (Air Liquide, UN 1066) and atmospheric air, taking into account the barometric pressure. The calibration was done with temperature compensation. For measurement of P_{O_2} inside the plastron, an optode was equipped with a 5 mm polyethylene tubing sheath (OD=0.60 mm, ID=0.25 mm) positioned such that the optode tip was inside the sheath about 1 mm from the end (Fig. 1 inset). The sheath was sealed to the optode at the proximal end with a thick layer of petroleum jelly.

The protocol for measuring plastron P_{O_2} involved submerging the optode under the water and placing a bubble of air or N_2 on the end of the sheath with a 50 µl gas-tight syringe (Hamilton Bonaduz, Switzerland). The bubble joined the air inside the optode sheath. The optode was then lowered until the bubble pushed against the ventral plastron of the bug, immediately over a spiracle. Successful measurements were characterised by immediate shrinkage of the bubble, which indicated that a gas contact was made between the bubble and the gas in the plastron. This was accompanied by a rapid change in optode P_{O_2}, particularly if the composition of the initial bubble on the optode was quite different from that in the plastron gas (Fig. 2). The initial volume of the bubble was important to control, in order to match the rate of bubble shrinkage to the rate of equilibration of the system. Bubbles that were initially 0.60 mm in diameter functioned well. As equilibration approached, the optode sheath was gradually lowered until it eventually rested almost flat on the plastron such that the gas around the optode was maximally exposed to plastron gas and minimally exposed to the ambient water. This situation did not last long, because the air connection between the plastron and optode quickly broke. This is the reason why continuous measurement of plastron P_{O_2} was not possible.

To evaluate how the bubble used to attach the optode to the plastron was affected by diffusion to or from the water, the sheathed optode was set up in an aquarium, just like the real experiment, except that the insect was replaced by a horizontal piece of solid styrene plastic. A bubble of atmospheric air was placed on the sheath and exposed to aquarium water equilibrated to a P_{O_2} of 31.7 kPa. This was performed with different-sized bubbles, different levels of convection and a range of exposure to the water (whole bubble and bubbles contacting the plastic to several extents, including those similar to the experimental contact with the plastron when the bubble was about to disappear).

Boundary layer thickness

All measurements were made at water P_{O_2}=20 kPa, nominally. A tapered optode without sheath was fixed to a micromanipulator and advanced so that the tip touched the surface of the plastron. After equilibration, it was pulled away in steps of 0.05 mm until the tip was 0.2 mm away and then in 0.10 mm steps until it reached 1.0 mm (outside the boundary layer). Boundary layer curves were obtained from the ventral (sternite 4; above the spiracle) and dorsal (tergite 2, about 30% from the midline) plastrons in stagnant water, from the ventral plastrons in the aquarium with moderate convection generated by the gas diffuser, and from ventral plastrons in a miniflume at two rates of water flow. The flume was milled from a piece of acrylic plastic 100 mm long×39 mm wide×35 mm high. The dimensions of the water course were 80 mm long, 20 mm wide and 16–19 mm deep (depending on flow rate). The first 28 mm contained a fabric screen and drinking straws to smooth the flow, followed by 52 mm of free water with the insect in the middle. A small pump from the aquarium flowed water through the flume and the flow rate was measured by collecting water in a graduated cylinder over a known period.

Respirometry

Closed-system respirometry was used to measure rate of oxygen uptake from individual bugs. This involved a 20 ml glass vial sealed with a thick rubber stopper designed to be pierced by hypodermic needles. The thin end of a Pasteur pipette was pushed through the stopper to create a tight fit. The vial was filled with aquarium water without any visible bubbles, the bug was admitted and then the vial was stoppered under water, which pushed a column of water into the pipette. With the vial lying horizontally, an optode was inserted into the pipette so that the tip was inside the chamber. There was little space between the optode covering and the glass, and this was filled with an approximately 5 cm length of water, so that diffusion was negligible. The chamber water was well mixed at the beginning of the run, and was mixed from time to time by the swimming insects. Nevertheless, at the end of each 60 min run, the vial was tilted repeatedly which caused the bug to fall back and forth, thus quickly mixing the water for the final measurement. A straight line was plotted between the P_{O_2} of the mixed water initially and finally in the chamber to obtain the rate of oxygen uptake according to the equation: $\dot{M}_{O_2}=V\beta(\Delta P_{O_2}/\Delta T)$ where $\dot{M}_{O_2}$ is the metabolic rate (µmol s^{-1}), V is the volume of the chamber (ml), β is the oxygen capacitance of the water (e.g. 0.013 µmol ml^{-1} kPa^{-1} at 23°C) and $(\Delta P_{O_2}/\Delta T)$ is the rate of change of P_{O_2} (kPa s^{-1}). Two blank runs were made at the beginning and end of the day on each chamber and the background respiration (~17% of the total respiration) was subtracted from the bug respiration. A second set of measurements was performed on six bugs at 20°C in a separate constant volume respirometry system. A glass syringe was equipped with a Clark-type oxygen electrode (E5047, Radiometer, Kopenhagen, Denmark) connected to a custom made amplifier. The cathode was covered with two 25 µm Teflon membranes in order to reduce the oxygen consumption of the electrode itself. The electrode was pushed forward in the syringe to reduce the volume of water to 2.65 ml. This small volume ensured a complete mixing during the measurement. The whole setup was submerged in a water bath kept at 20°C. The oxygen consumption rate of the aquarium water itself was determined to be less than 2 pmol s^{-1} (0.05 nmol g^{-1} s^{-1} for a 40 mg *Aphelocheirus*). There was no significant difference between the two techniques, so the data were combined.

Acknowledgements

We appreciate the technical assistance of Maik Kunert who helped with technical problems and Lisa Schilha and Ramona Voss who helped to catch *Aphelocheirus* in the field. The removal of the animals complied with the laws of the German federal state of Mecklenburg-Vorpommern.

Competing interests

The authors declare no competing or financial interests.

Author contributions

All authors designed the experiments, carried them out, analysed the results, drafted and edited the text.

Funding

This study was supported by the Alexander von Humboldt Foundation, the Australian Research Council and the Humboldt-Universität zu Berlin.

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