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The transition from water to air in aeshnid dragonflies is associated with a change in ventilatory responses to hypoxia and hypercapnia

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ABSTRACT

Dragonflies are amphibiotic, spending most of their lives as aquatic nymphs before metamorphosing into terrestrial, winged imagoes. Both the nymph and the adult use rhythmic abdominal pumping movements to ventilate their gas exchange systems: the nymph tidally ventilates its rectal gill with water, while the imago pumps air into its tracheal system through its abdominal spiracles. The transition from water to air is known to be associated with changes in both respiratory chemosensitivity and ventilatory control in vertebrates and crustaceans, but the changes experienced by amphibiotic insects have been poorly explored. In this study, dragonfly nymphs (*Anax junius*) and imagoes (*Anax junius* and *Aeshna multicolor*) were exposed to hypoxia and hypercapnia while their abdominal ventilation frequency and amplitude was recorded. Water-breathing nymphs showed a significant increase in abdominal pumping frequency when breathing hypoxic water (< 10 kPa O_2), but no strong response to CO_2, even in severe hypercapnia (up to 10 kPa CO_2). In contrast, both species of air-breathing imago increased their abdominal pumping amplitude when exposed to either hypoxia or hypercapnia, but did not show any significant increase in frequency. These results demonstrate that aquatic dragonfly nymphs possess a respiratory sensitivity that is more like other water breathing animals, being sensitive to hypoxia but not hypercapnia, while their air-breathing adult form responds to both respiratory challenges, like other terrestrial insects. Shifting from ventilating a rectal gill with water to ventilating a tracheal system with air is also associated with a change in how abdominal ventilation is controlled; nymphs regulate gas exchange by varying frequency while imagoes respond by varying amplitude.

1. Introduction

Amphibiotic animals that transition from water to air during their development must overcome the respiratory challenges associated with breathing two very different respiratory media. In vertebrates and crustaceans, this is achieved by transitioning from gills or cutaneous gas-exchange structures capable of extracting dissolved oxygen (O_2) directly from the surrounding water, to air-filled lungs that allow aerial gas exchange while limiting respiratory water loss (Dejours, 1981; Rahn, 1966). The physiochemical properties of air and water impose further constraints on gas exchange by differing widely in their density, viscosity, and capacity to act as a source of O_2 and sink for carbon dioxide (CO_2) (Dejours, 1988b). As a result, the aquatic life stage of an amphibiotic animal tends to be physiologically more like other obligate water-breathers (ventilation sensitive to aquatic hypoxia, low sensitivity to hypercapnia, low internal PCO_2) while the air-breathing adult is similar to other terrestrial air-breathing animals (sensitive to both hypoxia and hypercapnia, elevated internal PCO_2) (Dejours, 1988a; Randall et al., 1981). While the respiratory changes associated with the water-to-air transition of vertebrates and crustaceans have been studied extensively (Burggren and Doyle, 1986; Cameron and Mecklenburg, 1973; Dejours, 1988b; Randall et al., 1981; Torgerson et al., 1997), the physiological changes undergone by amphibiotic insects have been largely overlooked.

Hexapod insects are ancestrally an air-breathing group of animals (Pritchard et al., 1993) that have re-invaded the aquatic environment numerous times (Wootton, 1988). Because insects have secondarily returned to an aquatic existence, they can provide a unique perspective on the physiological constraints associated with adapting an air-breathing respiratory physiology to function in water (Graham, 1990). While some aquatic insects have remained air-breathers, relying on a bubble of air carried over their spiracles to act as an O_2 store or 'gas-gill' while submerged (Seymour and Matthews, 2013), many insects have also evolved the ability to breathe water directly. In all cases, the evolution of water-breathing is associated with an amphibiotic life cycle, where only the juvenile stage is aquatic, exchanging respiratory

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gases directly with the surrounding water across their cuticle, while the adult form reverts back to its ancestral air-breathing condition (Mill, 1974). While terrestrial insects are known to show a ventilatory response to both hypoxia and hypercapnia (Harrison et al., 2006; Harrison, 1997; Matthews and White, 2011), little is known about how the water-breathing juvenile life stages respond to these gases, and no study has followed the ventilatory responses of both the water-breathing and air-breathing life stages of a single amphibiotic insect species to hypoxia or hypercapnia.

The dragonflies (order Odonata, suborder Anisoptera) were among the very first insects to successfully divide their lifecycle between water and air, evolving an aquatic habit probably before the Permian period (Wootton, 1981, 1988). The adult dragonfly retains the functional spiracles and air-filled tracheal system common to all terrestrial insects, but the respiratory adaptations of the nymph revel it to be a water-breathing animal. Although the nymph still possesses spiracles and an air-filled tracheal system, their spiracles remain closed and non-functional for most of their development (Tillyard, 1915). In the absence of aerial gas exchange, the nymphs instead breathe water using their colon, the posterior section of which has become enlarged into a respiratory organ: the branchial basket or so-called rectal gill (Corbet, 1962; Tillyard, 1915, 1916). The gill is ventilated by rhythmic abdominal pumping movements that drive a tidal flow of water in and out through their anus (Hughes and Mill, 1966). All terrestrial dragonfly imagoes also display a continuous rhythmic pumping of their abdomen, ceasing this activity only briefly either before the start of flight or while actively struggling (Miller, 1962). This pumping is understood to move air in through their abdomen and out through their thoracic spiracles (Miller, 1962).

As both adult and juvenile dragonflies pump their abdomen as part of their ventilatory behaviour, they are an ideal candidate to investigate whether their sensitivity to O_2 and CO_2 changes as they move from water to air, and to examine if changing from a rectal gill to an open tracheal system alters how they regulate their ventilation. These questions were investigated by exposing aeshnid dragonfly nymphs and adults to environmental hypoxia and hypercapnia while measuring abdominal ventilation frequency and amplitude using video analysis.

2. Materials and methods

2.1. Animals

Dragonfly nymphs and adults were collected from around UBC's Point Grey campus and the UBC Botanical Gardens. Two dragonflies from the family Aeshnidae were used: *Anax junius* and *Aeshna multicolor*. *Anax junius* nymphs were collected during June 2015, while adult male *A. junius* and *Aeshna multicolor* dragonflies were caught between July and August 2017. The nymphs were collected from ponds using dip nets and were then transported to the lab and housed individually in 2 L glass jars connected to a recirculating filtration system at room temperature (20–23 °C). They were fed on a diet of frozen blood worms and a variety of locally caught aquatic invertebrates. Imagoes were netted on the wing, then immediately transferred into a 2 L glass jar that had been blacked-out using aluminium foil, and transported to the lab. A single imago was used each day, measured within ~2 h of capture on return to the lab.

2.2. Gas mixing

Hypoxic/normocapnic, and normoxic/hypocapnic gas blends were produced by combining N_2, O_2, and CO_2 from compressed cylinders of gas (Praxair, BC, Canada). Mixtures were created by metering the compressed gas through MC-50, MC-100, and MC-500SCCM-D mass flow controllers (CO_2, 0–50 $\mathrm{ml\,min^{-1}}$, O_2, 0–100 $\mathrm{ml\,min^{-1}}$, and N_2 0–500 $\mathrm{ml\,min^{-1}}$) that were controlled via a USB hub connected to a desktop PC running FlowVision MX gas mixing software (Alicat Scientific, AZ, USA). The total flow rate for these mixtures varied between 500 and 620 $\mathrm{ml\,min^{-1}}$. The flow rates (STPD) of all mass flow controllers were checked using a volumetric, positive displacement flow meter that had been calibrated using NIST standards (Bios DryCal Definer 220-L, Mesa Laboratories, Inc., CO, USA). All mass flow controllers produced flow rates that were between 0.9 and 2.8% above their set rate of flow, resulting in O_2 and CO_2 fractions that varied by a maximum of 0.2% below the percentage specified in the mixture. These gas mixtures were bubbled continuously through dechlorinated Vancouver tap water at room temperature (20–23 °C) in a 500 ml glass bottle using an air-stone. The humidified gas was then either flushed past an adult dragonfly in a closed chamber, or, in separate experiments, the gas-equilibrated water was pumped through a chamber containing a nymph.

In a separate series of measurements, the pH of the dechlorinated Vancouver tap water was measured following 25 min equilibration with the various PCO_2 levels used (0–10 kPa) using a SevenExcellence pH meter and InLab Expert Pro-ISM pH electrode (Mettler-Toledo Inc., Missisauga, ON, Canada) calibrated with standard pH buffers.

2.3. Nymph ventilation

Dragonfly nymphs were placed individually into a rectangular chamber (internal dimensions 75 × 20 × 18 mm) that was machined into the centre of a transparent Perspex block (Fig. 1). The back wall of the cavity was sealed with a plate of red transparent Perspex, while the removable front wall was clear. An inlet and outlet port at opposite ends of the cavity allowed water to be flushed through the chamber at 60 $\mathrm{ml\,min^{-1}}$ using a 12V 5000 RPM mini peristaltic pump (Adafruit, NY, USA). The peristaltic pump pulled gas-equilibrated water from the 500 ml glass bottle, through the chamber and back into the bottle in a continuous loop. Hypoxic (20, 15, 10, 5, 2.5, 1% O_2 with 0% CO_2, balance N_2) and hypercapnic (0, 0.5, 1, 2, 5, 7.5, 10% CO_2 with 21% O_2, balance N_2) gases were flushed through the water, in that order. A Fibox O_2 meter (Presens GmbH, Regensberg, Germany) connected to a fiber-optic sensor spot in the outflow from the chamber was used to monitor the dissolved O_2 level in the recirculating water. Nymphs were given 10–15 min to acclimate to a new gas mixture before being filmed for 3–4 min.

To record the abdominal pumping activity of a nymph, the nymphs were filmed side-on using a 60D DSLR camera in video mode with a EF-S 60 mm f/2.8 macro lens (Canon, Tokyo, Japan). The camera was positioned facing into the chamber, while the chamber itself was back-lit with red LEDs shining through a diffuser of white tissue paper. This produced a dark silhouette of the nymph on a red background (Fig. 1). Red light was chosen to illuminate the chamber as *Anax* nymphs are relatively insensitive to wavelengths longer than ~600 nm (Horridge, 1969; Sakamoto et al., 1998).The camera and chamber were then placed beneath a blacked out cardboard box to prevent the nymph from being disturbed while recording. Mean abdominal ventilation frequency was recorded by an observer watching the videos and counting the number of abdominal pumps in each video divided by the video duration. These values were subsequently checked against those obtained by video analysis using EthovisionXT ver. 9 (Noldus, Wagnenigen, The Netherlands). This software measured the projected profile area of the nymph (i.e., its silhouette) in each frame, allowing changes in area corresponding to the dorsoventral expansion and contraction of the abdomen to be measured for each frame. The dimensions of the frame were calibrated using the known 75 mm width of the chamber as a reference. This projected area data was then imported into LabChart software ver. 8 (ADInstruments Inc., Colorado Springs, CO, USA). The 1st order differential of the nymph's profile area was then calculated and analysed using the 'cyclic measurements' function configured in 'spike after threshold' mode to calculate mean abdominal pumping frequency. As the nymphs would often change position within the chamber, it was not possible to extract abdominal amplitude from the