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Consistent differences in tissue oxygen levels across 15 insect species reflect a balance between oxygen supply and demand and highlight a hitherto unknown adaptation for extracting sufficient oxygen from water

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ABSTRACT

Animals, including insects, need oxygen for aerobic respiration and eventually asphyxiate without it. Aerobic respiration, however, produces reactive oxygen species (ROS), which contribute to dysfunction and aging. Animals appear to balance risks of asphyxiation and ROS by regulating internal oxygen relatively low and stable, but sufficient levels. How much do levels vary among species, and how does variation depend on environment and life history? We predicted that lower internal oxygen levels occur in insects with either limited access to environmental oxygen (i.e., insects dependent on aquatic respiration, where low internal levels facilitate diffusive oxygen uptake, and reduce asphyxiation risks) or consistently low metabolic rates (i.e., inactive insects, requiring limited internal oxygen stores). Alternatively, we predicted insects with long life-stage durations would have internal oxygen levels > 1 kPa (preventing high ROS levels that are believed to occur under tissue hypoxia). We tested these predictions by measuring partial pressures of oxygen (PO_2) in tissues from juvenile and adult stages across 15 species comprising nine insect orders. Tissue PO_2 varied greatly (from 0 to 18.8 kPa) and variation across species and life stages was significantly related to differences in habitat, activity level, and life stage duration. Individuals with aquatic respiration sustained remarkably low PO_2 (mean = 0.88 kPa) across all species from Ephemeroptera (mayflies), Plecoptera (stoneflies), Trichoptera (caddisflies), and Diptera (true flies), possibly reflecting a widespread, but hitherto unknown, adaptation for extracting sufficient oxygen from water. For Odonata (dragonflies), aquatic juveniles had higher PO_2 levels (mean = 6.12 kPa), but these were still lower compared to terrestrial adults (mean = 13.3 kPa). Follow-up tests in juvenile stoneflies showed that tissue PO_2 remained low even when exposed to hyperoxia, suggesting that levels were down-regulated. This was further corroborated since levels could be modulated by ambient oxygen levels in dead individuals. In addition, tissue PO_2 was positively related to activity levels of insect life stages across all species and was highest in stages with short durations. Combined, our results support the idea that internal PO_2 is an evolutionarily labile trait that reflects the balance between oxygen supply and demand within the context of the environment and life-history of an insect.

Introduction

For animals, oxygen is a double-edged sword (Harrison et al., 2012). During oxidative phosphorylation of cellular respiration, electrons are transported in the electron transport chain and eventually transferred onto an oxygen molecule, the electron acceptor. Because oxygen is such an effective acceptor, it can release more energy from substrates than is possible with other electron acceptors. The majority of animals have come to rely on oxygen for respiration and asphyxiate when starved of it. Oxygen-based energy production was central to the evolution and diversification of modern eukaryotes and supports key aspects of modern ecological communities (Knoll 1992), many of which include large-bodied animals with energy-intensive lifestyles. Indeed, current interest in the mechanisms by which organisms sense and adapt to oxygen availability remains high, and work on this topic was recently awarded the 2019 Nobel Prize in Physiology or Medicine (Zhang et al.

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2019). At the same time, oxygen can be toxic. While respiring aerobically, mitochondria produce reactive oxygen species (ROS) (e.g., superoxide anion radical, hydroxyl radical, hydrogen peroxide), which damage cell components, including lipid bilayers and DNA, and whose effects contribute to disease and aging (Brieger et al. 2012; Davalli et al. 2016).

Animals thus need to balance risks of toxicity with those of asphyxiation (Verberk and Atkinson 2013), regulating the uptake of oxygen and its transport down the oxygen cascade so that enough is supplied to meet the oxygen demand of the mitochondria without triggering excess production of ROS. Thus, the partial pressure of oxygen, PO_2, tends to decrease across the steps of the oxygen transport cascade from ambient (~21 kPa at sea-level) via a convective distribution system (e.g., blood, hemolymph, or tracheal system) before the final diffusion-based step into the mitochondria. Holding internal PO_2 at low levels is likely beneficial because it increases the PO_2 gradient from atmosphere to mitochondria, which elevates rates of diffusive oxygen transport (Harrison et al. 2012).

Internal PO_2 also has consequences for rates of ROS production, although the relationship between ROS production and PO_2 is not straightforward (Zorov et al. 2014). While ROS production approaches zero when there is no oxygen, somewhat paradoxically, ROS production tends to increase under cellular hypoxia (e.g., < ~1 kPa) (Chandel et al. 1998; Guzy and Schumacker 2006; Murphy 2009; Semenza 2011; Zorov et al. 2014), despite being relatively stable across most other oxygen concentrations (Starkov 2008). In addition, ROS production tends to increase sharply during reoxygenation, leading to widespread cellular damage (Li et al. 2002; Millar et al. 2007; Wang et al. 2018). This suggests that organisms should hold internal PO_2 stable and low enough to support oxygen delivery but not so low that high levels of ROS are generated.

Data on mammals show that tissue PO_2 is indeed moderately low, in the range of 2.3 - 4.5 kPa in mammalian resting red muscle, with values somewhat lower around mitochondria (Wittenberg and Wittenberg 1989; Poole et al. 2020). Values in other mammalian tissues and organs (measured by a variety of techniques) are typically lower than 7 kPa, and sometimes as low as in red muscle (Vanderkooi et al. 1991). Fewer data have been obtained on insects, but those available suggest that hemolymph and intratracheal PO_2 typically lie between 5 and 18 kPa O_2 (Komai 1998; Kirkton 2007; Matthews and White 2011; Lehmann et al. 2019; Rowe et al. 2022), with substantial variation across body compartments (e.g., abdomen versus femur in grasshoppers; Kirkton 2007), between modes of breathing (e.g., continuous versus discontinuous; Rowe et al. 2022), or between levels of activity (e.g., preflight versus flight in hawkmoths; Komai 1998). Internal PO_2 tends to decrease with progressive atmospheric hypoxia and there is an attempt to minimize such decreases by increased ventilation rates once PO_2 of the metathoracic ganglion reaches 3 kPa (Harrison et al. 2020). Hetz & Bradley (2005) also reported active regulation of internal PO_2 levels. They measured intratracheal PO_2 in pupae of the silk moth *Attacus atlas* subjected to experimentally manipulated levels of external oxygen. Via spiracular opening and closing, pupae closely regulated intratracheal PO_2 to ~ 4 kPa, which the authors proposed was a mechanism for avoiding oxygen toxicity.

To date, published data on internal levels of PO_2 in insects have come from terrestrial species, which have ready access to high levels of oxygen in air. A significant proportion of insects, however, have aquatic juveniles, which extract oxygen from the surrounding water for respiration. These insects face fundamentally different oxygen challenges stemming from low concentrations of oxygen in water and the difficulty of extracting it (Lancaster and Downes 2013; Verberk and Bilton 2013). Many aquatic juveniles, moreover, have closed tracheal systems and thus cannot regulate oxygen levels via spiracular control (Buchwalter et al. 2020). We hypothesized that aquatic juvenile insects would sustain lower overall internal oxygenation for two reasons. First, for any given external PO_2 in water, sustaining the lowest possible internal PO_2 maximizes the driving gradient (ΔPO_2) for oxygen transport from environment to tissues. Second, high internal PO_2 is likely unnecessary for aquatic juveniles because they typically have stable, cool body temperatures and low activity levels that may be supported by small oxygen reservoirs and low metabolic rates. We thus also predicted that more active species would maintain higher levels of tissue PO_2 to provide larger reservoirs of oxygen for immediate use during rapid locomotion. Finally, we hypothesized that internal PO_2 levels reflect the need to avoid damage from ROS, something which should be a high priority for aquatic juvenile life stages which are typically longer lived, compared to either the adults they become or compared to terrestrial juveniles of other species. We thus predicted that life stages with long durations should have internal PO_2 above the threshold that is associated with cellular hypoxia (i.e. > 1 kPa), to avoid cellular damage, or perhaps alternative mechanisms for minimizing the effects of ROS production (e.g., enhanced antioxidant defenses).

Materials and methods

Insect collection and rearing

We measured the partial pressures of oxygen (PO_2) in juveniles and adults from nine orders of insects, five orders with terrestrial juveniles and adults (Coleoptera, Phasmatodea, Orthoptera, Diptera, Lepidoptera) and five orders with aquatic juveniles (Ephemeroptera, Plecoptera, Trichoptera, Odonata, Diptera). Obtaining juveniles and adults of the same species was often difficult, and more than one species was occasionally used from each order, for a total of fifteen species (Supplemental 1). Aquatic insects were all 'water-breathers' as juveniles, with closed tracheal systems and cutaneous gas-exchange. Aquatic insects that relied on exchange of aerial oxygen, e.g. by surfacing regularly to replenish air stores, on compressible physical gills, or on respiratory siphons at any point in development were not used. Adults of aquatic insects were air-breathers with functional spiracle systems. Terrestrial insects were air-breathers with open-spiracle systems both as juveniles and adults.

Insects were either collected near Missoula, Montana, USA from summer 2021 to spring 2022 or obtained from collaborators or commercially from a variety of sources (Supplemental 1). Field-collected insects were maintained in the laboratory at the University of Montana for up to two days until PO_2 measurements were made. Aquatic insects were held in buckets (10 L) with de-chlorinated tap water with an air stone to provide oxygen and to stir the water. Insects were held at roughly the mean temperature of the water source from which they were collected at the time of collection. Temperature was controlled by either placing the bucket of insects inside a refrigerator (5 °C) or cooling the water in the bucket with a recirculating water bath (10 - 20 °C) (ActiveAqua AACH10HP). Field-collected terrestrial insects were held in a plastic container (2 L) with a mesh lid until measurements were made. PO_2 of most insects from rearing sources were measured immediately upon arrival at the laboratory. Terrestrial Diptera and Lepidoptera were received from rearing sources as larvae and then reared to adulthood in the lab. Larvae of *Calliphora vicina* (Diptera) were held in a plastic container (2 L) with a mesh lid filled with food (wood shavings) at room temperature (~ 20 °C) until they began to pupate (~ 1 week). Once pupated, individuals were transferred to a refrigerator and held at 5 °C to prevent them from drying out in the lab. Caterpillars of *Manduca sexta* (Lepidoptera) were reared on commercial artificial diet until wandering, at which point they were transferred to wooden blocks with pre-drilled pupation chambers. Pupae were held at room temperature until eclosion.

After PO_2 measurements were taken, field-collected individuals were identified in the laboratory using a dissecting microscope and dichotomous keys (Tripplehorn and Johnson, 2004; Merritt et al. 2008). Data on the duration of each stage of species (juvenile or adult) were retrieved from a variety of peer-reviewed papers, non-peer-reviewed but

reputable online sources, and personal communications with experts who have unpublished data on study species' lifespans from laboratory rearing programs. Peer-reviewed sources could not be used in every case because of limited published data. For some species, no stage duration data were available, in which case stage durations of closely related species were substituted (Supplemental 1). No data were available on the temperature-sensitivity of stage duration, and so no adjustments could be made for rearing temperature. In addition, activity levels of 1 or 2 were assigned to each species based on our knowledge of their lifestyles and after carefully observing each in the laboratory under controlled temperatures, with a value of 1 being slow and inactive (e.g., stick insect) and a value of 2 being fast and active (e.g., flesh fly adult) (Supplemental 1). Subjecting individuals to activity level assays to collect more quantitative data was impractical as major, categorical differences in activity level were always apparent and because measurements would have overly stressed individuals prior to internal PO_2 measurements, necessitating time-intensive collections of many more individuals from the field.

Because differences in activity levels may be associated with variation in metabolism, metabolic rates were estimated for each species and stage. Resting metabolic rate data were retrieved from peer-reviewed papers or published, online datasets. For several species or stages, no metabolic data were available, and we then substituted data from the closest related species for which data were available (i.e., usually the same genus or family, sometimes order). When no metabolic data were available for species within the same order, a metabolic rate value was not assigned (Supplemental 1). Mean mass-specific metabolic rate was then calculated for each species and stage. All values were then converted to units of Watts g^{-1}. Because values reported in the literature were measured on different individuals, differing in mass and measurement temperatures, we derived temperature and mass corrected metabolic rates by fitting a linear model (R function: *lm*) of $\log_{10}$(metabolic rate) $\sim \log_{10}$(mean mass) + temperature. This model was used to derive mass specific metabolic rate at 20 °C, using the mass scaling coefficient and thermal dependency from the model (1.032 and 0.268 eV, respectively).

Oxygen measurements

PO_2 was measured using either Clark-style oxygen microelectrodes or an oxygen-sensitive optode. Oxygen microelectrodes (Unisense, 100 μm tip, with guard electrode; optimal measuring range 0 to 21 kPa) were connected to a picoammeter (Unisense, PA-2000) and calibrated at room temperature (~ 20 °C) using a custom glass apparatus holding tap water into which we bubbled pure N_2 gas (0 kPa O_2) or room air (19 kPa O_2, since Missoula is situated at an elevation of 978 m above sea level). Analog outputs from the picoammeter were sent to a Sable Systems acquisition system (UI-2, Expedata software) and visualized in real time during measurements. The optode was housed in a needle (Pyroscience, OXR430, 430 μm tip; optimal measuring range 0 to 50 kPa) and was connected to a meter (Pyroscience, FireSting O_2). The meter sent the outputs to a computer, where they were processed and visualized via the Firesting recording software. The optode was calibrated at room temperature using a plastic cup holding tap water into which we bubbled pure N_2 gas (0 kPa O_2) or room air (19 kPa O_2). Readings for both the electrodes and optodes were reliable, providing similar readings during calibration on different days with each method, and we performed the calibration procedure every day that measurements were taken.

PO_2 was measured inside the thorax and abdomen of individuals (n = 118) while they were still alive: Ephemeroptera (n = 8 and n = 3 for juveniles and adults, respectively), Plecoptera (n = 8, n = 6), Trichoptera (n = 8, n = 4), Odonata (n = 4, n = 3), Diptera (n = 9, n = 8); terrestrial orders: Coleoptera (n = 8, n = 8), Phasmatodea (n = 5, n = 5), Orthoptera (n = 7, n = 7), Diptera (n = 7, n = 3), Lepidoptera (n = 8, n = 7). Measurements were taken by securing each individual to a plastic Petri dish with rubber bands fed through holes cut in the dish. A small hole was made in the cuticle of the thorax and abdomen of each individual, the exact location being haphazardly chosen within each body segment. An electrode or optode was mounted to a micro-manipulator secured to the lab bench-top, which was used to carefully insert the sensor tip into the hole cut in the insect as viewed through a stereomicroscope (Nikon SMZ 1500). Electrodes were used to measure internal PO_2 in most species, which were small or weakly sclerotized. Optodes were used to measure PO_2 in two larger, more heavily sclerotized species (*Trypoxylus* beetles and *Phyllium* stick insects). Optodes were used in these instances because the moving limbs of the insects would easily break the microelectrodes. Because juveniles of aquatic fly larvae (*Tipula* sp.) repeatedly wiggled out of the rubber band mount, individuals were instead cut in half transversely. The abdomen and thorax were then inserted onto the tip of the electrode mounted to the micro-manipulator. PO_2 was recorded based on the values shown on the acquisition system or computer program as soon as the reading stabilized (i.e., was constant ± ~ 2 %) for ~ 2 s. Measurements were made soon after initial incisions were made in the body cavity, usually < 1 min. Based on the rapidity of measurements, changes to internal PO_2 due to diffusion of atmospheric air through holes or body openings, or due to reduced rates of ventilation post-injury, were considered to be negligible.

For each method, measurement locations and depths varied from individual to individual in an attempt to avoid biasing measurements. This is because little was known about the spatial variation of structures likely to support different internal PO_2 levels among species – i.e., the location of air sacs and large tracheae (with high PO_2), areas of high muscle fiber density (with low PO_2), etc. In addition, taking measurements in the same location or structure type (e.g., tissue or hemolymph) was problematic because most individuals were small (median body mass: 0.3 g), inhibiting our ability to take measurements in specific locations with certainty. However, for all individuals, measurements were made "deep" inside the body cavity (roughly 1/8 to 1/2 of the depth of each segment) and away from the center of the segment to avoid the gut lumen. Notably, analyses showed that internal PO_2 was no different in the thorax and abdomen for all species, suggesting that measurements were indeed representative of internal (tissue-hemolymph) oxygen levels, rather than specific body compartments.

Different methods and equipment used for measuring PO_2 were considered equivalent, based on preliminary PO_2 measurements on giant salmonfly nymphs (Plecoptera: *Pteronarcys californica*), in which measurements were made by inserting an optode through a hole cut in the cuticle (nine individuals, 16 measurements) or by inserting it into the abdomen and thorax after it has been cut transversely (one individual, two measurements). Mean PO_2 values from both methods using the optode were 0.14 kPa and were similar to measurements on the same species using the electrode in the main dataset (all were 0.0 kPa). Optodes and electrodes both showed nearly identical values when measuring the PO_2 of air-bubbled water near saturation and nitrogen-bubbled water near anoxia. Validity of our methods are also corroborated by similar mean internal PO_2 measurements made by Lee & Matthews (2024) on Odonata nymphs (6.2 and 6.3 kPa, respectively).

In a separate experiment, aquatic juveniles of the stonefly, *Pteronarcys californica*, were exposed to either normoxic (19 kPa O_2; n = 7) or hyperoxic (60 kPa; n = 7) water at 10 °C for 24 h. Internal PO_2 was measured twice inside the thorax of each individual. For the first measurement, a small hole was cut in the thorax of a haphazardly chosen location. The tip of an electrode was inserted into the hole using a micromanipulator and dissecting microscope. For the second measurement, the thorax was cut from the abdomen, and the thorax was inserted onto the tip of the electrode. For both measurements, we recorded the value shown on the acquisition system as soon as the reading stabilized (± ~ 2 %) for ~ 2 s, as above.

As an additional control, we measured internal PO_2 in the thorax of *P. californica* juveniles preserved in vials of 70 % ethanol at room temperature for at least one year. Here, the expectation was that biological processes affecting internal PO_2 would be negligible and should cause

internal PO_2 to track that of the external environment. Any differences in PO_2-change between exposures of live versus dead nymphs should thus reflect an effect of an unmeasured biological process (e.g., regulation of PO_2 levels). Before measurements were made, individuals were separated into unique vials with 5 ml of 70 % 'used' ethanol from the vial in which each juvenile was originally stored. Individuals were then assigned to either a normoxic (19 kPa O_2; $n = 7$) or hyperoxic (60 kPa; $n = 7$) treatment group. However, individuals from the same vial of origin were assigned to different treatments to avoid biasing the results based on slight differences in the percentage of ethanol, and therefore the solubility of oxygen, in each original storage vial (i.e., percentages of ethanol in preserved samples can change after storage as water from specimen bodies is drawn out into the solution) (Shchukarev and Tolmacheva 1968). For the normoxic treatment, thoracic PO_2 was measured for each individual at room temperature. Manipulating the PO_2 of the ethanol by bubbling in air was unnecessary because ethanol PO_2 was near saturation (~ 19 kPa O_2) in each vial. For the hyperoxic treatment, O_2 gas was bubbled into each vial until it reached 60 kPa, at which point the vial was tightly closed and stored for 24 h at room temperature. After exposure, we measured the PO_2 in both the ethanol and the thorax of each individual. For both treatments, one thoracic measurement was made for each nymph, in which a small hole was cut in the thorax of a haphazardly chosen location. The tip of an electrode was then inserted into the hole using a micromanipulator and dissecting microscope and the value shown on the acquisition system was recorded as soon as the reading stabilized for ~ 2 s, as above.

Statistical analysis

Tissue PO_2 data were zero-inflated, and we added 1 kPa to each measurement value and subsequently performed a log 10 transformation to improve normality. Although data transformation improved the distribution without yielding a truly normal distribution, we proceeded to use linear mixed-effects models (function: lmer; package: lme4) to analyze the effects of covariates on tissue PO_2 (Bates et al. 2015). To assess the performance of our models, we analyzed residuals and then checked the robustness of our linear mixed-effects models by reanalyzing our models using censored regression, which is designed for zero-inflated, semi-continuous data (function: censReg; package: censReg). Additionally, because strong correlations existed among some explanatory variables (Supplemental 2), results of each model were checked with residual regression – a common method for analyzing colinear data – following recommendations of Graham (2003). See additional details below.

In each linear mixed effects model, we included species as a random effect to account for effects of species identity and to isolate effects of habitat, life stage, activity and lifespan on PO_2. In preliminary analyses, we also tested for differences in location (i.e., on the thorax and abdomen), and body mass. Because both location and body mass did not contribute to the model fit and were non-significant as main effects nor in interaction with stage and habitat, we dropped them from further analyses and included measurements of both thoracic and abdominal PO_2 in our models. Because explanatory variables (habitat, life stage, activity and lifespan) could not be included in a single model without causing the fitting procedure to fail (likely due to strong collinearity among variables), we tested six more simplified candidate models, which, in total, included all possible combinations of covariates (Supplemental 3). Interactions between explanatory variables were only included when collinearity was low ($r < 0.30$) and models with explanatory variables that were highly correlated ($r > 0.30$) were checked with residual regressions (see additional details below). We calculated R^2 and AIC for use in model selection via the R functions, r.squaredGLMM (package: MuMIn) (Barton 2009) and aictab (package: AICcmodavg) (Mazerolle 2017).

Although R^2 and AIC did not agree on the single best model (Supplemental 3), model 1 ($\log_{10}(PO_2) \sim$ stage × habitat + activity level) was strongly supported by both selection criteria and was presented in the results. Residuals of model 1 were calculated using the R function, *residuals* (R Core Team, 2022), and visual analysis suggested that they were approximately normally distributed (Supplemental 3). However, because model 1 did not include stage duration, which had significant effect on tissue PO_2 in several of the candidate models, we ran an additional linear mixed effect model (i.e., model 7) with stage duration as the sole predictor ($\log_{10}(PO_2)$ + stage duration). Although model 7 had a considerably poorer fit than the other models (Supplemental 3), the effect of stage duration was significant, suggesting that patterns of internal PO_2 in insects can be explained by a single, alternative explanatory variable. In addition to model 1, we therefore also present the results from model 7 in the results. As indicated above, outputs of all candidate models were checked with supplemental censored regression (Supplemental 2) and residual regression models (Supplemental 4). Outputs of linear mixed effects models were similar to those from the supplemental models and were therefore deemed reliable.

Because many of the explanatory mechanisms are rooted in metabolic rate, we also ran an additional analysis in which we tested whether mass-specific metabolic rate affected tissue PO_2 using linear mixed-effects models (R function: lmer). Mass-specific metabolic rate had no effect on tissue PO_2 and was thus dropped from subsequent analyses (Supplemental 5). In addition, we used linear mixed effects models, as above, to analyze the effect of insect order and clade (i.e. Paleoptera, Polyneoptera, and Holometabola) on internal PO_2, and no significant effects were found (Supplemental 6). We thus deemed phylogenetic effects to be negligible and further phylogenetic analyses unnecessary.

To test whether internal PO_2 levels varied with external oxygenation levels (i.e., normoxia vs. hyperoxia) and internal oxygen consumption we measured internal PO_2 of live and dead salmonfly (Plecoptera: *Pteronarcys californica*) nymphs. For live salmonfly nymphs, we used a linear mixed effects model (function: *lmer*; package: lme4). Before analysis, we removed a single, extreme outlier ($> Q3 + 3 * IQR$) from the dataset. As above, measurement values were zero-inflated and strongly right-skewed, and we added 1 kPa to each value and transformed each using $\log_{10}$. Data transformation improved the distribution but did not yield a truly normal distribution. We nevertheless proceeded to use linear mixed-effects models (function: lmer; package: lme4) to analyze the effect of water oxygenation, and later checked the results using censored regression (Tobit) models with mixed effects, as above (function: censReg; R-package: censReg). The method used (i.e., cutting a small hole in the insect vs. cutting in half) and mass had no effect on internal PO_2, and these covariates were dropped from the analysis. Supplementary censored regression models yielded similar results to the linear mixed effects models, and outputs of linear mixed effects models were, therefore, considered valid. For the analysis of the effect of external oxygenation levels (i.e., normoxia vs. hyperoxia) on internal PO_2 of dead nymphs stored in ethanol, linear mixed effects models were used (R function: lme; package: lme4). Vial of origin was included as a random effect to account for any differences in the percentage of ethanol in which nymphs were stored. Mass has no effect in tissue PO_2 and was dropped from subsequent analyses.

Results

Across the 15 insect species and 9 orders examined, internal PO_2 ranged between 0 and 18.8 kPa (0.0–16.0 kPa, 5th and 95th percentile), with substantial variation among species and stages (Table 1). Tissue PO_2 of live insects was best explained by a model that included interactions between life stage and habitat (model 1: $P < 0.0001$) and activity level (model 1: $P < 0.0001$) (Fig. 1A) (Table 2). The interaction was driven by the remarkably low internal PO_2 values in aquatic juveniles (mean = 0.88 kPa), which were several times lower than in juveniles or adults of fully terrestrial species (mean = 3.85 and 5.33, respectively) and an order of magnitude lower compared to the air-breathing adults of the same or similar species (mean – 11.83 kPa).

Table 1
Table of the number of replicates and the mean and standard deviation of internal PO_2 (kPa) for each species-stage, with the order and habitat to which they belong.

Summary of internal PO2 for each species-stage						
Order	Taxon	Stage	Habitat	N	Mean PO2	Sd PO2
Ephemeroptera	Drunella grandis	immature	aquatic	8	0.00	0.00
Ephemeroptera	Drunella grandis	adult	aquatic	2	11.95	1.20
Ephemeroptera	Rhithrogena sp.	adult	aquatic	1	17.73	0.43
Diptera	Tipula sp.	immature	aquatic	9	0.43	1.60
Diptera	Tipula sp.	adult	aquatic	8	7.76	6.74
Plecoptera	Pteronarcys californica	immature	aquatic	8	0.04	0.11
Plecoptera	Pteronarcys californica	adult	aquatic	6	14.17	1.99
Odonata	Aeshna palmata	immature	aquatic	4	6.13	4.88
Odonata	Aeshna palmata	adult	aquatic	2	14.71	1.38
Odonata	Sympetrum danae	adult	aquatic	1	7.84	NA
Trichoptera	Parapsyche sp.	immature	aquatic	8	0.52	0.67
Trichoptera	Brachycentrus sp.	adult	aquatic	4	13.72	4.97
Coleoptera	Trypoxylus dichotomus	immature	terrestrial	8	1.34	0.73
Coleoptera	Trypoxylus dichotomus	adult	terrestrial	8	0.92	0.24
Phasmatodea	Phyllium philippinicum	immature	terrestrial	5	1.44	0.61
Phasmatodea	Phyllium philippinicum	adult	terrestrial	5	1.17	0.32
Orthoptera	Acrididae	immature	terrestrial	7	8.21	4.27
Orthoptera	Melanoplus bivittatus	adult	terrestrial	3	14.25	2.30
Orthoptera	Trimerotropis verruculata	adult	terrestrial	4	7.93	4.51
Diptera	Calliphora vicina	immature	terrestrial	7	2.64	4.35
Diptera	Calliphora vicina	adult	terrestrial	3	9.60	3.98
Lepidoptera	Manduca sexta	immature	terrestrial	8	5.12	5.84
Lepidoptera	Manduca sexta	adult	terrestrial	7	6.19	4.28

Aquatic juveniles also generally displayed low activity levels (mean = 1.1), contrasting with adults of aquatic insects which displayed high activity levels (mean = 2.0) (Fig. 1B). Internal PO_2 was also significantly affected by the duration of life stages (model 7: $P < 0.0001$) (Table 2), with aquatic juveniles exhibiting the longest stage durations (> 300 days) (Fig. 1C), again contrasting with adults that exhibited the shortest stage durations (mean = 10 days).

In addition, internal PO_2 of live salmonfly nymphs was not significantly affected by the PO_2 of the water (19 kPa vs. 60 kPa) during a 24 h exposure to hyperoxia ($P = 0.89$) (Fig. 2A) (Table 3). However, dead stoneflies preserved in ethanol had ~ 2 times higher tissue PO_2 after being exposed to hyperoxic ethanol (60 kPa) for 24 h than those exposed to normoxic ethanol (19 kPa) ($P < 0.0001$) (Fig. 2B) (Table 3).

Discussion

We observed impressive variation in internal levels of oxygen across the 15 insect species and 9 orders examined. Most notably, aquatic juveniles had remarkably low internal levels of oxygen, far lower than air-breathing adult insects or fully terrestrial species. Variation in PO_2 could be explained from differences in life stage, habitat, activity, and life-stage duration, indicating that insects likely actively regulate internal oxygen levels to balance competing functions (Fig. 1). While active regulation of PO_2 has long been known to occur in holometabolous, terrestrial insects (Hetz and Bradley 2005; Chown et al. 2006), it has been little-explored in aquatic species. Nevertheless, active regulation in aquatic insects is further supported by our experimental results, in which internal oxygen levels of juvenile stoneflies (*Pteronarcys californica*) remained low even when individuals were exposed to strong hyperoxia (~ 60 kPa) (Fig. 2). In contrast, internal oxygen levels of dead juvenile stoneflies had elevated internal oxygen levels (~ 18 kPa) in ambient normoxia and these levels were further elevated (~ 32 kPa) in hyperoxia, further supporting our conclusion that live aquatic juveniles actively regulate internal PO_2 to low levels.

What about life in water explains the low tissue oxygen levels in aquatic juveniles? One possibility is that low internal PO_2 evolved to facilitate oxygen uptake from water by maximizing the environment-to-mitochondria PO_2 gradient, which ultimately drives oxygen transport. In water (compared to in air), rates of oxygen flux are orders of magnitude lower because water imposes much lower oxygen diffusion coefficients (Woods, 1999; Verberk et al., 2011). In addition, water holds 20–30-fold less oxygen than air and has a higher (dynamic) viscosity, which generates thicker boundary layers around insect respiratory surfaces and greatly increases the energetic costs of ventilation (Denny 1993; Verberk and Atkinson 2013; Woods and Moran 2020). Indeed, aquatic insects have evolved a suite of morphological (gills), physiological (oxygen-carrying proteins), and behavioral adaptations (exploiting high flow microhabitats) to help mitigate this biophysical problem (Hynes 1970; Harrison et al. 2012; Buchwalter et al. 2020; Frakes et al., 2021; Birrell and Woods, 2023). Our finding of low PO_2 in juveniles across four orders of aquatic insects nevertheless suggests that holding PO2 to low levels is a widely used mechanism for increasing oxygen uptake. This innovation is likely important for aquatic juveniles to sustain sufficiently high basal metabolic rates, which we show are no different than those of air-breathers, despite much lower levels of oxygen availability in water than in air (Supplemental 5). It also suggests that oxygen fluxes in many but not all aquatic insects are well-approximated by indices of oxygen availability derived from external oxygen conditions, which often assume internal PO_2 to be zero (such as the OSI in Verberk et al. 2011). Exceptions in our data set include dragonfly nymphs, which, despite being aquatic, sustained relatively high mean PO_2 of 6.2 kPa. These results agree with those of Lee and Matthews (2024), who recently showed that nymphs of a dragonfly sustained hemolymph PO_2 of 6.3 kPa in normoxic water.

Maintaining low internal PO_2 may also require aquatic insects to have low activity levels. Across all stages and species, estimated activity level was positively correlated with tissue PO_2, with immature stages of aquatic insects generally displaying the lowest tissue PO_2 and activity levels. If insects are to regulate internal oxygen levels, rates of uptake and use must be matched, at least over timescales of minutes or longer. Indeed, many species of terrestrial, holometabolous insects regulate internal PO_2 by opening and closing their spiracles (Hetz and Bradley 2005; Chown et al. 2006), though to much higher levels than those observed here for aquatic juveniles. Thus, high internal PO_2 may support generally higher levels of activity by terrestrial insects, or greater variation in levels of activity. However, inactive insects may not require large reserves of oxygen for use during sustained bursts of locomotion – e.g., winged flight – and thus, hold tissue PO_2 at lower levels. Living life in the slow lane should thus allow insects to maintain sufficiently low internal PO_2 to support gas exchange, and may also help prevent accumulation of ROS (see below) and increase energy savings (Hetz and Bradley 2005; Speers-Roesch 2018). Indeed, energy savings could be

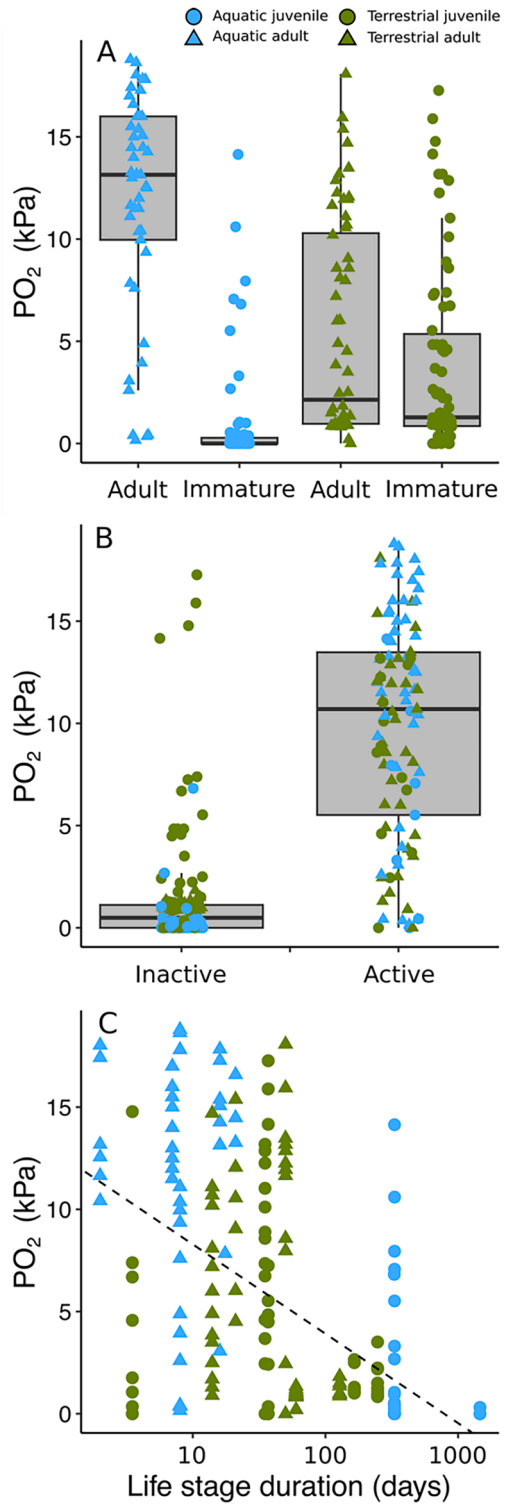

Fig. 1. Boxplot of tissue PO_2 of live insects from different life stages (adult vs. immature), habitats (aquatic vs. terrestrial) (A) and with different activity levels (B), and stage durations (C). Raw data are shown instead of $\log_{10} + 1$ transformed data used in final analyses for easier interpretation. The regression line in panel C was derived from a linear model (R function: lm), which showed a similar result to model 7, with a significant effect of life stage duration ($P < 0.001$).

paramount in long-lived aquatic stages that have access to only intermittent food (e.g., leaf shredders over an annual cycle). Nevertheless, the causal relationship between internal PO_2 and activity levels in

Table 2
Linear mixed effects models of the effects of stage, habitat, activity level and stage duration on tissue PO_2 of live insects. For models with categorical predictors, reference level is shown in brackets.

PO_2 in live insects – model 1					
Predictor	Estimate	Std. Error	Df	T-value	P-value
Intercept [reference: aquatic, adult]	0.511	0.079	22.911	6.503	< 0.001
Stage [Immature]	−0.423	0.076	68.025	−5.596	< 0.001
Habitat [Terrestrial]	−0.174	0.070	23.228	−2.486	0.021
Activity level [2]	0.541	0.060	24.466	9.025	< 0.001
Stage [Immature] * Habitat [Terrestrial]	0.503	0.079	189.453	6.400	< 0.001
PO_2 in live insects – model 7					
Predictor	Estimate	Std. Error	Df	T-value	P-value
Stage duration	−0.001	< 0.001	245.400	−12.189	< 0.001

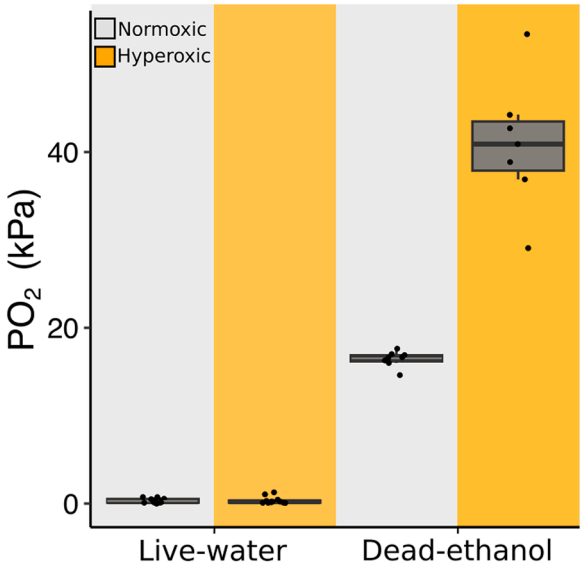

Fig. 2. Boxplots of tissue PO_2 of both live and dead juvenile salmonflies. Measurements on live individuals made after 24hr exposure to normoxia (19 kPa) and hyperoxia (60 kPA). Measurements on dead individuals in ethanol at normoxia (19 kPa) and 24 h exposure to hyperoxia (60 kPa).

Table 3
Linear mixed effects models of the effect of treatment (hyperoxia vs. normoxia) on tissue PO_2 of live salmonflies in water and dead salmonflies in ethanol.

PO_2 in live salmonflies in water					
Predictor	Estimate	Std. Error	Df	T-value	P-value
Treatment	−0.005	0.038	23.000	−0.141	0.889
PO_2 in dead salmonflies in ethanol					
Predictor	Estimate	Std. Error	Df	T-value	P-value
Treatment	24.434	2.635	13.000	9.272	< 0.001

aquatic juveniles is uncertain, as low levels of oxygen supply in water may instead *constrain* activity levels by requiring low tissue PO_2 for gas exchange, as discussed above.

We found no support for the alternative hypothesis that hypoxia-induced production of reactive oxygen species (ROS) prevents aquatic insects from having extremely low internal PO_2 levels (< 1 kPa). ROS, produced in mitochondria as a by-product of cellular respiration, are a major driver of cellular damage, disease, and aging (Boveris et al. 1973; Costa et al. 1993; Zorov et al. 2014; Shields et al. 2021). Because rates of

ROS production *rise* under both cellular hypoxia and reoxygenation (Murphy 2009; Wang et al. 2018), high levels of ROS damage under hypoxia could be expected to prevent aquatic juveniles from maintaining extremely low internal PO_2, especially in those with longer stage durations. We found, however, that insects with longer stage durations had significantly *lower* tissue PO_2, including many PO_2 values below 1 kPa, and this pattern was partly driven by aquatic juveniles, which had both the longest life stages and the lowest PO_2. This result, along with the taxonomic breadth of low internal PO_2 in aquatic juveniles, suggests that the benefits of improved gas exchange outweigh the costs of increased ROS production under cellular hypoxia and that aquatic juveniles employ a variety of mechanisms to minimize either the production or deleterious effects of ROS. Indeed, aquatic insects employ a host of antioxidant systems (Felton and Summers 1995; Xie et al. 2009; Sanz et al. 2017), yet their capacities to minimize ROS damage remains understudied compared to work on vertebrate or holometabolous insect model species. One possibility, however, is that aquatic juveniles reduce activity levels to consistently low levels, as shown by our data, to avoid excessive ROS production during activity-related reoxygenation (e.g., Wang et al. 2018).

In conclusion, using the first comparative dataset of tissue PO_2 from juvenile and adult insects from both aquatic and terrestrial environments, we show that aquatic juveniles across four orders have unexpectedly low internal oxygen levels. Because of the centrality of oxygen to both metabolic energy generation and oxidative damage causing senescence, this outcome likely reflects that competing priorities play out in fundamentally different ways in aquatic versus terrestrial environments. The mechanisms by which aquatic insects regulate internal oxygen levels provide fruitful direction for follow-up research. Potential mechanisms likely include shifts in tracheal morphology and the density and conductance of tracheoles (Wigglesworth 1983). In addition, we encourage additional studies on how the mitochondria of aquatic juveniles function under low internal oxygen levels and whether and how they avoid ROS production and damage during cellular hypoxia. More broadly, our data suggest that the problem of understanding patterns and mechanisms of oxygen regulation across insects would benefit from both focused and comparative studies utilizing species from a greater diversity of phylogenetic and ecological backgrounds.

CRediT authorship contribution statement

Jackson H. Birrell: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Wilco C.E.P. Verberk:** Formal analysis, Writing – review & editing. **H. Arthur Woods:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data and R scripts used in this study have been made publicly available on the Zenodo repository and can be accessed at: https://doi.org/10.5281/zenodo.12745828.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2024.100095.

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