Oxygen limited thermal tolerance is seen in a plastron breathing insect, and can be induced in
a bimodal gas exchanger

Wilco C.E.P. Verberk¹,²,*, David T. Bilton²

¹ Department of Animal Ecology and Ecophysiology, Institute for Water and Wetland
Research, Radboud University, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands.

²Marine Biology and Ecology Research Centre, School of Marine Science and Engineering,
University of Plymouth, Davy Building, Drake Circus, Plymouth PL4 8AA, United Kingdom

*To whom correspondence should be addressed: wilco@aquaticecology.nl
Abstract

Thermal tolerance has been hypothesized to result from a mismatch between oxygen supply and demand. However, the generality of this hypothesis has been challenged by studies on various animal groups, including air-breathing adult insects. Recently, comparisons across taxa have suggested that differences in gas exchange mechanism could reconcile the discrepancies found in previous studies. Here we test this suggestion by comparing the behavior of related insect taxa with different gas exchange mechanisms, with and without access to air. We demonstrate oxygen limited thermal tolerance in air-breathing adults of the plastron exchanging water bug *Aphelocheirus aestivalis*. *Ilyocoris cimicoides*, a related, bimodal gas exchanger, did not exhibit such oxygen limited thermal tolerance and relied increasingly on aerial gas exchange with warming. Intriguingly, however, when denied access to air, oxygen limited thermal tolerance could also be induced in this species. Patterns in oxygen-limited thermal tolerance were found to be consistent across life history stages in these insects, with nymphs employing the same gas exchange mechanisms as adults. These results advance our understanding of oxygen limitation at high temperatures; differences in the degree of respiratory control appear to modulate the importance of oxygen in setting tolerance limits.

Keywords

Global warming; Heat tolerance; Hypoxia; Multi stressor; OCLTT; Respiration physiology
Introduction

Of the many effects of temperature on the physiology of ectotherm animals, it has been argued that thermal limits are set by oxygen limitation at the level of the whole organism (Winterstein, 1905; Brett, 1971; Pörtner, 2006; 2010), since with increasing temperatures there is a progressive mismatch between oxygen supply and demand (see Verberk et al., 2011). The resulting oxygen deficiency initially causes animal performance to decline, and -at more extreme temperatures- aerobic metabolism can no longer be maintained, resulting in heat coma and death due to a lack of oxygen (Pörtner, 2010). This oxygen limitation at the whole-organism level is seen to set in before other physiological systems become thermally impaired (Pörtner, 2006). Oxygen limitation is thus seen as a mechanism which integrates performance across physiological systems, something which can assist our understanding and prediction of thermal effects on growth, reproduction and ultimately survival.

Perhaps due to the inherent attractiveness of such a general principle the hypothesis has become very popular, but has also attracted considerable criticism (e.g. Clark et al., 2013; Norin et al., 2014; Ern et al., 2014; Wang et al., 2014). The prediction that thermal tolerance limits may depend on oxygen delivery has been supported in a number of species (e.g. Frederich & Pörtner, 2000; Mark et al., 2002; Davenport & Davenport, 2007). However, the generality of this mechanism has been perhaps most strongly challenged by studies on terrestrial insects, many of which provide little or no evidence for oxygen limited thermal tolerance (Klok, 2004; Stevens, 2010; Molich et al., 2012; Neven et al., 2014). This contrasts with the situation in a number of aquatic insects, whose heat tolerance is apparently much more strongly affected by ambient oxygen availability (Whitney, 1939; Verberk and Calosi, 2012; Verberk et al., 2013a; Verberk and Bilton, 2013).

The oxygen requirements of animals and their capacity to deliver oxygen are frequently matched. Fluctuations in both the oxygen requirements of an animal and the environmental availability of oxygen require animals to regulate oxygen delivery in order to match supply to demand. This is important as both a shortage and an excess of oxygen are problematic, leading to asphyxiation and poisoning respectively. This ability of an organism to regulate oxygen uptake to balance the risks of toxicity and asphyxiation, or its respiratory control, is inherently different between species performing gas exchange in either water or air (Verberk and Atkinson, 2013). In this context, the better respiratory control afforded by breathing air may have driven the evolutionary colonization of land by decapods (see e.g. Giomi et al. 2014). A
recent comparative study employing species from different insect orders has suggested that interspecific differences in ability to regulate gas exchange (i.e. the degree of respiratory control) could reconcile these apparently contrasting results between aquatic and terrestrial insects in the degree to which their heat tolerance is set by oxygen limitation (Verberk and Bilton, 2013). However, the eight species investigated in that study differ in more than their mode of respiration, meaning that the contrasting results do not necessarily arise solely from differences in respiratory control, but could also result from other biological differences. Moreover, some of the reported differences in thermal tolerance between aquatic (mostly juvenile stages) and terrestrial insects (mostly adult stages) could also be due to ontogenetic differences in thermal physiology (see e.g. Bowler and Terblanche, 2008; Voorhees and Bradley, 2012) and thus unrelated to respiratory capacity. Here we compare respiration and thermal tolerance in detail for both juveniles and adults in each of two species of aquatic bugs, one of which is a plastron breather, *Aphelocheirus aestivalis* (Fabricius, 1794), and the other a bimodal gas exchanger that is capable of extracting oxygen from air and water, *Ilyocoris cimicoides* (Linnaeus, 1758) (see Fig. 1). Moreover, we experimentally manipulate the degree of respiratory control in adults by studying their responses with and without access to air (see below). Such intraspecific comparisons allow more direct testing of the hypothesis that differences in respiratory control govern the extent to which thermal tolerance limits depend on oxygen availability.

Respiration is much more challenging in water compared to air, owing to the lower solubility and diffusivity of oxygen in water, whilst the higher density and viscosity of water increase the cost of breathing (Dejours, 1981; Verberk and Atkinson, 2013). With this in mind, we can derive several hypotheses regarding the responses of the study organisms based on their mode of respiration. With respect to oxygen consumption, we expect that of the plastron breather to be lower than that of the bimodal breather, owing the greater challenge of breathing under water than in air. Furthermore, difference in oxygen consumption between both species are expected to become larger at higher temperatures, with the bimodal breather relying more and more on aerial gas exchange. With respect to temperature, we expect the extent of oxygen limited thermal tolerance to be a function of the capacity of an animal to regulate oxygen uptake. If the plastron breather is indeed worse at regulating oxygen uptake than the bimodal breather, we expect its heat tolerance to be more oxygen dependent, with a greater impact of both hypoxia and hyperoxia. Furthermore, juveniles and adults of a given species should perform similarly in heating trials if their gas exchange mechanisms are comparable. Finally,
and most crucially, we predict that the heat tolerance of the bimodal breather becomes much more oxygen dependent when it is denied access to air, as restricting such access would reduce its respiratory control.

Results

Metabolism increased with both temperature (Fig. 2) and body size, and for a given body size, the bimodal breather *I. cimicoides* consumed more oxygen than the plastron breather *A. aestivalis* (Table 1). Also, in warmer conditions, *I. cimicoides* increased its oxygen consumption more than *A. aestivalis* (Fig. 2; GLM species * temperature F$_{1,74}$ = 8.65; *P* = 0.0044). In warmer conditions, *I. cimicoides* relied increasingly on aerial gas exchange; proportionally more oxygen was consumed from air with increasing temperatures (Fig. 2; linear regression Beta = 0.015; F$_{1,33}$ = 20.25; *P* < 0.001), whereas, oxygen consumption from water did not change (linear regression Beta = 0.0019; F$_{1,33}$ = 0.575; *P* = 0.454). Thus there are clear differences between both species in their rates of oxygen consumption and the thermal dependence of this; *I. cimicoides* consumes more oxygen and can increase its consumption more with increasing temperatures by relying on more frequent aerial gas exchange.

Heat tolerance was also different between the two species, with *I. cimicoides* showing higher heat tolerance than *A. aestivalis* at each oxygen tension tested (Fig. 3; GLM species F$_{1,128}$ = 270.78, *P*< 0.001). Oxygen affected heat tolerance of the two species to differing degrees (GLM species * oxygen interaction F$_{1,128}$ = 17.25, *P*< 0.001), having a much more pronounced effect in *A. aestivalis*.

In adults of *I. cimicoides*, hypoxia did not significantly reduce heat tolerance (Fig. 3A; *t* = -1.93; *P* = 0.060) so long as animals had access to air. However, when *I. cimicoides* was deprived of air, heat tolerance was reduced significantly under hypoxia by 4.6°C (*t* = -3.84; *P* < 0.001). In contrast, adults of *A. aestivalis* showed both reduced heat tolerance under hypoxia and improved heat tolerance under hyperoxia (Fig. 3A). Providing adult *A. aestivalis* access to air did not improve heat tolerance under hypoxia (Fig. 3A), as *A. aestivalis* never attempted to surface in any experimental treatment.

Juveniles and adults did not differ in heat tolerance across the two species (Fig. 3B; GLM Stage, F$_{1,99}$ = 1.419, *P* = 0.237). Nevertheless, including juveniles in the analyses did affect the
statistical significance compared to the analyses on adults only. There was a modest overall effect of hypoxia on heat tolerance in *I. cimicoides* (*t* = -2.490, *P* = 0.0144), whilst the effect of hyperoxia in *A. aestivalis* was marginally non-significant (*t* = 1.758, *P* = 0.082). Nevertheless, in general the results were highly similar to those for only adults (Fig. 3B), showing much stronger effects of oxygen in the plastron breathing *A. aestivalis*.

**Discussion**

A mechanistic understanding is essential to predict the impacts of environmental stressors, and comparisons of species attributes or traits offer a promising approach to unravel such mechanisms (Huey et al., 2012; Chown, 2012; Verberk et al. 2013b). With respect to temperature, a primary driver of species distributions (e.g. Root *et al.*, 2003; Calosi *et al.*, 2010; Sunday *et al.*, 2010), the thermal tolerance of a species has been argued to be one of the key traits predicting biological responses to a rapidly changing climate (Chown, 2012). Our study supports the view that the extent to which the mechanism of oxygen limited thermal tolerance applies, is context dependent, and related to differences in respiratory control. As the critical lethal temperatures reported here will be higher than sublethal limits to growth and reproduction, these cannot be related directly to the habitat conditions in which these bugs live. However, our study provides a clear proof of principle that the extent to which oxygen limits thermal tolerance is a function of the respiratory control of an animal, rather than oxygen limited thermal tolerance being universally applicable. Such a result may offer a way forward in the current debate between proponents and opponents of the generality of this conceptual framework (e.g. Clark *et al.*, 2013; Pörtner 2014).

Differences in respiratory control arise in part from the respiratory medium. Underwater gas exchange has higher ventilation costs than aerial exchange, as water is more dense and viscous than air, a problem which is further compounded by lower oxygen content and much lower diffusion rates in liquid media (Dejours, 1981; Verberk and Atkinson, 2013). This difficulty of breathing in water rather than air is also exemplified by the finding of Jones (1961) who observed pulmonate snails to increase their reliance on aerial oxygen under aquatic hypoxia. Likewise, we found that the bimodal gas exchanger *I. cimicoides* increasingly relied on aerial gas exchange under warmer conditions (Fig. 2). The plastron breather *A. aestivalis* did not show such a response, however, and was less efficient in obtaining oxygen at higher
temperatures. This latter species also showed clear evidence for oxygen limited thermal tolerance, with heat tolerance being greatly compromised under hypoxic conditions, whilst hyperoxia somewhat improved heat tolerance (Fig. 3).

Finding that hypoxia depresses CT_{max} does not in itself imply that oxygen also limits thermal tolerance under normoxia (e.g. Molich et al., 2012; Neven et al., 2014). Therefore, elevated CT_{max} under hyperoxia is usually seen to constitute a stronger test of the hypothesis that oxygen limits thermal tolerance. Here it has to be born in mind that we only assessed short-term responses to acute heating, whereby animals did not have to actively perform, but rather meet minimum oxygen requirements for survival. Studies have shown that oxygen can become more readily limiting when animals have oxygen requirements beyond standard metabolism (due to activity, feeding, growing etc.). For example, the dragonfly *Erythemis simplicicollis* was found to be oxygen limited during flight even in normoxia (Harrison and Lighton, 1998). Thus, when animals have to actively perform they may become oxygen limited and the likelihood of this happening could be approximated by the decrease in CTmax under hypoxia (assuming that a more challenging oxygen uptake under hypoxia is equivalent to a greater requirement of oxygen in active animals under normoxia). From this perspective, the bimodal gas exchanger *I. cimicoides* seems unlikely to be oxygen limited when having access to air. In a similar manner the increased respiratory control afforded by aerial gas exchange appears to increase heat tolerance in some intertidal decapods (Giomi et al. 2014; Fusi et al. 2015). If not set by oxygen, thermal limits can arise due to other processes, such as the loss of protein function due to denaturation, loss of membrane stability and neuronal dysfunction (Somero, 1995; Feder and Hofmann 1999; Klok et al. 2004; Verberk et al. 2013a). If the critical temperatures for these other processes are only slightly higher than the temperature at which oxygen becomes limiting, then hyperoxia will only have minor effects, alleviating constraints on oxygen delivery, but with the next process in line breaking down at a temperature very close to the CT_{max} observed under normoxia. From this perspective even a slight increase in thermal tolerance under hyperoxia observed for the plastron breather could, together with the large decline in thermal tolerance under hypoxia, indicate that it may become oxygen limited even under normoxic conditions. Thus, in addition to tegument and gill breathing aquatic insects, we can add that plastron breathing insects are also likely to be vulnerable to oxygen limitation. This is the second example of an insect in which hyperoxia results in improved heat tolerance (see Verberk and Bilton, 2011 for the first case), and the first case for insect species with an open tracheal system. Contrary to the conclusions of Klok et al. (2004), it seems that adult
insects with open tracheal systems can indeed suffer from oxygen limited thermal tolerance, at least if, like plastron breathers, they have limited respiratory control. In short, animals that have difficulty in regulating oxygen consumption are more likely to become oxygen limited, irrespective of whether they are tracheates or otherwise.

Previous comparisons between plastron breathers and aerial gas exchangers have shown the former to be especially vulnerable to warming and hypoxia, presumably because of their poor respiratory control (Verberk and Bilton, 2013). Here this pattern is confirmed and extended to hyperoxia (Fig. 3). More importantly, our experimental manipulations enable us to isolate the effect of respiratory control within a single species. By denying the bimodal breather access to air, we reduce the respiratory control of *I. cimicoides* and this resulted in a clear reduction of heat tolerance under hypoxia by as much as 4.6 °C. This makes it very likely, that respiratory control is the driving factor underlying the extent to which oxygen limits thermal tolerance, explaining the differences in support across a range of species (Verberk and Bilton, 2013).

From our results it is also unlikely that ontogenetic differences in thermal biology play a large role. Differences in thermal tolerance between life stages could be inherent, irrespective of differences in their gas exchange mechanism, arising since life-stages may differ in their ability to thermoregulate (e.g. Bowler and Terblanche, 2008; Voorhees and Bradley, 2012). In holometabolous insects, larvae frequently differ from adults in their gas exchange mechanisms, making it difficult to disentangle the potential effects of ontogenetic shifts in thermal physiology and the effect of respiratory control. In the hemimetabolous bugs studied, comparing juveniles and adults, keeping the gas exchange mechanism constant, showed very similar levels of heat tolerance for both life stages, suggesting that differences between instars may not result from ontogenetic differences in their thermal physiology (Verberk and Bilton, 2011). A similar heat tolerance across life stages also implies that the capacity to increase oxygen consumption does not differ markedly with body size in these insects. For terrestrial insects, critical PO$_2$ (the oxygen tension below which metabolic rate is depressed) was found to be indeed size-independent (Greenlee et al., 2007; Harrison et al., 2014), as a result of compensatory changes in tracheal conductance (e.g. Loudon, 1989). Our results suggest that across the 2-3 fold size range, the two hemipterans studied here could maintain capacity for oxygen delivery with increasing size. In tegument-breathing stonefly larvae, a modest but significant effect was found for body size on heat tolerance (Verberk et al., 2013a). This could be related to the larger 8-fold size range in the stonefly nymphs and their poor respiratory control associated with tegument breathing.
In conclusion, our study significantly advances our understanding of oxygen limitation at warm conditions, demonstrating that respiratory control dictates the importance of oxygen in setting heat tolerance limits. As differences in respiratory control will often be tightly linked to their respiratory medium (air vs water), this finding reconciles the contrasting results on thermal tolerance limits previously reported for terrestrial and aquatic insects. Based on our results, we predict that the vulnerability of species to the interactive effects of warming and hypoxia will be dictated by their respiratory control. Thus, within water breathers, thermal generalists are expected to have excellent respiratory control (Ern et al. 2014). Conversely, species with poor respiratory control are predicted to be highly vulnerable. Species in running waters are more likely to fall into this category as their habitat does not require good respiratory control: water flow greatly reduces the ventilation effort needed, while the resultant mixing of air and water ensures consistently high oxygen saturation. Thus, respiratory control is a key trait which must be included in studies which aim to predict biological responses to a rapidly changing climate.

Materials and Methods

Study species

Animals were collected in SW England. *Aphelocheirus aestivalis* (Fabricius, 1794) was collected from riffles in the River Torridge, Devon, UK (50.9094, -4.0743), a medium to fast flowing river with a stony bed and little vegetation. In the River Torridge, temperature ranges from 6.2-19.2 °C with an average of 10.9 °C (data obtained from ongoing monitoring by the UK Environment Agency). *Ilyocoris cimicoides* (Linnaeus, 1758) was collected from South Drain, Somerset Levels, UK (51.1806, -2.8803), a slow flowing, drainage canal with abundant riparian and aquatic macrophytes. In the South Drain, temperature ranges from 10.1-24.6 °C with an average of 16.7 °C (Dalesman and Rundle, 2010). After collection, animals were maintained in the Marine Biology and Ecology Research Centre in a constant temperature room at 10±1 °C in a 12 L:12 D regime. They were fed chironomid larvae *ad libitum* and were kept in aquaria, containing artificial pond water (ASTM 1980), buffered and diluted to reflect the pH and conductivity of the field site. All nymphs were acclimated for at least 7 d to lab conditions all to reduce variability in thermal history (see Terblanche et al. 2007), before respiration trials and trails to assess critical temperatures were undertaken.
The plastron breathing heteropteran *Aphelocheirus aestivalis* does not need to surface to renew its air supply. Instead it relies on diffusion of oxygen from the water into the thin film of air which is in contact with its tracheal system via the spiracles (see Fig. 1). A pile of small hairs covers much of the cuticle and prevents the air film from collapsing (Thorpe, 1950). As it resists changes in volume, oxygen consumed from the air film is renewed by inward oxygen diffusion and the animal can maintain respiration indefinitely under normal conditions. In comparison to the other heteropteran, *Ilyocoris cimicoides*, *A. aestivalis* has a larger surface area to volume ratio facilitating oxygen diffusion (see Fig. 1). Respiration rates are relatively low, which seems to be a general feature of plastron breathing insects (Kölsch and Krause, 2011). Body weights (mg dry weight) ranged from 5.3-9.3 mg, 11.4-17.3 mg, and 14.9-24.7 mg for juveniles, males and females, respectively (measured to the nearest 0.0001 g), with dry weight making up 44.9% of fresh weight.

The other heteropteran investigated, *Ilyocoris cimicoides*, is very similar in morphology and diet. Moreover, recent work on the phylogeny of these hemipterans suggests that the families to which both species belong (Aphelocheiridae and Naucoridae respectively) are sister taxa (Fig. 3 in Hua et al. 2009), with the Naucoridae being morphologically similar to Aphelocheiridae (Hebsgaard et al., 2004). *Ilyocoris cimicoides* has an air store under its hemi-elytra and it also carries a thin film of air on the ventral side which functions like a physical gill, but unlike *Aphelocheirus aestivalis* the bubble cannot be maintained indefinitely and the species therefore needs to surface to renew its air stores (Fig. 1). This species is consequently a bimodal breather, using the thin layer of air as a physical gill and surfacing periodically to replenish its air stores. Being hemimetabolous, juveniles of both species employ gas exchange mechanisms identical to those of the adults. Body weights (mg dry weight) ranged from 7.8-21.8 mg, 22.3-36.6 mg, and 23.0-54.8 mg for juveniles, males and females, respectively (measured to the nearest 0.0001 g), with dry weight making up 34.9% of fresh weight.

*Respiration measurements*

To measure respiration rates in the bimodal breather, *Ilyocoris cimicoides*, we used cylindrical respiratory chambers (115mm high, 27 mm inner diameter) with a cone shaped top (sides started tapering 15 mm from the top). These chambers were fully filled with water before animals were inserted. Immediately after an animal was inserted, we injected 2ml of air that was water vapor saturated and temperature equilibrated. The resulting air bubble was held at
the conical top of the respiratory chamber (hereafter called air compartment) and proved sufficiently spacious for the animal to perform aerial gas exchange. Animals were given between 1-1.5 h to acclimate. Next, oxygen consumption was measured by measuring the oxygen tension in the water and air compartment, using a micro optodes connected to a Microx TX3 fiber-optic oxygen meter (PreSens instruments, Regensburg, Germany). This micro-optode was situated in the air compartment. Oxygen tensions in the water compartment were measured at the start and end of each respiration trial by carefully inverting the respiratory chamber to displace the air bubble and allow the optode to come into contact with the water. Water was stirred throughout the respiration, using submersible stirrers (Cole-Palmer Instrument Company Ltd., London, UK). A specifically constructed mesh separated the stirring bar from the animal. This also provided animals something to cling on thus minimizing activity. Oxygen tensions in the air compartment were measured multiple times and a linear regression line was fitted to calculate oxygen consumption. During the experiment, oxygen could freely diffuse between the air and water compartment (i.e there was no paraffin barrier added to the water). As the oxygen tension in the air compartment was usually lower than that of the water compartment, such diffusion of oxygen across the air-water interface could underestimate the oxygen consumed from air and overestimate the oxygen consumed from the water. We therefore corrected for this by empirically measuring the diffusive flux over a range of differences in pO\(_2\) at different temperatures. Corrected and uncorrected values were highly similar (e.g. for aerial consumption, corrected values were on average 7% higher than uncorrected values and highly correlated; R\(^2\)=0.996), indicating that the contribution of diffusion of oxygen across the air-water interface was relatively modest in comparison to oxygen consumption by the bugs. Respiration trials lasted between 113-182 minutes, in accordance with the rates of oxygen consumption of the animal, which differed with size and temperature. On average oxygen saturation dropped to 90% at the end of a respiration trial, but never below 71%. Oxygen consumption was corrected for background respiration which was assessed by blanks.

To measure respiration rates in the plastron breather, *Aphelocheirus aestivalis*, we used cylindrical respiratory chambers without an air compartment and which were of smaller size (50mm high, 15 mm inner diameter). Water was stirred throughout the respiration, using submersible stirrers (Cole-Palmer Instrument Company Ltd., London, UK). A specifically constructed mesh separated the stirring bar from the animal. An additional piece of mesh was added to give animals something to adhere to and thus prevent activity. Oxygen concentrations
were measured with using an optode connected to a Fibox 3 LCD oxygen meter (PreSens instruments, Regensburg, Germany), at both the start and again at the end of each respiration trial. Respiration trials lasted between 66-241 minutes, in accordance with the rates of oxygen consumption of the animal, which differed with size and temperature. On average oxygen saturation dropped to 96% at the end of a respiration trial, but never below 82%. To minimize background respiration, respiratory chambers were cleansed with 100% ethanol before use, and thoroughly washed and dried afterwards. Oxygen consumption was corrected for background respiration which was assessed by blanks. For both species, respiration rates were measured at three temperatures: 5 °C, 10 °C, and 15 °C. To this end, respiratory chambers were submerged in water maintained at 5 °C, 10 °C or 15 °C by a Grant R5 water bath with a GP200 pump unit (Grant Instrument Ltd, Cambridge, UK). Volume of the respiratory chambers was determined gravitationally from the difference in weight when empty or completely filled with water.

Assessing CTmax

In order to assess critical temperatures, we employed the same methods as previously described (Verberk and Bilton 2011; Verberk and Calosi 2012). Briefly, individual nymphs were placed in five parallel flow-through chambers (70x70x30 mm; flowrate 0.016 L x s⁻¹) and water was supplied to these chambers by gravity from a 25L header tank after having passed through a tubular counter-current heat exchanger. Water in the header tank was of the same composition as that used to maintain animals and was bubbled with a mixture of 20% O₂ and 80% N₂, obtained using a gas-mixing pump (Wösthoff, Bochum, Germany). Individuals were left resting for 1 h at the equilibration temperature of 10 °C, after which temperature in the experimental chambers was increased at 0.25 °C min⁻¹, using a Grant R5 water bath with a GP200 pump unit (Grant Instrument Ltd, Cambridge, UK), connected to the heat exchanger. Temperatures were logged using a HH806AU digital thermometer (Omega Engineering Inc., Stamford, USA).

The critical maximal temperature, CTmax was defined as loss of all movement. At this temperature animals no longer showed any body movement or muscular spasms and could be most reliably determined across the different taxa. It is considered a comparable endpoint as animals in this state lose their ability to escape from the conditions that will lead to its death (Lutterschmidt and Hutchison, 1997). At lower temperatures, animals initiated in repeated swimming behaviour (interpreted as attempts to escape experimental conditions) and fell upon
their backs (loss of equilibrium) and an onset of spasms was observed which eventually prevented the animal from coordinated swimming strokes with the two hind legs.

CTmax was assessed at normoxia, hypoxia and hyperoxia. Different levels of oxygenation were achieved by changing the O2-N2 gas mixture obtained using the gas-mixing pump (Wösthoff, Bochum, Germany). The gas mixture was adjusted 10 mins after placing the animals in the small flow-through chambers, to allow for gradual exposure to hypoxic and hyperoxic conditions during the 1 h resting period. To prevent equilibration with the atmosphere, the header tank was sealed using an 18 mm thick expanded polystyrene sheeting and other openings were closed off with plastic material. During the 1 h resting period, O2 levels in the outflow water from the chambers were measured approximately every 15 mins, to verify that the O2 levels had stabilized to hypoxic (5 kPa), normoxic (20 kPa) and hyperoxic (60 kPa) conditions at the onset of warming. Because some equilibration with the atmosphere could not be prevented, nominal output values from the gas mixer were slightly more extreme (3 kPa for hypoxia and 65 kPa for hyperoxia) in order to achieve the desired O2 conditions in the test chambers.

Adults were assessed with and without access to air, while juveniles were assessed with access to air for *I. cimicoides* and without access for *A.aestivalis*. To assess CTmax with the animals having access to air, we used flow-through chamber with a small head space holding a layer of air. The air in this compartment could be flushed via a narrow opening that was sealed from the atmosphere by the capillary action of water. Using a thin needle connected to the gas-mixing pump (Wösthoff, Bochum, Germany) we could flush the head space of the flow-through chamber with different gas mixtures of oxygen and nitrogen. Equilibration of the head space with the atmosphere was prevented by the capillary action of water which sealed off the opening immediately after the needle was retracted. To ensure that the air and water compartments held the same gas mixture at the onset of the warming, we flushed the head space of the flow-through chamber with air of the appropriate gas mixture, both 10 min after the start of the resting period (when water oxygen content was also adjusted) and at the end (when warming was initiated).
Data analysis

Fitted residuals for the respiration data deviated slightly from a normal distribution. Visual inspection revealed that the deviance of the residuals increased with higher predicted values (funnel shaped error distribution). To evaluate the robustness of the model outcome, we therefore also fitted a GLM using a gamma distribution. As the results from both analyses were qualitatively highly similar, we only presented those of the simpler linear regression. Moreover, respiration of the same individual was measured at different temperatures. To account for the non-independence of data points from the same individual, we also ran a mixed effect linear regression using individual as a random factor. Again, this analysis confirmed the results from the simpler linear regression (higher metabolism at higher temperatures, which was stronger for *I. cimicoides*).

Preliminary analysis on heat tolerance revealed differences between both species in their heat tolerance, in how oxygen conditions affected their heat tolerance and in how the experimental setup interacted with this effect of oxygen on heat tolerances (see Table A1). As these latter three-way interactions are difficult to interpret, we decided to analyze the effects of the experimental setup (access or no access to air) in two analysis for each species separately (see Table A2 and A3). Also, since we only manipulated access to air for adults we restricted this analysis to adults. To assess if heat tolerance was consistently different between juveniles and adults we conducted a third analysis whereby we included data for both species (Table A4), including data on juveniles and adults that were assessed at the same experimental conditions (no access to air for the permanently submerged plastron breather and access to air for the bimodal breather).
Acknowledgements

We gratefully acknowledge Andrew Atfield, Roger Haslam, Rick Preston, Peter Russell and Richard Ticehurst for technical assistance.

Competing interests

The authors declare no competing financial interests.

Author contributions

Both authors conceived and designed the experiments. W.C.E.P.V. performed the experiments and analyzed the data. Both authors wrote the paper.

Funding

This study was supported by the Netherlands Organization for Scientific Research (NWO-RUBICON fellowship no. 825.09.009), the European Union (Marie-Curie Fellowship no. FP7-PEOPLE-2009-IEF and FP7-PEOPLE-2012-CIG) and an Erasmus Staff training grant.

References


Tables
Table 1.
Anova table for linear regression on differences in respiration between species and as a function of body size and temperature.

<table>
<thead>
<tr>
<th>Factor</th>
<th>df</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log Body mass</td>
<td>1</td>
<td>158.3491</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Species</td>
<td>1</td>
<td>10.3410</td>
<td>0.0019</td>
</tr>
<tr>
<td>Temperature</td>
<td>1</td>
<td>29.1054</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Species * Temperature</td>
<td>1</td>
<td>8.6509</td>
<td>0.0044</td>
</tr>
<tr>
<td>Residuals</td>
<td>74</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figures**

*Ilyocoris cimicoides*  
(surface exchanger and physical gill)

![Diagram of Ilyocoris cimicoides](image)

1 cm

*Aphelocheirus aestivalis*  
(plastron breather)

![Diagram of Aphelocheirus aestivalis](image)

1 cm
The bimodal breather *Ilyocoris cimicoides* surfaces periodically to replenish the air that is stored under its hemi-elytra and that is carried on the ventral side. Air is inhaled via the posterior thoracic spiracles (placed dorsally) and the abdominal spiracles (placed ventrally, except for the first abdominal spiracles) and exhaled by the anterior thoracic spiracles (placed ventrally). While submerged the air stored on the ventral side functions as a physical gill. In contrast, the plastron breather *Aphelocheirus aestivalis*, does not surface and relies solely on diffusion of oxygen from the water. A pile of small hairs covers much of its cuticle and thus a very thin air film is captured and prevented from collapsing. The tracheal spiracles are in open contact with this air film and as it resists changes in volume, oxygen consumed from the air film is renewed by inward oxygen diffusion. *A. aestivalis* is smaller, more flattened and more rounded, giving it a large surface area to volume ration which facilitates oxygen diffusion.
Figure 2. Differences between *Aphelocheirus aestivalis* (yellow) and *Ilyocoris cimicoides* (green) in mass specific oxygen consumption (per gram dry weight) at different temperatures. For the bimodal breather *I. cimicoides*, aerial respiration (red line) and aquatic respiration (blue line) are indicated separately. Error bars indicate s.e.m. and different letters indicate significant differences in aerial respiration (*P* < 0.05) between temperatures (note that aquatic respiration did not differ between temperatures for *I. cimicoides*).
Figure 3. **Differences in heat tolerance between *Aphelocheirus aestivalis* (red) and *Ilyocoris cimicoides* (blue) at different temperatures.** Differences for adults with and without access to air (A) are shown separately from differences between adults and juveniles (B). For ease of comparison, the line for adults is repeated in both panels. Error bars indicate s.e.m.
Table S1. ANOVA table for linear regression on CTmax data for all individuals

Response: data$CTmax1

<table>
<thead>
<tr>
<th></th>
<th>Df</th>
<th>Sum Sq</th>
<th>Mean Sq</th>
<th>F value</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O2</td>
<td>1</td>
<td>286.55</td>
<td>286.55</td>
<td>69.5651</td>
<td>1.01e-13 ***</td>
</tr>
<tr>
<td>Species</td>
<td>1</td>
<td>1115.40</td>
<td>1115.40</td>
<td>270.7805</td>
<td>&lt; 2.2e-16 ***</td>
</tr>
<tr>
<td>Expsetup</td>
<td>1</td>
<td>15.03</td>
<td>15.03</td>
<td>3.6481</td>
<td>0.05837 .</td>
</tr>
<tr>
<td>log10(DW)</td>
<td>1</td>
<td>0.28</td>
<td>0.28</td>
<td>0.0674</td>
<td>0.79560</td>
</tr>
<tr>
<td>Stage</td>
<td>1</td>
<td>7.98</td>
<td>7.98</td>
<td>1.9382</td>
<td>0.16628</td>
</tr>
<tr>
<td>O2:Species</td>
<td>1</td>
<td>71.06</td>
<td>71.06</td>
<td>17.2517</td>
<td>5.94e-05 ***</td>
</tr>
<tr>
<td>O2:Expsetup</td>
<td>1</td>
<td>24.09</td>
<td>24.09</td>
<td>5.8477</td>
<td>0.01700 *</td>
</tr>
<tr>
<td>Species:Expsetup</td>
<td>1</td>
<td>15.20</td>
<td>15.20</td>
<td>3.6908</td>
<td>0.05694 .</td>
</tr>
<tr>
<td>O2:Species:Expsetup</td>
<td>1</td>
<td>19.02</td>
<td>19.02</td>
<td>4.6165</td>
<td>0.03355 *</td>
</tr>
<tr>
<td>Residuals</td>
<td>128</td>
<td>527.26</td>
<td>4.12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table S2. Summary of linear regression on CTmax data for Ilyocoris adults

lm(formula = CTmax1 ~ oxygen + Expsetup + log10(DW) + Expsetup:oxygen, data = subset(ilyo, ilyo$Stage != "juv"))

Residuals:
  Min 1Q Median 3Q Max
-5.0109 -0.4133  0.1690 0.5986 2.5541

Coefficients:

|                  | Estimate | Std. Error | t value | Pr(>|t|) |
|------------------|----------|------------|---------|----------|
| (Intercept)      | 47.23300 | 5.57956    | 8.465   | 6.15e-11 *** |
| oxygen0.05       | -1.06163 | 0.54952    | -1.932  | 0.059541 . |
| oxygen0.6        | -0.23786 | 0.55757    | -0.427  | 0.671654 |
| Expsetupnoair    | 0.05135  | 0.70832    | 0.072   | 0.942520 |
| log10(DW)        | -2.32237 | 2.22779    | -1.042  | 0.302648 |
| oxygen0.05:Expsetupnoair | -3.59189 | 0.93629    | -3.836  | 0.000379 *** |
| oxygen0.6:Expsetupnoair | 0.78553  | 1.06128    | 0.740   | 0.462956 |

---

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Table S3. Summary of linear regression on CTmax data for _Aphelocheirus_ adults

```
lm(formula = CTmax1 ~ O2 + Expsetup + log10(DW) + Stage + O2:Expsetup +
    Expsetup:O2, data = aphe)

Residuals:
            Min       1Q     Median       3Q      Max
-4.9723 -0.6602  0.0136  0.7151  4.4284

Coefficients:
                      Estimate     Std. Error     t value     Pr(>|t|)     
(Intercept)          29.1112     4.2081     6.918     2.42e-09 *** 
O20.2                 4.9774     0.9341     5.328     1.33e-06 *** 
O20.6                 6.4267     0.9416     6.825     3.53e-09 *** 
Expsetupnoair -0.4826     0.7525   -0.641     0.524
log10(DW)             0.9482     1.8867     0.503     0.617
Stagejuv              0.3786     0.9576     0.395     0.694
O20.2:Expsetupnoair   1.0179     1.0413   0.977     0.332
O20.6:Expsetupnoair   0.3254     1.0648   0.306     0.761
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Table S4. ANOVA table of linear regression on CTmax data for adults and juveniles of both the permanently submerged plastron breather _Aphelocheirus_ (assessed without access to air) and the bimodal breather _Ilyocoris_ (assessed with access to air)

```
Response: CTmax1
           Df  Sum Sq Mean Sq  F value    Pr(>F)     
oxxygen    2  340.14  170.07 95.4400 < 2.2e-16 ***
Species    1  978.04  978.04 548.8599 < 2.2e-16 ***
Stage      1   2.53   2.53  14.186   0.02365 
oxxygen:Species 2 160.85  80.42 45.1327 1.077e-14 ***
Residuals 100 178.20  1.78
---
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```