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RESEARCH ARTICLE

Changes in hemolymph total CO_2 content during the water-to-air respiratory transition of amphibiotic dragonflies

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ABSTRACT

Dragonflies (Odonata, Anisoptera) are amphibiotic; the nymph is aquatic and breathes water using a rectal gill before metamorphosing into the winged adult, which breathes air through spiracles. While the evolutionary and developmental transition from water breathing to air breathing is known to be associated with a dramatic rise in internal CO_2 levels, the changes in blood-gas composition experienced by amphibiotic insects, which represent an ancestral air-to-water transition, are unknown. This study measured total CO_2 (TCO_2) in hemolymph collected from aquatic nymphs and air-breathing adults of *Anax junius*, *Aeshna multicolor* (Aeshnidae), *Libellula quadrimaculata* and *Libellula forensis* (Libellulidae). Hemolymph P_{CO_2} was also measured *in vivo* in both aeshnid nymphs and marbled crayfish (*Procambarus fallax*. f. *virginalis*) using a novel fiber-optic CO_2 sensor. The hemolymph TCO_2 of the pre- and early-final instar nymphs was found to be significantly lower than that of the air-breathing adults. However, the TCO_2 of the late-final instar aeshnid nymphs was not significantly different from that of the air-breathing adults, despite the late-final nymphs still breathing water. TCO_2 and P_{CO_2} were also significantly higher in the hemolymph of early-final aeshnid nymphs compared with values for the water-breathing crayfish. Thus, while dragonfly nymphs show an increase in internal CO_2 as they transition from water to air, from an evolutionary standpoint, the nymph's ability to breathe water is associated with a comparatively minor decrease in hemolymph TCO_2 relative to that of the air-breathing adult.

KEY WORDS: Blood-gas composition, Insect, TCO_2, P_{CO_2}, Tracheal gill, Aquatic

INTRODUCTION

Insects were among the first terrestrial animals to appear, some 479 million years ago (Misof et al., 2014). This transition to life on land likely occurred when the insects' ancestors evolved the ability to extract oxygen (O_2) directly from the atmosphere using an air-filled tracheal system that developed from invaginations of the cuticle (Pritchard et al., 1993). However, while insects have come to inhabit all terrestrial environments, some lineages have also secondarily reinvaded the aquatic environment, evolving the ability to exchange respiratory gases directly across their cuticle with the surrounding water. It is currently thought that between 100,000 and 1 million described insect species live in aquatic habitats (Lancaster and Downes, 2013), with five insect orders, the Ephemeroptera, Megaloptera, Odonata, Plecoptera and Trichoptera, all being predominantly amphibiotic: breathing water as larvae before metamorphosing into air-breathing adults (Pritchard et al., 1993). Thus, insects are one of the few examples of an ancestrally air-breathing animal lineage that has repeatedly evolved the ability to breathe water across multiple different orders. But how the respiratory physiology of these amphibiotic insects changes as they transition from water to air is almost completely unknown.

Any animal transitioning from breathing water to breathing air faces serious challenges because of the very different physiochemical properties of these two media. Compared with air, water is a poor source of O_2, containing only 3.8% of the O_2 found in an equivalent volume of air (Ultsch, 1996). The low O_2 availability in water inevitably means that an aquatic animal must ventilate a large volume of water to extract the O_2 required to satisfy its aerobic metabolic demands. This high convective requirement, in combination with water's approximately 28 times greater solubility for carbon dioxide (CO_2) than for O_2 (Rahn, 1966), enhances CO_2 excretion and results in a low internal P_{CO_2} (Ultsch, 1996). From this, it may be concluded that water-breathing animals must have a lower internal P_{CO_2} than their air-breathing counterparts (Dejours, 1989; Erasmus et al., 1970; Howell et al., 1973). A rise in internal P_{CO_2} associated with the respiratory transition from water to air can be seen across evolutionary time, i.e. from water-breathing to obligate air-breathing vertebrates (Howell, 1970) and crustaceans (Howell et al., 1973), as well as across ontogeny in the case of vertebrate amphibians (Erasmus et al., 1970).

The amphibiotic insects represent yet another group of animals that undergo a switch from aquatic to aerial gas exchange during their development. However, unlike the amphibians, these insects derived their water-breathing life stage from an air-breathing ancestor rather than vice versa. As such, water-breathing insects might have had to adapt to lower blood CO_2 levels over evolutionary time, contrary to the challenge faced by ancestrally water-breathing animals. But, unfortunately, comparatively little is known regarding the typical range of CO_2 levels in most air-breathing insects [with the exception of grasshoppers/locusts (e.g. Harrison et al., 1990; Harrison, 1988) and moth pupae (e.g. Buck and Keister, 1958; Levy and Schneiderman, 1966)] and even less is known regarding the CO_2 levels in water-breathing insects (Sutcliffe, 1962). As such, investigating the respiratory changes that occur in amphibiotic insects could reveal new insights into both the respiratory and evolutionary constraints associated with transitioning between water and air.

Members of the suborder Anisoptera (order Odonata), particularly species of the large-bodied Aeshnidae, are an ideal group in which to investigate how transitioning from water to air affects the internal CO_2 levels of an insect. Fossil evidence suggests that members of the Odonata evolved their amphibiotic life cycle some time prior to the divergence of the Zygoptera (damselflies) and Protoanisoptera (dragonflies), which had occurred by the lower Permian (Wootton, 1988). Thus, the nymph life stages of this group

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Received 23 March 2018; Accepted 5 June 2018

Journal of Experimental Biology (2018) 221, jeb181438. doi:10.1242/jeb.181438

have been living as water breathers for at least 300 million years. The gas exchange structures of the Anisoptera are also well studied, with the nymphs breathing water using a tidally ventilated rectal gill which they then discard when they undergo metamorphosis, while the air-breathing adult dragonflies that emerge from the exuviae of the final instar nymphs share the same open tracheal system as all other terrestrial insects (Tillyard, 1915).

To determine whether amphibiotic insects undergo the same dramatic increase in internal CO_2 as observed in other animal lineages that transition from water to air, hemolymph samples from the aquatic and terrestrial life stages of aeshnid and libellulid dragonflies were analyzed for total CO_2 content (TCO_2), while a novel optical P_{CO_2} microsensor was used to measure *in vivo* P_{CO_2} directly in the dragonflies' hemocoel. As a procedural control, the same techniques were used to obtain measurements of TCO_2 and *in vivo* P_{CO_2} from freshwater marbled crayfish in order to provide results that could be compared with previously published values in the literature.

MATERIALS AND METHODS

Animals

Dragonfly nymphs

Aeshnid and libelullid dragonfly nymphs were captured from ponds at the University of British Columbia, Point Grey campus using aquatic sweep nets. *Anax junius* (Drury 1773) and *Aeshna multicolor* Hagen 1861 dragonflies were the most common adult aeshnid species flying around these ponds, and subsequent identification using photographs of the captured nymphs revealed that they were indeed a mix of *Anax* and *Aeshna* species (Cannings and Stuart, 1977). However, not all sampled nymphs were photographed, and as it was not possible to further identify them to the species level, these nymphs were treated as a single aeshnid group. The libellulid dragonfly nymphs were all identified as being *Libellula quadrimaculata* Linnaeus 1758 (Cannings and Stuart, 1977).

Captured nymphs were brought back to the lab and housed individually in 11×11×17 cm square glass aquaria filled with dechlorinated Vancouver tap water. Each aquarium contained a pea gravel substrate. These aquaria were connected to a re-circulating system which consisted of a pump inside a 76 l sump that supplied a gentle flow of water in through the top of each aquarium. A drain port on one side of each aquarium directed the overflow water through a filter before discharging it back into the sump. The water in the sump was completely replaced with fresh dechlorinated Vancouver tap water as required. Nymphs that were small enough to enter the drain ports were housed in 600 ml polypropylene containers with a pea gravel substrate. The water in these containers was completely replaced with fresh dechlorinated Vancouver tap water once a week. The nymphs were maintained at lab temperature (20–23°C) on a 12 h:12 h daylight cycle, fed on a variety of aquatic and terrestrial invertebrates, and allowed to acclimate for at least 2 weeks prior to measurement. All nymphs were starved for 24 h prior to measurement. The same dechlorinated Vancouver tap water was used in all subsequent experiments.

Dragonfly adults

Four species of dragonflies from two families were caught using aerial nets between June and August 2016–2017 from around the same ponds where the nymphs were collected. They were *Anax junius*, *Aeshna multicolor* (Aeshnidae), *Libellula quadrimaculata* and *Libellula forensis* Hagen 1861 (Libellulidae). Captured adults were immediately placed in blacked-out glass jars that were covered with aluminium foil and lined with fine plastic mesh on the inside. The adults remained at rest in the jars for at least 2 h prior to measurement. Following the experiments, they were released back into the wild.

Marbled crayfish

Parthenogenic marbled crayfish (*Procambarus fallax* f. *virginalis*; Martin, Dorn, Kawai, van der Heiden and Scholtz 2010) were purchased from a commercial supplier (JLAquatics, Vancouver, BC, Canada) and acclimated to lab conditions (20–23°C, 12 h:12 h daylight cycle) in a 20 l aquarium filled with dechlorinated Vancouver tap water and fed *ad libitum* on frozen bloodworms and spirulina tablets for 1 week prior to measurement. All individuals were starved for 24 h prior to measurement.

Body mass

All animals were weighed to 0.01 mg on an electronic balance (XPE205DR, Mettler-Toledo Inc., Missisauga, ON, Canada) directly before use in experiments (Table 1).

Classification of dragonfly nymph developmental stage

In this study, the dragonfly nymphs were divided into three stages: pre-, early- or late-final instar. Nymphs with small, incompletely developed wing-buds were collectively termed pre-final instars (Fig. 1). Early-final instar nymphs were characterized by large wing-buds with both pairs of wings lying flat on the dorsal surface. In addition, the hindwing-buds were collapsed on top of the forewing-buds such that the forewing-buds were not visible. Late-final instars were identified by wing-buds that spread apart such that both the forewing- and hindwing-buds were clearly visible, in addition to possessing more pronounced venation. These 'late-final' changes occurred in the absence of a molt and were accompanied by cessation of eating, indicating that the nymphs were beginning to prepare for metamorphosis (Corbet, 1962).

Measuring hemolymph TCO_2

Hemolymph TCO_2 content was measured using a custom-built gas sparging system coupled with a two-channel LI-7000 infra-red CO_2 gas analyzer (LI-COR, Lincoln, NE, USA). Compressed N_2 from a gas cylinder (99.998% pure, Praxair, Mississauga, ON, Canada) was connected to a 0–100 ml min^{-1} mass flow controller (GFC17, Aalborg, Orangeburg, NY, USA) set to a flow rate of 20 or 100 ml min^{-1} standard temperature and pressure dry (STPD). This N_2 stream then passed through cell A of the two-channel LI-7000 to provide a constant zero-CO_2 reference. The cell A outlet was then

Table 1. Average body mass data for individuals used in each experiment

Experiment	Species/group	*n*	Mass (g)
Hemolymph TCO_2	*Procambarus fallax* f. *virginalis*	6	1.6±0.2
	Pre-final aeshnid	7	0.47±0.03
	Early-final aeshnid	11	0.98±0.05
	Late-final aeshnid	8	1.42±0.02
	Early-final *Libellula quadrimaculata*	8	0.37±0.02
	Anax junius‡	8	0.95±0.03
	Aeshna multicolor‡	8	0.57±0.03
	Libellula quadrimaculata‡	7	0.37±0.02
	Libellula forensis‡	7	0.52±0.03
Time-wise TCO_2	0.5 min air exposure	3	0.35±0.04
	3.2 min air exposure	3	0.39±0.02
Hemolymph P_{CO_2}	*Procambarus fallax* f. *virginalis*	5	1.4±0.2
	Early-final aeshnid	5*	0.60±0.01

Mass data are means±s.e.m. TCO_2, total CO_2.
*Mass available for 5 out of 6 individuals; ‡adult dragonflies.

Journal of Experimental Biology (2018) 221, jeb181438. doi:10.1242/jeb.181438

Fig. 1. Photographs of pre-final (left), early-final (center) and late-final (right) instar Aeshnidae dragonfly nymphs. These three instar stages were differentiated based on their wing-bud morphology, with pre-final instars having short underdeveloped wing-buds, early-final instars having long and collapsed wing-buds and late-final instars having long and spread apart wing-buds. The grid is 5×5 mm.

connected to the bottom of a custom-made gas sparging column that consisted of a cylindrical glass chamber (4.7 ml internal volume). Gas entering from the bottom of the column passed up through a sintered glass disc, then bubbled through an acid solution: 1 ml of 0.01 mol l^{-1} HCl mixed with 1 µl Antifoam 204 (Sigma-Aldrich, St Louis, MO, USA) to prevent excessive frothing during sample injections. After passing through the acid solution, the N_2 gas and any liberated gaseous CO_2 was passed through cell B of the infra-red CO_2 gas analyzer for analysis. The CO_2 concentration (µmol mol^{-1}) in the N_2 stream was logged at 5 Hz using a desktop PC running LI-7000 software (v2.0.3, LI-COR). A gas-tight injection port with a PTFE-lined septum mounted on the side of the sparging column allowed samples to be injected directly into the acid solution for analysis.

To generate a TCO_2 calibration curve, a series of sodium bicarbonate standard solutions were prepared by dissolving 1.26 g of $NaHCO_3$ in 500 ml of distilled water to create a 30 mmol l^{-1} stock solution. This stock solution was then diluted to produce additional standard solutions of 10, 15, 20 and 25 mmol l^{-1}. Then, a 26s-gauge 10 µl Hamilton syringe was primed with CO_2-free distilled water (that had been purged with 99.998% pure N_2) by repeatedly pumping the plunger while the needle tip was kept submerged in the water, thereby expelling any air from the needle lumen (hereafter referred to as 'primed'). This primed syringe was then used to withdraw and inject a 5 µl sample of each standard solution into the gas sparging column through the PTFE-lined septum. The resulting reaction between the bicarbonate and HCl led to the liberation of gaseous CO_2, which was recorded by the infra-red CO_2 gas analyzer as a CO_2 pulse in µmol mol^{-1}. The CO_2 pulse was first converted to instantaneous $\dot{V}_{CO_2}$ (ml min^{-1}) using the formula:

$$\dot{V}_{CO_2} = CO_2/1{,}000{,}000 \times \dot{V}_{in}, \quad (1)$$

where CO_2 is the CO_2 pulse trace (µmol mol^{-1}) and $\dot{V}_{in}$ is the incoming flow rate (ml min^{-1}, STPD). The area under the recorded CO_2 pulse was then integrated against time using the area approximation formula:

$$V_{CO_2} = [(Y_2 + Y_1)/2] \times (X_2 - X_1), \quad (2)$$

where V_{CO_2} is the volume of CO_2 liberated (ml), Y_2 and Y_1 are instantaneous $\dot{V}_{CO_2}$ (ml min^{-1}) and X_2 and X_1 are time (min). The CO_2 volume (ml) was converted to liters, then divided by the molar volume constant 22.414 l mol^{-1}. The resulting moles of CO_2 was converted to millimoles then divided by the volume of sample injected to produce the final TCO_2 in mmol l^{-1}. This series of standard solution injections was repeated 2–3 times, and the true CO_2 value was fitted against the average measured value in order to create a calibration curve.

Hemolymph TCO_2 was measured using samples extracted directly from the insect's hemocoel. Nymphs were removed from their aquaria and quickly patted dry with paper towels, after which they were gently restrained in a groove cut into an expanded polystyrene block. A 26s-gauge 10 µl Hamilton syringe was again primed, and the needle tip was inserted into the nymph between the dorsal abdominal tergites of segments 7 and 8 to either the left or right side of the midline, depending on the position of the nymph. A 5 µl sample of hemolymph was withdrawn from the animal and injected immediately into the gas sparging column. Sodium bicarbonate standard solutions were run before and after each series of hemolymph sample injections to provide direct reference to known values, and CO_2-free distilled water blanks were run between each injection to ensure no residual CO_2 remained in the syringe. The average time that the nymphs spent out of water was 1 min.

The hemolymph TCO_2 of adult dragonflies was measured using the same protocol as above, except that the adults were restrained ventral-side down on an expanded polystyrene board using insect pins, and a primed syringe was used to pierce the mid-dorsal surface of the mesothorax to extract a 5 µl hemolymph sample directly from the aortic diverticula (Jensen, 1976).

Verification of the experimental approach

To determine whether the removal of nymphs from water during the extraction procedure had any effect on hemolymph TCO_2, measurements were taken from two additional groups of nymphs. One group had the hemolymph extracted as quickly as possible, while the other group had the hemolymph extracted after being left in air for 2 min.

As a procedural control for the TCO_2 protocol, hemolymph TCO_2 was measured from marbled crayfish using the same technique, thereby providing data that could be compared with published TCO_2 values for other crustaceans. The crayfish were removed from the aquarium, quickly patted dry using paper towels, and held in place with one hand while a primed 26s-gauge 10 µl Hamilton syringe was inserted into the hemocoel surrounding the heart. A 5 µl sample of hemolymph was obtained and analyzed for TCO_2 as described previously. The average time that the marbled crayfish spent out of water for each extraction was 0.5 min.

Measuring hemolymph P_{CO_2}

The syringe-mounted P_{CO_2} microsensors (NTH-CDM1, PreSens, Regensburg, Bavaria, Germany) were calibrated in a 0.154 mol l^{-1} NaCl solution equilibrated with various known P_{CO_2} levels. A 100 ml glass bottle was filled with the saline solution and an aquarium air stone connected to a gas mixing system was threaded through one of two holes in the bottle's lid. The bottle was then placed in a temperature-controlled water bath (F33-ME, Julabo, Seelbach, Baden-Württemberg, Germany) set to 21°C. The P_{CO_2} microsensor was inserted into the calibration bottle through the second hole in the lid, and its sensor tip was extended out from the hypodermic needle into the solution. A series of P_{CO_2} levels were generated using two 0–500 ml l^{-1} mass flow controllers

(MC-500SCCM-D/5M, Alicat Scientific, Tucson, AZ, USA) to combine compressed gases (99.998% N_2 and 5% CO_2 balance N_2; Praxair, Mississauga, ON, Canada). Flow rates were verified using a Bios DryCal Definer 220-L primary flow meter that had been calibrated using NIST standards (Mesa Laboratories, Inc., Lakewood, CO, USA). Gas mixing software (Flow Vision v1.3.13.0, Alicat Scientific) running on a PC controlled the mass flow controllers to generate 0, 0.5, 1, 2 and 3 kPa P_{CO_2} gas mixtures at a total combined flow rate of 500 ml min^{-1} STPD. The P_{CO_2} sensor was allowed to equilibrate for 1 h at 0 kPa P_{CO_2} and for 30 min at all other P_{CO_2} levels. P_{CO_2} readings were recorded once every 5 min using a micro fiber-optic CO_2 meter (pCO_2 micro, PreSens) and control software (pCO_2 micro view v1.0.0, PreSens). Following calibration, the sensors were back-checked at 0.5 and 1 kPa P_{CO_2} before being placed in a saline storage solution until use.

Calibrated P_{CO_2} probes were implanted into early-final aeshnid nymphs that had been starved for at least 24 h. Before implantation, the nymph was first cold-anesthetized in ice water for 4 min. Following removal from the ice water, its dorsal surface was quickly patted dry with paper towels. The nymph was then attached to a plastic harness using a low-viscosity polyvinylsiloxane dental impression material (President light body, Coltene Whaledent, Cuyahoga Falls, OH, USA). The concave bottom surface of the harness was coated with the impression material, which was then pressed down over the wing-buds of the nymph and allowed to cure. During the curing period, the nymph's abdomen was kept submerged in dechlorinated Vancouver tap water to allow it to breathe. The harness was then attached to a micromanipulator (M3301, World Precision Instruments, Sarasota, FL, USA) and positioned on top of a Plexiglas platform inside a 25×16.7×8 cm polypropylene container, such that the ventral abdominal surface of the nymph was approximately 3 mm above the platform. The polypropylene container was filled with water until the tip of the nymph's abdomen was submerged. A 1 ml hypodermic syringe with a 20-gauge needle, mounted on a second micromanipulator, was used to make a small hole in the middle of the cuticle between the first and second thoracic tergites. The syringe was subsequently removed and replaced with a previously calibrated P_{CO_2} microsensor. This allowed the sensor to be inserted through the hole, approximately 2 mm into the nymph's hemocoel, while monitoring the P_{CO_2} reading to ensure a successful implantation. The hole and sensor were sealed in place using the dental impression material. The polypropylene container was then filled to the brim with dechlorinated Vancouver tap water, continuously bubbled with room air, and covered with an opaque mesh to reduce the visual agitation of the nymph. The container was maintained at 21°C by being placed within a larger acrylic water bath, which was kept thermally stable using a temperature-controlled water bath (F33-ME, Julabo). The hemolymph P_{CO_2} was recorded every 5 min. The experiment lasted for 24 h, after which the sensor was retrieved and checked in CO_2-equilibrated saline for signal drift. It was not possible to measure *in vivo* P_{CO_2} in the libellulid nymphs because of their small size, which did not allow enough room for both the harness and the microsensor.

The P_{CO_2} readings recorded using the pCO_2 micro view software were exported into Microsoft Excel for analysis. The raw percentage CO_2 traces were first converted to P_{CO_2} by multiplying each value by 101.3 kPa. The P_{CO_2} values were then plotted against time and the values from 12 to 24 h were averaged to calculate mean P_{CO_2} for each individual. These individual means were then averaged together to calculate the grand mean P_{CO_2} for the nymphs.

Verification of the experimental approach

Given that *in vivo* P_{CO_2} was measured using an experimental P_{CO_2} sensor in this study, it was considered prudent to also measure hemolymph P_{CO_2} in an animal group that has been measured previously to provide a comparison between our measured values and those in the literature. Marbled crayfish were selected for this purpose. The protocol for measuring P_{CO_2} in these crustaceans was largely the same as the one used for dragonfly nymphs except that following the 24 h starvation period, the individuals were cold-anesthetized in ice water for 20 min, and after removal from the ice water a 20-gauge needle was used to make a small hole in the carapace approximately 3 mm to the left of the mid-dorsal line. The crayfish were then attached to the plastic harness while ensuring the hole was not occluded by the impression material, and after transfer to the measurement setup, the P_{CO_2} sensor tip was inserted into the pre-existing hole and sealed in place, again using dental impression material.

Statistical analyses

Data were analyzed in R v3.4.1 (R Core Team 2017). A one-way ANOVA was performed to test for any statistical differences between the mean TCO_2 of dragonfly nymphs, adults and marbled crayfish, and two-sample *t*-tests were performed to test for any changes in the mean TCO_2 of the two treatment groups exposed to either short or long emersion, as well as for any differences between the mean P_{CO_2} of dragonfly nymphs and marbled crayfish. Data are shown as means±s.e.m. unless otherwise stated.

RESULTS

Total hemolymph CO_2

TCO_2 measurements were made on pre-final, early-final and late-final aeshnid nymphs, early-final *L. quadrimaculata* nymphs, and adults of *Anax junius*, *Aeshna multicolor*, *L. quadrimaculata* and *L. forensis*, as well as on marbled crayfish (Fig. 2). The pre-final and early-final aeshnid nymphs had statistically the same TCO_2 of 15.6 ±1.2 and 16.0±0.7 mmol l^{-1}, respectively (one-way ANOVA, F=36, d.f.=8, P<0.001). However, there was a significant increase in TCO_2 to 21.3±1.3 mmol l^{-1} in the late-final aeshnid nymphs. This high TCO_2 was not statistically different from the values seen in either species of adult aeshnid. The *A. junius* and *A. multicolor* adults had TCO_2 levels that were not significantly different (23.2±0.3 and 22.1±1.3 mmol l^{-1}, respectively), but *A. junius* individuals had noticeably less variation in their measured TCO_2 compared with all other groups. The early-final *L. quadrimaculata* nymphs had a mean TCO_2 of 19.6±1.0 mmol l^{-1}, which was not significantly different from the values in all the water-breathing and air-breathing aeshnid groups. However, the early-final *L. quadrimaculata* TCO_2 was significantly lower than the mean TCO_2 of both adult libellulid species. Adults of *L. quadrimaculata* and *L. forensis* had TCO_2 levels that did not differ significantly (29.2±1.3 and 30.6± 1.4 mmol l^{-1}, respectively). However, both were significantly higher than the values from *A. junius*, *A. multicolor* and the late-final aeshnid nymphs. Marbled crayfish had a mean TCO_2 of 8.4± 1.0 mmol l^{-1}, which was lower than that of all dragonflies, aquatic or terrestrial.

Effect of emersion on TCO_2

TCO_2 measurements were made on two groups of pre-final aeshnid nymphs (Table 2). The average emersion duration for group 1 was 0.5 min, while the average duration for group 2 was 3.2 min. The lowest and highest values in the 0.5 min group were 17.7 and 28.9 mmol l^{-1}, respectively, while the corresponding values in

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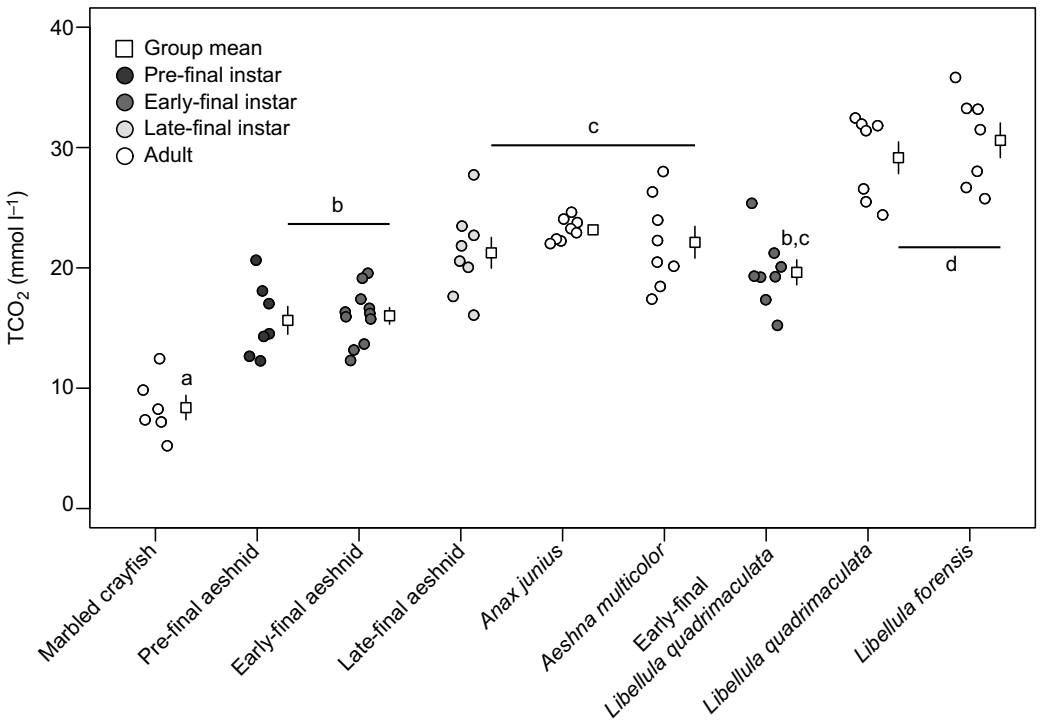

Fig. 2. Individual and mean hemolymph total CO_2 (TCO_2) of the study species. From left to right: marbled crayfish *Procambarus fallax* f. *virginalis* (*n*=6), pre-final (*n*=7), early-final (*n*=11) and late-final (*n*=8) aeshnid nymphs, adult *Anax junius* (*n*=8) and *Aeshna multicolor* (*n*=8), early-final *Libellula quadrimaculata* nymphs (*n*=8), and adult *Libellula quadrimaculata* (*n*=7) and *Libellula forensis* (*n*=7). Points with different letters are significantly different (one-way ANOVA, *P*<0.001, Tukey's HSD). Data are presented as means±s.e.m.

the 3.2 min group were 14.0 and 24.7 mmol l^{-1}, respectively. Comparing the mean TCO_2 values showed that there was no significant difference between the two groups (two-sample Student's *t*-test, d.f.=4, *P*=0.9).

Hemolymph P_{CO_2}

P_{CO_2} measurements were made on early-final aeshnid nymphs and marbled crayfish (Fig. 3). The mean P_{CO_2} of early-final nymphs was 0.9±0.1 kPa, which was significantly higher than the 0.38±0.05 kPa mean P_{CO_2} of marbled crayfish (two-sample Student's *t*-test, d.f.=9, *P*=0.002). The P_{CO_2} always started high for both the dragonfly nymphs and marbled crayfish (mean 2.1±0.4 and 1.0±0.1 kPa, respectively) (Fig. 4). As the experiment progressed, the P_{CO_2} began to decrease such that all animals had reached a stable plateau by 12 h into the experiment. Therefore, the P_{CO_2} readings from 12 h to the end of the experiment were considered to be physiologically relevant and were used to calculate mean hemolymph P_{CO_2} for each animal.

Table 2. Hemolymph TCO_2 of pre-final aeshnid nymphs exposed to two different emersion durations

Emersion duration (min)	Individual	TCO_2 (mmol l^{-1})
0.5	1	17.7
	2	18.0
	3	28.9
	Average	21.5±3.7
3.2	4	23.5
	5	24.7
	6	14.0
	Average	20.7±3.4

Where applicable, values are shown as means±s.e.m. (*n*=3). The average TCO_2 in the two emersion groups did not differ significantly (Student's *t*-test, *P*=0.9).

DISCUSSION

Dragonfly TCO_2 in water-breathing nymphs and air-breathing adults

Findings from the current study indicate that hemolymph TCO_2 increases when dragonflies undergo the transition from breathing water as nymphs to breathing air as adults. This is not entirely

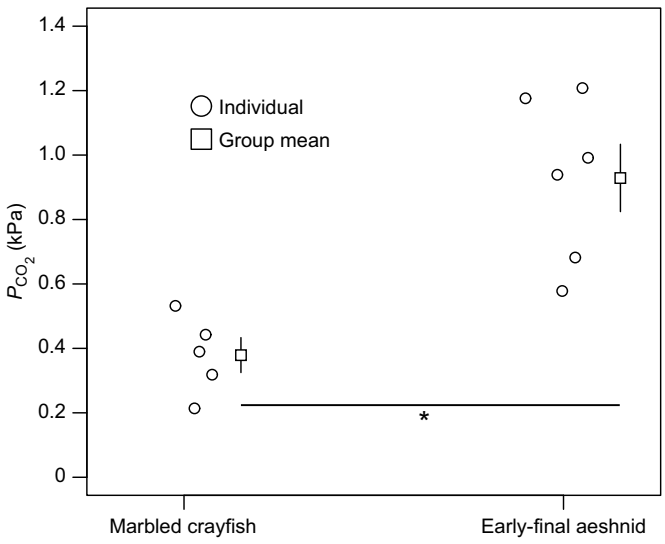

Fig. 3. Comparison of hemolymph P_{CO_2} in marbled crayfish *P. fallax* f. *virginalis* (*n*=5) and early-final aeshnid nymphs (*n*=6). *Significant difference (Student's *t*-test, *P*=0.002). Data are presented as means±s.e.m.

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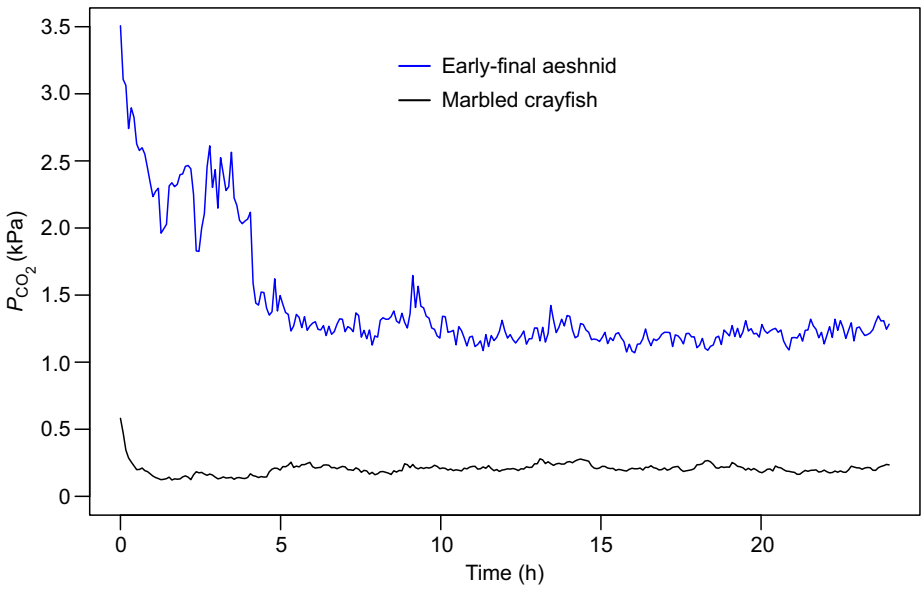

Fig. 4. Representative graph showing hemolymph P_{CO_2} against time for early-final aeshnid nymphs and marbled crayfish *P. fallax* f. *virginalis*. Time 0 was the start of the experiment. In all individuals, P_{CO_2} started high, then slowly stabilized to a lower level by 12 h, and the steady-state level was maintained for the rest of the 24 h experimental period.

unexpected, and suggests that the nymphs are pressed by their need to extract sufficient amounts of O_2 from water, which results in high ventilation rates and greater CO_2 excretion in water relative to air. However, the observed increase in TCO_2 does not coincide with the nymph leaving the water, but precedes metamorphosis. As such, the aquatic late-final aeshnid nymphs have a hemolymph TCO_2 that is significantly higher than that of both the pre- and early-final instars, but not significantly different from that of the air-breathing adult (Fig. 2). One possible explanation for this unexpected result is that as late-final instar nymphs begin to modify their physiology in anticipation of metamorphosis, the conductance of their rectal gill declines and/or they become less reliant on their gills for gas exchange. This is plausible, as the mesothoracic spiracles of final instar nymphs become functional prior to metamorphosis, giving them the ability to breathe air (Corbet, 1962; Gaino et al., 2007). The nymphs of the primitive Petaluridae family are known to possess functional thoracic spiracles during all their later instars (Green, 1977; Wolfe, 1953). This trait, combined with amphibious habits, allows them to hunt on land in a manner that is reminiscent of the ancestral Odonata (Corbet et al., 1960). Interestingly, the air-breathing final instar petalurid nymphs of *Uropetala carovei* possess a hemolymph TCO_2 of 28.0±6.0 mmol l^{-1} (Bedford and Leader, 1975), a value that is far higher than those measured from all aeshnid nymphs and adults, but similar to the values recorded from the adult Libellulidae (29.2–30.6 mmol l^{-1}; Fig. 5).

Comparing *A. junius* and *A. multicolor* with *L. quadrimaculata* and *L. forensis* shows that the adult Libellulidae have significantly higher hemolymph TCO_2 levels than those of the adult Aeshnidae. Differences between these two dragonfly families can also be seen from the water-breathing nymph stages, where, although not statistically significant, the TCO_2 of the early-final aeshnid nymphs is lower than the value from the early-final *L. quadrimaculata*. A previously reported value for hemolymph HCO_3^- concentrations in a mixed-age sample of *A. grandis* (Aeshnidae) nymphs (15.0 ±2.5 mmol l^{-1}; Sutcliffe, 1962) is also in good agreement with the aeshnid nymph TCO_2 values presented here (Fig. 5), suggesting family-specific differences in internal CO_2 levels. Understanding the phylogeny, ecology and behavior of these two common dragonfly families can be helpful in interpreting the above trend. The Aeshnidae and Libellulidae families are evolutionarily distant (Misof et al., 2001; Saux et al., 2003), and have diverged substantially in their morphology and behavior. Nymphs belonging to the Aeshnidae are typically active hunters that crawl through aquatic vegetation, while libellulid nymphs are sluggish and live on the substrate, often in warmer, eutrophic waters (Cannings and Stuart, 1977). Thus, the libellulid nymphs are more likely to be exposed to hypoxia and hypercapnia than the aeshnids. The adults of these two families, too, show markedly different flight behaviors, with the Aeshnidae spending most of their time on the wing, while the Libellulidae fly intermittently from a selected perch, chasing after prey and conspecifics (Corbet, 1962). These different flight strategies are reflected in the dragonflies' circulatory systems, in that *A. junius* and *A. multicolor* aeshnids have much larger hearts and hemolymph volumes than the similarly sized libellulid *Libellula saturata* (Heinrich and Casey, 1978). Thus, the comparatively poor hemolymph circulation in the Libellulidae may well impede CO_2 excretion in comparison to the Aeshnidae, resulting in elevated TCO_2 levels. A phylogenetic approach examining the relationship between hemolymph TCO_2 and adult flight strategies across a wide range of dragonfly families would be a powerful way to test the hypothesis that internal TCO_2 is mechanistically linked to flight behavior within the dragonflies.

Dragonflies compared with other animals

The dragonfly nymph values presented here are comparable with those few published hemolymph HCO_3^- values from the aquatic larvae of other developmentally amphibiotic insect lineages. In particular, dragonfly nymph TCO_2 is similar to the HCO_3^- concentration seen in larvae of the alderfly *Sialis lutaria* (Shaw, 1955), but is substantially higher than that of the larval caddisfly *Limnephilus stigma* (Sutcliffe, 1962) (Fig. 5). Both caddisfly and alderfly larvae breathe water using external tracheal gills, but while caddisfly larvae are generally apneustic, alderfly and other Megalopteran larvae possess functional spiracles along the length of their body, allowing them to breathe air (Barclay et al., 2005). When compared with that of air-breathing insects, the CO_2 content in the dragonfly nymphs is similar to values in the aquatic (but air-breathing) water boatman *Cenocorixa blaisdelli* (Cooper et al., 1987), but is noticeably higher than the values measured from terrestrial grasshopper and locust species such as *Melanoplus*, *Romalea* and *Schistocerca* (Gulinson and Harrison, 1996; Harrison et al., 1990; Harrison, 1988, 1989; Krolikowski and Harrison, 1996).

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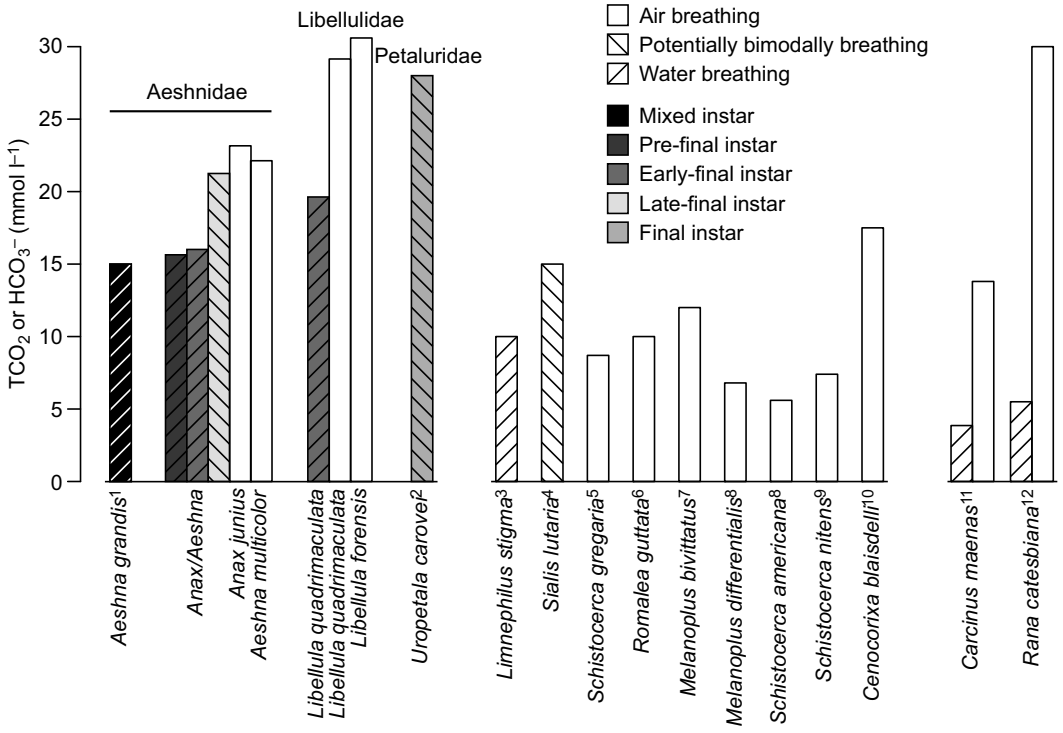

Fig. 5. Hemolymph TCO_2 in the nymphs and adults of Aeshnidae and Libellulidae dragonfly families. The data from the current study are compared with the hemolymph HCO_3^- content in nymphs of additional dragonfly species ([1]Sutcliffe, 1962; [2]Bedford and Leader, 1975), other water-breathing and air-breathing insects ([3]Sutcliffe, 1962; [4]Shaw, 1955; [5]Harrison et al., 1990; [6]Gulinson and Harrison, 1996; [7]Harrison, 1988; [8]Krolikowski and Harrison, 1996; [9]Harrison, 1989; [10]Cooper et al., 1987), decapod crustaceans ([11]Truchot, 1975) and amphibians ([12]Erasmus et al., 1970). Open bars represent air-breathing animals/life stages, while diagonal dashes represent water-breathing or potentially bimodally breathing animals/life stages. The various dragonfly nymph developmental stages are represented by shades of gray. Values are presented as means.

While hemolymph TCO_2 is a useful indicator of blood-gas composition that includes both HCO_3^- and dissolved gaseous CO_2 contributions, the direct measurement of *in vivo* hemolymph P_{CO_2} in dragonfly nymphs further suggests that these insects do not have the same low P_{CO_2} as seen in most other water breathers. For example, while lower than the P_{CO_2} of various air-breathing orthopterans (Gulinson and Harrison, 1996; Harrison et al., 1990; Krolikowski and Harrison, 1996), the early-final aeshnid nymphs have a significantly higher hemolymph P_{CO_2} than the water-breathing marbled crayfish (Fig. 3), and this is also much higher than the CO_2 tension in rainbow trout *Oncorhynchus mykiss* (Eddy et al., 1977) (Fig. 6) and other 'typical' fish (Ultsch, 1996).

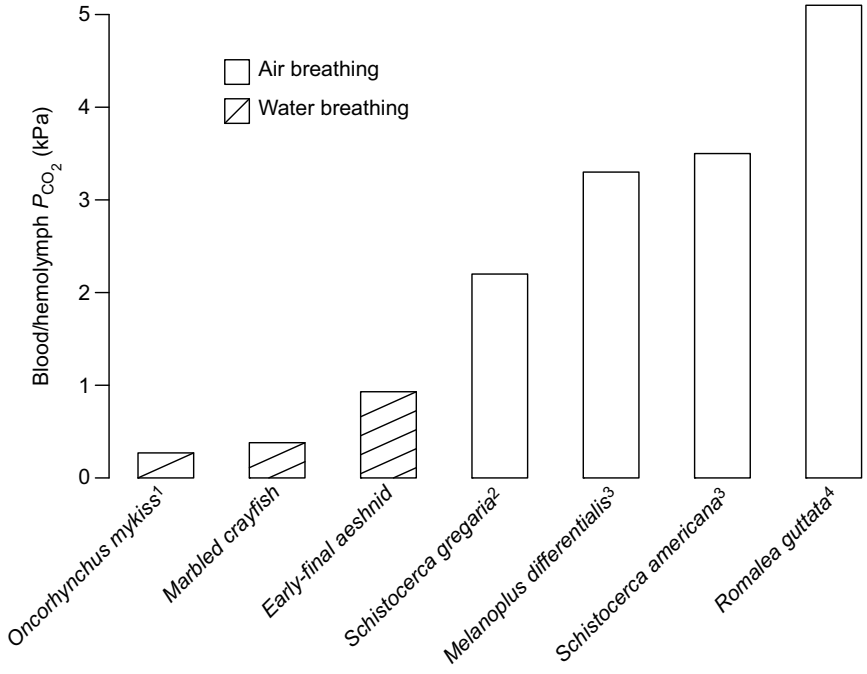

Fig. 6. Hemolymph P_{CO_2} in early-final Aeshnidae nymphs and in the marbled crayfish *P. fallax* f. *virginalis*. The data from the current study are compared with the blood P_{CO_2} of a water-breathing fish ([1]Eddy et al., 1977) and hemolymph P_{CO_2} of air-breathing orthopterans ([2]Harrison et al., 1990; [3]Krolikowski and Harrison, 1996; [4]Gulinson and Harrison, 1996). Values are represented as means.

In addition to an overall higher internal TCO_2 and P_{CO_2} in the water-breathing dragonfly nymphs relative to those of other water-breathing animals, the increase in TCO_2 that occurs during their transition from water to air breathing is fairly modest: an increase of only 45% and 48% from early-final instar to adult in the Aeshnidae and Libellulidae, respectively. Ancestrally aquatic animals making this same transition show a far higher increase. For example, the tadpole to bullfrog transition of *Rana catesbiana* results in a 445% increase in blood HCO_3^- (Erasmus et al., 1970), while the crab *Carcinus maenas* experiences a rise of 258% when moving from water to air (Truchot, 1975). These trends suggest that, as a consequence of adapting their ancestral air-breathing respiratory system to function in water, dragonfly nymphs have evolved gas exchange strategies that limit CO_2 excretion and result in greater internal CO_2 accumulation. One possible explanation could lie in the efficiency of the rectal gill as a gas exchange organ. However, it has also been recognized that insects lack carbonic anhydrase in their hemolymph and gills (Harrison, 2001; Kohnert et al., 2004). As a result, a disequilibrium between CO_2 and HCO_3^- could arise in the hemolymph (Cooper, 1994), which may impede CO_2 excretion across the gills.

Verification of the experimental protocol

One criticism of the protocol used in the current study relates to the removal of dragonfly nymphs from the water during hemolymph extraction, during which time they are unable to excrete CO_2. This may lead to an artificially elevated TCO_2 which would confound the measurements in this study. However, this is unlikely to be the case, as the nymphs that had been out in the air for 0.5 min did not have significantly different TCO_2 compared with those that were out in the air for 3.5 min (Table 2). Given that the actual extraction only lasted 1 min, the measured TCO_2 is unlikely to be artificially elevated. The marbled crayfish TCO_2 also suggests the protocol did not confound the measurements, as they were treated and sampled in the same way as the nymphs, and their TCO_2 values were both significantly lower than those of the dragonfly nymphs and also comparable to values from other freshwater crayfish such as the European crayfish *Astacus astacus* (Jensen, 1990).

Conclusions

Amphibiotic dragonflies show a significant increase in hemolymph TCO_2 as they transition from breathing water to breathing air. However, when compared with ancestrally aquatic animal groups that undergo dramatic increases in blood HCO_3^-, the increase in TCO_2 experienced by dragonflies is relatively small, suggesting that they have undergone a comparatively minor decrease in internal CO_2 content upon adapting their ancestrally air-breathing respiratory system to function underwater. Further research is needed in order to assess whether this is a trend that is common to all amphibiotic insect lineages.

Acknowledgements

We thank the University of British Columbia (UBC) Botanical Garden, UBC Museum of Anthropology and Dolph Schluter for allowing us to collect insects in and around their ponds, Ramandeep Ubhi for assisting with insect collection, and two anonymous reviewers for their helpful comments on the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.J.L., P.G.D.M.; Methodology: D.J.L., M.G., F.M.F., P.G.D.M.; Software: M.G., F.M.F.; Investigation: D.J.L.; Resources: M.G., F.M.F.; Writing - original draft: D.J.L.; Writing - review & editing: D.J.L., M.G., F.M.F., P.G.D.M.; Supervision: P.G.D.M.; Funding acquisition: P.G.D.M.

Funding

This work was supported by the Natural Sciences and Engineering Research Council of Canada [Discovery grant: RGPIN-2014-05794 to P.G.D.M., CSG-M to D.J.L.].

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