Flow increases tolerance of heat and hypoxia of an aquatic insect

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.5416978.

1. Introduction

For aquatic insects, one of the primary challenges of living in water is balancing oxygen supply and demand [1–3]. Water contains substantially less oxygen compared to air and is dense and viscous, making respiratory ventilation difficult and energetically expensive [2,4,5]. This problem has profoundly shaped the physiology, morphology, distribution and behaviour of aquatic insects [1,6–11].

The oxygen problem also plays a central role in explaining the effects of another key aquatic variable—temperature. Upper thermal limits of many aquatic ectotherms appear to be caused by oxygen limitation [4,12–14], although there is controversy about the significance and magnitude of oxygen’s roles [15]. Although warmer temperatures modestly increase the supply of oxygen to aquatic insects by increasing the diffusion coefficient of oxygen and decreasing water viscosity [4,16–19], they also elevate organismal metabolic demand for oxygen. Consequently, demand for oxygen can exceed supply at high water temperatures [4,12–14,20–23] and in some cases underlie the decline or failure of organismal performance [24].

A key parameter that can influence oxygen supply, and therefore thermal tolerance, is flow. Flow and turbulence both promote gas exchange between water and atmospheric gases and, at the organismal scale, increase rates of oxygen delivery to insect respiratory surfaces (e.g. tracheal gills or thin sections of the cuticle) [12,25]. Specifically, by altering the thickness of boundary layers, i.e. layers of slow-moving fluid that envelop an insect’s body and gills [26], variation in flow can enhance or restrict oxygen delivery to organisms [12,27–30].

At the body surface, the flow velocity is zero, and velocity within the boundary layer increases steadily with distance from the body until it reaches the free stream velocity [31] (figure 1). The boundary layer typically is defined as the distance from the surface at which flow velocity reaches 90 or 95% of free stream velocity. In thick boundary layers, formed in slow-moving water, oxygen levels
adjacent to respiratory surfaces can become more depleted by metabolic demand than they do in thin boundary layers, which form in fast, turbulent flows [4,12,32,33]. We therefore expect that heat and hypoxia tolerance limits of organisms, which depend on temperature–oxygen interactions, should be strongly influenced by variation in water flow velocity [12,33–36]. Although previous studies have found support for this prediction [36–38], flow is rarely included in more recent studies on the tolerance limits of aquatic ectotherms, even though flow regimes are predicted to change significantly with climate change. A better understanding of how flows affect aquatic insect survival will be crucial to forecasting their responses to climate change [12].

Here, we measured the influence of flow on the thermal and hypoxia tolerance of giant salmonfly nymphs (Plecoptera, *Pteronarcys californica*) (figure 2a), a common stonefly in western North American rivers. We predicted that nymphs would better tolerate high temperatures and hypoxia in flowing than in standing water. We tested this prediction by measuring the onset of two behavioural stress responses in nymphs subjected to ramps of temperature (increasing) or oxygen (decreasing) with or without flow. The first measured stress response was the onset of ventilatory movements (push-ups, hereafter), which facilitate oxygen uptake in these stoneflies enabling them to prevent oxygen stress [10]. The second measured response was the loss of righting response (LRR; *sensu* [39]) which is often used when determining the critical thermal maximum (*CT*max; [40,41]).

## 2. Material and methods

### (a) Insect collection and acclimation

We collected medium-sized *Pteronarcys californica* nymphs (mean 0.60 g, range 0.098–1.38 g) using a screen barrier net (91 x 91 cm) in the autumns of 2018 and 2019 from lower Rock Creek, near Clinton, Montana (46.6980, −113.6695), a mid-sized tributary of the Clark Fork River in western Montana (elevation = 1100 m). Summertime mean (15.5 ± 2.2°C) and maximum (18.5°C) water temperature was calculated over 3 years (2016–2019) using July and August daily statistics from USGS hydrologic unit number 17 010 202 (46.7223, −113.6830). Using an oxygen optode system (PyroScience Model FireStingO2), we took point measurements of dissolved oxygen on 8 July 2019 in a nearby reach of Rock Creek. At this time, oxygen saturation was consistently above air saturation (mean = 103% ± 0.26%) in both riffles and pools. In the laboratory, nymphs were housed in aerated buckets of stream water and acclimated in incubators for one week (Percival Scientific I-66LLC8) at a similar temperature and photoperiod to that of Rock Creek at the time of collection, 4.5°C, and a 12 L : 12 D h cycle. *P. californica* are shredders, i.e. they feed on microbial colonies living on decaying leaves in streams. Thus, once per week, we collected decaying leaves and fed them to the captive stoneflies.

### (b) Experimental set-up

We examined the effects of flow on heat and hypoxia tolerances by subjecting nymphs to ramps of either temperature or oxygen with and without flow. Ramps were carried out in a custom-built Plexiglas flume (45 x 15 x 7.5 cm), partially submerged in a temperature-controlled water bath (VWR Scientific Model 1160A) (electronic supplementary material, figure S1) and fitted with a Plexiglas lid to minimize gas exchange with the surrounding air. We placed stonefly nymphs on one side of the flume, along with small, flat rocks for a substrate. The nymphs were positioned in a 20 cm arena between two stacks of 100 glued straws (each straw 5.5 cm long, 5 mm diameter glued 10 across and 10 tall, making a 6 x 6 cm honeycomb structure). The honeycomb was used to restrict stonefly movement and to create more laminar flow.

Flow was manipulated by turning on or off a small water pump (PULACO PL-118, 50 gal h⁻¹) situated on the opposite side of the flume. In preliminary experiments, we measured water velocities from video recordings of red dye injected into the experimental section of the flume at the surface of the substrate. The pump generated flow rates of approximately 10 cm s⁻¹, which is a typical flow velocity within stream cobbles (personal observation). When the pump was turned off, flow rates dropped to <0.1 cm s⁻¹ although water was stirred slightly by gas bubbling, which helped to homogenize conditions in the test section. In all cases, oxygen levels in the flume were controlled by bubbling room air (barometric pressure 93 kPa), or mixtures of air and nitrogen, through air pumps and aquarium air stones. During ramps, temperature and oxygen levels were sampled throughout the experiment (at 1 Hz) using a FireSting Probe (PyroScience FS02-2, 3 mm probe diameter, response time 21 s).

In each trial, a haphazardly chosen set of three *P. californica* nymphs were placed together in the 20 cm arena in the flume and monitored simultaneously. Nymphs were allowed to rest for 5 min before the temperature or oxygen ramps began. During each ramp, we monitored nymphs continuously and recorded the onset of two events—LRR, which occurred when nymphs lost the ability to right themselves after being gently placed on their backs using forceps [41] and push-ups, in which nymphs swayed vigorously from side to side (see electronic supplementary material, video). Each nymph was tested only once. At the end of each trial, the tested nymph was blotted dry and weighed.
To determine heat tolerance, we held dissolved oxygen at constant levels between 100% and 22% of air saturation while ramping temperature up from 7°C at 1.1°C min⁻¹ using a water heater (VWR Scientific Model 1160A). Trials were carried out either with (N = 21 individuals) or without (N = 21 individuals) flow, and we recorded temperatures at which nymphs initiated push-ups and displayed LRR.

To measure hypoxia tolerance, we held water temperatures constant at different values between 4 and 26°C while ramping dissolved oxygen down from 100% of air saturation by bubbling nitrogen gas into the flume. Ramps were repeated with (N = 25 individuals) and without flow (N = 21 individuals). Rates of oxygen depletion in the flume ranged from 1.9 to 6.65% of air saturation min⁻¹. Here too, hypoxia tolerance was recorded in two ways: the oxygen level at which nymphs started ventilatory push-ups and at LRR.

Analyses were done in R [42]. Preliminary analyses included mass as a covariate, but since it had no significant effect in any analysis (possibly due to the small size range of around one order of magnitude), we excluded it from the analyses presented below (electronic supplementary material, figure S2). To examine the effects of oxygen and flow on heat tolerance (figure 2b; electronic supplementary material, figure S3), we used linear mixed-effects models (implemented in the nlme package in R) [43] to model the temperatures at LRR and the initiation of push-ups as a function of oxygen level, flow and their interaction. Because experiments were conducted on up to three stoneflies at once, individual ramping trials were treated as a random effect. We performed a chi-squared test on the counts of initiated push-ups in the temperature ramping experiment (electronic supplementary material, figure S3).

To analyse the effects of temperature and flow on hypoxia limits characterized by LRR, which appeared to contain breakpoints, we used piecewise regression implemented in the R package segmented [44] (figure 2c). Piecewise regressions were
first fit to flow and no-flow datasets separately. To analyse the effects of flow, we then split both datasets (flow versus no flow) above and below their respective breakpoints and compared the combined data using linear mixed-effects models (again, treating individual ramping experiments as a random effect, with 1–3 stoneflies per trial) [43].

To examine the effect of temperature and flow on hypoxia limits characterized by the push-up response (figure 2d), we used a linear mixed-effects model (individual ramping experiments were treated as a random effect with 1–3 individuals per trial) [43] to model the dissolved oxygen levels at the onset of push-ups as a function of temperature, flow and their interaction.

To ensure that the differences in heat and hypoxia tolerances between flow and no-flow treatments were due to variation in flow and not an artefact of sampling year (flowing trials were conducted in 2018 while standing water trials took place in 2019), we performed another round of temperature ramping experiment at 100% oxygen saturation repeated in flowing and standing water performed another round of temperature ramping experiment at 100% oxygen saturation repeated in flowing and standing water with nymphs collected in 2021. The same methods were followed as in previous experiments, with six nymphs in each treatment. A t-test showed that $CT_{max}$ of nymphs in flow was significantly elevated. These findings are consistent with those of our prior experiments, supporting that the differences in heat and hypoxia tolerances of nymphs collected in 2018 and 2019 were due to variation in the flow instead of sampling year (electronic supplementary material, figure S4).

3. Results

(a) Temperature ramps

Nymphs tolerated higher temperatures when oxygen levels were higher ($F_{(1,10)} = 115.54, p < 0.0001$) (figure 2f; electronic supplementary material, table S1), and this effect was strongly modified by the flow. Across the range of oxygen levels, nymphs in flowing water tolerated temperatures that were approximately 4°C higher than did those in standing water ($F_{(1,10)} = 9.40, p = 0.0119$). By contrast, oxygen level did not affect the onset temperature of push-ups ($F_{(1,5)} = 2.368, p = 0.1845$), while the oxygen $\times$ flow interaction showed borderline significance ($F_{(1,5)} = 5.92, p = 0.0591$) (electronic supplementary material, table S1 and figure S3). Significantly fewer nymphs initiated push-ups in the flowing trials of the temperature ramping experiment ($N = 4$, at approx. $15^\circ C$), than in standing water, where 19 nymphs initiated push-ups (at approx. $7^\circ C$) ($X^2 = 9.78, p = 0.0017$).

(b) Oxygen ramps

Nymphs tolerated lower oxygen concentrations at lower temperatures, and those in flowing water tolerated 10–20% less dissolved oxygen than those in standing water (figure 2c). Separate piecewise regressions identified breakpoints of 14.99 ± 2.62°C (with flow, mean ± s.e.) and 11.47 ± 9.24°C (without flow). These breakpoints are similar to the recorded mean summertime high water temperatures in Rock Creek. To examine the effects of flow statistically, we analysed data from above and below the breakpoints separately. In both cases, flow was highly significant (below breakpoints: $F_{(1,123)} = 307.88, p < 0.0001$; above breakpoints: $F_{(1,10)} = 15.75, p = 0.0026$; figure 2c; electronic supplementary material, table S1). The temperature $\times$ flow interaction was significant only below the temperature breakpoints ($F_{(1,8)} = 10.1, p = 0.0191$; figure 2c; electronic supplementary material, table S1). Nymphs initiated push-ups at higher dissolved oxygen levels when exposed to high temperatures ($F_{(1,15)} = 22.29, p = 0.0003$) (figure 2d), and this response was also modified by flow ($F_{(1,19)} = 27.40, p < 0.0001$). Specifically, nymphs initiated push-ups at higher levels of oxygen (by about 30% of air saturation) in standing compared to flowing water across temperatures.

4. Discussion

Flow profoundly influenced the ability of stonefly nymphs to tolerate high temperatures and hypoxia. These effects likely arose from the influence of flow on oxygen transport. In faster flows, boundary layers are thinner and pose less of a barrier to oxygen transport from the environment to the respiratory surfaces (figure 1). In turn, faster oxygen transport across thinner boundary layers supports higher metabolic demand arising at higher temperatures and can support ongoing activity in deeper layers of environmental hypoxia found at high elevation or in eutrophic conditions [12, 45, 46]. In the ongoing discussions about the mechanistic causes of upper thermal limits in aquatic systems [12, 15], our results support the idea that oxygen is important and suggest that renewed attention should be paid to oxygen transport in relation to physical aspects of flow.

Critical limits are a widely used metric for assessing the vulnerability of organisms to climate change. Yet, methodology strongly affects outcomes; for example, fast ramping rates or high start temperatures can result in higher $CT_{max}$ estimates [47]. Our study highlights the importance of flow in influencing thermal limits and suggests that higher flows may raise estimated values of $CT_{max}$. We thus propose that studies of thermal limits should routinely measure and report flow conditions and that a more nuanced understanding of vulnerability to temperature extremes will depend on varying flow experimentally. Ideally, flow regimes during $CT_{max}$ experiments should span the range of flows experienced naturally by the study organism.

The results of our study have important ecological implications. Climate change and anthropogenic disturbances are causing many streams and rivers to warm, to become more hypoxic and to show greater overall variability in flow, resulting in periods of low flow that may coincide with warm conditions during dry summers [48–52]. Although water temperature and dissolved oxygen receive much attention, flows vary more in time and space, with strong effects on aquatic insects [45]. For example, flow directly affects the size and shape of individual macroinvertebrates [53], where they choose to position themselves [54, 55], how readily they can find and consume food [56] and whether they can obtain sufficient oxygen [29, 30, 33, 57, 58]. Variation in flow also influences other characteristics of stream habitats relevant to aquatic insects, including temperature, chemistry and rates of sedimentation [59–61]. Thus, long-term shifts in flow regimes will have systemic effects on stream and river ecosystems [62]. In temperate streams, lower seasonal flows and warmer water temperatures have become increasingly frequent [49, 63] primarily because the annual snowpack is decreasing and drought severity is increasing in many regions [45, 64]. Our results, in particular, suggest that low flows will magnify the climatic risks of oxygen limitation stemming from previously recognized interactions between temperature and oxygen [12, 45, 65].

Ethics. We followed the University of Montana guidelines for working with invertebrates.
Data accessibility. Data and the corresponding R script have been made publicly available from the Dryad Digital Repository at: https://doi.org/10.5061/dryad.mgqnk98x6d [66]. The data are provided in the electronic supplementary material [67].

Authors’ contributions. J.J.F., J.H.B. and H.A.W. conceived and designed experiments; J.J.F. and J.H.B. conducted the experiments; J.J.F., A.A.S. and H.A.W. analysed the results, and J.J.F. and J.H.B. wrote the manuscript with contributions from A.A.S. and H.A.W. All authors agree to be held accountable for the content of our findings and have approved the final version on the manuscript.

Competing interests. We declare we have no competing interests.

Funding. A.A.S. was supported by a National Science Foundation Postdoctoral Fellowship (DBI-1807694).

Acknowledgements. We are grateful to Bret Tobalske for providing the flume for experiments, to Sean Kellogg and Reed Traynor for helping to collect insects, and to C. Riley Nelson for the image of a salmonfly nymph used in figure 1. We thank Wilco Verberk for comments on a draft and two anonymous referees for comments on the manuscript.

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