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HOW AQUATIC INSECTS MITIGATE TEMPERATURE-OXYGEN CHALLENGES VIA BEHAVIORAL, MORPHOLOGICAL, AND PHYSIOLOGICAL PLASTICITY

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How aquatic insects mitigate temperature-oxygen challenges via behavioral, morphological, and physiological plasticity

Chairperson: Dr. H. Arthur Woods

Abstract

How do organisms respond to environmental challenges and to environmental change? These questions occupy a central place in ecology and answering them will help us to understand why species live where they do, how organisms are affected by human activities, and, ultimately, how to choose among alternative conservation strategies. These questions are difficult, however, for two reasons. First, environmental challenges often involve multiple, interacting stressors. Second, individual responses can be modified by behavioral, morphological, and physiological plasticity. My dissertation investigates how interactions between temperature and oxygen influence the performance and survival of aquatic insects and how plasticity allows individuals to mitigate temperature-oxygen challenges.

Understanding temperature-oxygen interactions is important for aquatic insects because, in water, oxygen availability is very low (compared to in air). This oxygen problem can be exacerbated by warming because rising temperatures cause metabolic demand for oxygen to increase exponentially. Risings temperature can thus cause oxygen demand to surpass supply, depressing performance and survival. Yet, how well nymphs can mitigate the effects of temperature-oxygen challenges via plasticity remains poorly understood.

In **chapter one**, I demonstrate the importance of 'the oxygen problem' for aquatic insects by showing that tissue oxygen levels are far lower in aquatic than terrestrial insects. Results suggest that levels of internal oxygen are actively regulated by aquatic insects to establish stronger oxygen gradients and higher rates of oxygen flux, necessary for living in water. In **chapter two**, I present a literature review that examines how climate change threatens insects in high-elevation streams. Overall, the outlook is bleak due to both discrete and interacting challenges from warming temperatures, shifting flow regimes, and increasing levels of ultraviolet radiation and salinity. However, populations may also cope with changing conditions via plasticity and local adaptation. In chapters three and four, I use laboratory experiments to investigate the capacity of aquatic insects to mitigate temperature-oxygen challenges via plasticity. In **chapter three**, I presented aquatic insects with gradients in temperature, oxygen, and flow and measured how nymphs relocated among them. Nymphs readily moved to microclimates with higher flows when ambient conditions depressed oxygen availability or increased oxygen demand. In **chapter four**, I exposed nymphs to long-term normoxic or hypoxic temperature ramps, with ecologically relevant ramping rates and diel thermal variation and measured nymph performance, survival, and morphological and physiological plasticity. Overall, both temperature and exposure duration, but not oxygen, affected the long-term performance and survival of nymphs. Individuals acclimated strongly throughout the long-term ramp, however, allowing them to mitigate the effects of exposure duration and to cope better with chronically hot and hypoxic conditions. **Collectively**, my research shows that while temperature-oxygen interactions can reduce the survival of aquatic insects under some scenarios, plasticity allows nymphs to strongly mitigate their effects. Indeed, because of plasticity, individuals may be more resilient to immediate consequences of oxygen-limitation than previously recognized.

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Introduction

Life on earth is challenging. To survive, organisms must cope with significant environmental stressors, from extreme-heat in tropical deserts to chaotic flows in marine intertidal zones (e.g., Denny et al. 1985; Shi et al. 2015). Even the basic ingredients of life – oxygen, sunlight, water – are harmful if not provided in optimal doses (e.g., Zorov et al. 2014). Human themselves often present additional challenges by altering conditions by amounts or at paces to which organisms are unaccustomed. One of the fundamental goals of ecology is thus to understand how environmental challenges shape the lives of organisms. How does the environment affect the survival and distribution of species? And what capacities do individuals have to mitigate challenges that overwise would kill them? Answering these questions can help us understand why species live where they do, how organisms are affected by human disturbances, and how to prioritize conservation strategies to protect biodiversity (Helmuth et al. 2005).

Accurately predicting the effects of environmental challenges is difficult, however, for two reasons. First, 'the environment' rarely, if ever, represents a single, isolated stressor (Sinclair et al. 2016). Instead, environmental challenges are complex, and often involve multiple, interacting challenges (Todgham & Stillman 2013). Organisms in tropical deserts, for example, may need to endure both heat and drought; those in marine intertidal zones may need to endure strong waves and also rapid changes in temperature and humidity. Humans too rarely disturb environments in univariate ways. Climate change, for example, is not only a temperature problem – because it also alters patterns of precipitation, ocean currents, sun exposure, seasonality, and more (IPCC 2018). Second, individual responses to environmental challenges can be mediated at different temporal scales by behavioral (seconds to hours) and physiological and morphological plasticity (days to months). Indeed, plasticity can provide powerful opportunities for individuals to escape or modify local conditions (e.g., moving among or altering local microclimates) or to increase tolerance limits (e.g., acclimation) (Angilletta 2009). Nevertheless, studies of interacting challenges are rare, especially those that provide organisms the opportunity to mitigate challenges via plasticity during experiments.

Temperature-oxygen problems

In this dissertation, I investigate how interactions between temperature and oxygen influence the performance and survival of aquatic insects, and how plasticity allows individuals to mitigate temperature-oxygen challenges. Understanding temperature-oxygen interactions is especially salient for aquatic insects because life in water is shaped by the scarcity of oxygen (Hynes 1970). Oxygen is essential for nearly all animals because it facilitates efficient energy production through aerobic cellular respiration. In water, however, oxygen availability is low, and costs of ventilating respiratory surfaces are high (compared to in air) (Verberk & Bilton 2013; Verberk et al. 2016a). This is because water imposes high diffusion coefficients, holds relatively little oxygen (20-30 times less than air), and generates thick boundary layers (i.e., layers of slow-moving fluid that 'stick' to submerged solids) around respiratory surfaces due to its high dynamic viscosity (Denny 1993; Verberk & Atkinson 2013; Woods and Moran 2020). Indeed, low oxygen availability is reflected in the many behavioral, morphological, and physiological adaptations of aquatic insects to facilitate gas exchange (Lancaster & Downes 2013).

Oxygen availability in water can be lowered even further, however, when oxygen concentrations or local flow velocities are low (Verberk et al. 2011). Strong hypoxia is relatively rare in clean, cold-water systems, yet can occur (particularly at night) when large nutrient inputs lead to increased biomass and, hence, oxygen consumption of benthic and pelagic communities (i.e., eutrophication) (Allan & Castillo 2007). In addition, low flows (and low turbulence) decrease gas exchange between atmosphere and water, which can further depress oxygen concentrations (Denny 1993). At the organismal scale, they also reduce rates of oxygen flux to respiratory surfaces by increasing boundary layer thicknesses (Verberk et al. 2011). In thick boundary layers, oxygen levels adjacent to respiratory surfaces are readily depressed by metabolic demand because oxygen diffuses too slowly to replenish thick, slow-moving boundary layers (Pinder & Feder 1990).

In addition, the low-oxygen problem of water can be exacerbated via interactions with temperature (Harrison et al. 2018). In isolation, rising temperatures threaten insects by rapidly increasing biological rates and demand for resources, altering body sizes and rates of development, and, under extreme warming, denaturing proteins and cell membranes (Harrison et al. 2012). However, warming temperatures can also result in oxygen limitation by causing metabolic demand for oxygen to rise exponentially, such that rates of oxygen demand surpass rates of supply (Woods 1999; Jacobsen et al. 2003; Verberk et al. 2011). This can lead to decrements in aerobic scope and performance and may underlie upper thermal limits, range limits, and vulnerabilities of aquatic ectotherms across diverse taxonomic groups (Pörtner & Knust 2007; Deutsch et al. 2015; Verberk et al. 2016a,b; Rubalcaba et al. 2020).

However, substantial evidence shows that temperature-induced oxygen-limitation may be important primarily during exposure to acute, extreme temperatures (Brijs et al. 2015; Ern et al. 2016; Verberk et al. 2016a; Kim et al. 2017, Jutfelt et al. 2017). Indeed, individuals have been shown to overcome short-term oxygen limitation by altering behaviors, morphologies, and underlying physiological systems (Verberk & Bilton 2013; Malison et al. 2022). However, there are few current examples from the literature, and we still understand little about the mechanisms and capacities of aquatic insects to solve temperature-oxygen problems. How well individuals adjust to long-term temperature-oxygen problems also is poorly known. Additional experiments are thus needed to understand how strongly interactions among temperature and oxygen drive patterns of performance and survival under conditions that allow plasticity.

Filling these knowledge gaps is particularly important for aquatic insects, as their habitats are undergoing widespread warming and decreasing oxygen availability from the combined effects of climate change, de-watering, river damming, and eutrophication (Lancaster & Downes 2016). In addition, many aquatic insects, such as stoneflies, mayflies, and caddisflies are especially sensitive to human disturbance (Merritt et al, 2008). Yet, they remain understudied (Strayer 2006), and we simply do not understand yet how they will respond to rising temperatures and falling oxygen availabilities (Verberk et al 2016a). Filling these gaps will aid in forecasting their responses to human disturbances and for prioritizing conservation strategies (Helmuth et al. 2005; Sinclair 2016). Indeed, the conservation of aquatic insects is particularly important because they represent the majority of aquatic biodiversity and constitute the core of stream and river food webs (Merritt et al. 2008; Dijkstra et al. 2013). They also provide numerous ecosystem services and sustain economically important recreational and commercial fishing industries (Macadam & Stockan 2015).

Chapter summaries

In chapter one, I investigate how tissue oxygen levels of insects from aquatic and terrestrial ecosystems reflect oxygen challenges from their respective environments and life history characteristics. Because oxygen is necessary for aerobic cellular respiration, yet also creates harmful reactive oxygen species (ROS) as a byproduct of respiration, animals appear to balance risks of asphyxiation and ROS by regulating internal oxygen levels to low levels (Hetz & Bradley 2005). I predicted that internal oxygen levels should occur in insects with either limited access to environmental oxygen (i.e., aquatic insects, with low internal levels facilitating diffusive oxygen uptake by creating stronger environment-tissue oxygen gradients, reducing asphyxiation), low metabolic rates (i.e., inactive insects, requiring limited internal oxygen stores), or long life-stage durations (reducing the toxicity from ROS production). I tested these predictions by measuring oxygen partial pressures (PO_2) in tissues of aquatic and terrestrial juveniles and adults from 15 species comprising nine orders. Tissue PO_2 was highly variable (from 0 to18.8 kPa), and this variation was significantly related to differences in insect habitat, life stage, activity level, and life stage duration. Aquatic juveniles consistently had remarkably low PO_2 (mean = 0.88 kPa), which appears to reflect active regulation, as levels remained low in living but not dead juvenile stoneflies exposed to hyperoxia. These results reflect the importance of 'the oxygen problem' for aquatic insects and suggest that low internal PO_2 may be a universal, necessary trait for aquatic insects to establish sufficiently strong oxygen gradients and higher rates of oxygen flux, necessary for living in water. Tissue PO_2 was also positively related to activity levels of insect life stages across all species and was lowest in stages with long durations, suggesting that internal PO_2 levels also reflect relative levels of oxygen demand and the need to avoid ROS across long stage durations among insects. These results suggest that internal PO_2 is an evolutionarily labile trait that reflects the balance between oxygen supply and demand within the context of the life-history of an insect.

In chapter two, I present a literature review that examines how climate change threatens aquatic insects in high-elevation streams and how insects may respond to future challenges. High-elevation streams are one of the most extreme ecosystems on earth. They are often covered by snow and ice for prolonged periods, and they tend to be heavily fragmented and isolated, with cold, fasting-flowing water, low salinity, low oxygen availability, and high levels of ultraviolet radiation (Jacobsen & Dangles 2017). Despite their harshness, high-elevation streams support high insect biodiversity (Hotaling et al., 2017). Climate change is, however, rapidly altering conditions in high-elevation streams, with largely unknown effects on aquatic insects. My review focuses on three key topics: 1) how current and future multivariate challenges and interactions among challenges affect aquatic insects, 2) how abiotic stressors influence the long-term performance of individual insects, 3) the capacity of aquatic insects to mitigate future challenges via plasticity and adaptation. Overall, the outlook is bleak due to both individual and interacting challenges from warming temperatures, shifting flow regimes, and increasing levels of ultraviolet radiation and salinity. However, populations may also cope with changing conditions via strong behavioral, morphological, and physiological plasticity and via local adaptation. At present, however, current knowledge gaps impede accurate predictions.

In chapters three and four, I begin to fill these knowledge gaps by investigating the capacity of aquatic insect nymphs to mitigate temperature-oxygen challenges in laboratory experiments via behavioral, morphological, and physiological plasticity. In my experiments, I used nymphs of a large stonefly, *Pteronarcys californica* – the giant salmonfly – as a study species.

P. californica is an ideal study species because they are abundant in western Montana and are easy to house and feed in the laboratory. *P. californica* is one of the largest stoneflies in the world and is native to Western North America. They inhabit medium to large mountain streams with cold, fast-flowing water and large, unconsolidated cobbles. As nymphs, *P. californica* help cycle in-stream nutrients by shredding leaves (Merritt et al. 2008). They also make up a substantial component of the diets of economically important fish, like trout, especially during their large, synchronous adult emergences (Nehring et al. 2011). For this reason, *P. californica* emergences are followed closely by anglers. Adult emergences also transfer large pulses of carbon to terrestrial ecosystems, supporting riparian insectivores (Walters et al. 2018). Like many other aquatic insects, however, *P. californica* is also sensitive to environmental change (Barbour et al. 1999). They are declining across much on the northern Rockies, yet little is known about why (Anderson et al. 2019; Birrell et al. 2019). New knowledge about how *P. californica* respond to temperature-oxygen problems will, thus, be useful for predicting responses of this and other sensitive stream insects to future disturbances and for understanding the mechanisms of aquatic insect distributions and declines.

In chapter three, I investigate the capacity of *P. californica* nymphs to mitigate oxygen limitation via behavioral plasticity – i.e., by moving among local microclimates. Whether stream insects can make meaningful choices, however, depends on: i) how the abiotic conditions that influence organismal demand and supply of oxygen (i.e., temperature and oxygen levels and flows, respectively) vary at microspatial scales and ii) the ability of insects to sense and exploit that variation. I first measured microspatial variation in temperature, oxygen saturation, and flow velocity within riffles of two local streams in Montana (USA). In the lab, I then examined preferences of *P. californica* nymphs to experimental gradients based on values measured in the field. Temperature and oxygen level varied little within stream riffles. In contrast, flow velocity varied strongly – often by more than 125 cm/s within riffles and 44 cm/s around individual cobbles. Exploiting micro-variation in flow is, thus, likely the most reliable option by which aquatic insects can alter rates of oxygen transport. In support of this prediction, the laboratory experiments revealed that *P. californica* nymphs weakly exploit gradients in temperature and oxygen but readily exploit micro-variation in flow – they consistently moved to higher flows when exposed to were warmer temperatures, hypoxia, or slow flows. Overall, these data show that *P. californica* can use behavior (local movements) to exert relatively strong control over functional oxygen availability. If such behaviors are shared widely among other species (e.g., Kovalak 1976; Wiley & Kohler 1980), they may facilitate survival, despite rising temperatures and decreased flows and oxygen levels from climate change and other anthropogenic disturbances.

In chapter four, I investigate the capacity of *P. californica* nymphs to mitigate long-term exposure to rising temperatures and variable oxygen levels via morphological and physiological plasticity. Mechanisms driving responses to long-term, multivariate challenges have been rarely studied together, and expectations about ectotherm responses have been unclear (Sinclair et al. 2016). Indeed, while exposure duration is often hypothesized to *lower* long-term thermal tolerances by causing prolonged sublethal stress (Rezende et al. 2014), individuals can also *raise* their thermal limits via plasticity (Angilletta 2009). Whether plasticity can overcome the effects of duration, however, likely depends on patterns of thermal variation. Most experiments measuring insect responses to temperature and oxygen expose organisms to constant-stressful conditions or fast ramping rates (e.g., Rezende et al. 2014). However, such thermal regimes may not allow enough time, or provide appropriate conditions, for individuals to adjust via morphological or physiological plasticity. In contrast, slow ramping rates and diel thermal variation, which are common in nature, may facilitate plasticity by providing opportunities to experience successively higher temperatures along with chances to recover during relatively cool nights. In this chapter, I examine how the performance and survival of *P. californica* nymphs depend on interactions between warming temperatures and variable oxygen availabilities and how responses are mediated by plasticity under long-term, ecologically relevant thermal regimes.

I did this by exposing nymphs to higher temperatures over three months at warming rates similar to those in nature, with realistic levels of diel thermal variation in both normoxia and hypoxia. I measured feeding, growth, molting, and survival, as well as changes in gill morphology and upper critical thermal limits. Overall, temperature and exposure duration, but not oxygen, had a significant effect on the performance and survival of nymphs. Nymphs were, however, remarkably resilient, tolerating chronically high temperatures more than 10 °C above typical stream temperatures in both normoxic and hypoxic treatments. High long-term thermal limits and a weak effect of hypoxia likely arose via plasticity, as the length of gill filaments and upper thermal maxima of nymphs increased after long-term exposure to warming and hypoxia. Overall, this study shows that, in ecologically realistic warming scenarios, plasticity may overshadow the negative effects of duration and temperature-induced oxygen-limitation. Further, it suggests that in ectotherms with strong acclimation abilities, temperature may only rarely kill organisms outright, indicating that long-term decrements in performance may be the most important mechanism of mortality under natural thermal regimes.

Collectively, my research shows that while temperature-oxygen interactions can reduce the survival of aquatic insects under some scenarios, plasticity allows nymphs to strongly mitigate their effects. Indeed, given conditions that facilitate plasticity, individuals may be more tolerant of immediate temperature-oxygen challenges than previously recognized. Long-term, sublethal effects of temperature-oxygen stress maybe more important and deserve more attention in future studies.

References

Allan, D. J. and Castillo, M. M. (2007). *Stream ecology: structure and function of running waters*. Dordrecht: Springer Science & Business Media.

Anderson, H. E., Albertson, L. K. and Walters, D. M. (2019). Water temperature drives variability in salmonfly abundance, emergence timing, and body size. *River Res. Appl.* 35, 1013–1022.

Angilletta Jr., M. J. (2009). *Thermal Adaptation: a theoretical and empirical synthesis*. New York: Oxford University Press.

Arthur Woods, H. and Moran, A. L. (2020). Reconsidering the oxygen-temperature hypothesis of polar gigantism: Successes, failures, and nuance. *Integr. Comp. Biol.* 60, 1438–1453.

Barbour, M. T., Faulkner, C. and Gerritsen, J. (1991). Rapid Bioassessment Protocols For Use in Streams and Wadeable Rivers: Periphyton, Benthic Macroinvertebrates, and Fish Second Edition http://www.epa.gov/OWOW/monitoring/techmon.html By: Project Officer. *Georg. Gibson (ed). Biol. criteria Res. Regul. Proc. a Symp. (December 1213, 1990 Arlington, Virginia).* 174.

Birrell, J. H., Meek, J. B. and Nelson, C. R. (2019). Decline of the Giant Salmonfly Pteronarcys Californica Newport , 1848 (Plecoptera : Pteronarcyidae) in the Provo River, Utah, USA. *Illiesia* 15, 83–97.

Brijs, J., Jutfelt, F., Clark, T. D., Gräns, A., Ekström, A. and Sandblom, E. (2015). Experimental manipulations of tissue oxygen supply do not affect warming tolerance of European perch. *J. Exp. Biol.* 218, 2448–2454.

Denny, M. W. (1993). *Air and water: the biology and physics of life's media*. Princeton: Princeton University Press.

Denny, M. W., Daniel, T. L. and Koehl, M. A. R. (1985). Mechanical Limits to Size in Wave-Swept Organisms. *Ecol. Monogr.* 55, 69–102.

Deutsch, C., Ferrel, A., Seibel, B., Portner, H.-O. and Huey, R. B. (2015). Climate change tightens a metabolic constraint on marine habitats. *Science (80-.).* 348, 1132–1135.

Dijkstra, K.-D. B., Monaghan, M. T. and Pauls, S. U. (2013). Freshwater Biodiversity and Aquatic Insect Diversification. *Annu. Rev. Entomol.* 59, 143–163.

Ern, R., Norin, T., Gamperl, A. K. and Esbaugh, A. J. (2016). Oxygen dependence of upper thermal limits in fishes. *J. Exp. Biol.* 219, 3376–3383.

Harrison, J. F., Greenlee, K. J., Verberk, W. C. E. P., Frazier, M. R., Henry, J. R., Kaiser, A., Klok, C. J., Rascón, B., Greenlee, K. J. and Verberk, W. C. E. P. (2018). Functional Hypoxia in Insects: Definition, Assessment, and Consequences for Physiology, Ecology, and Evolution. *Annu. Rev. Entomol.* 63, 303–325.

Harrison, J. F., Woods, H. A., and Roberts, S. P. (2012). Ecological and environmental physiology of insects. Oxford: Oxford University Press.

Helmuth, B., Kingsolver, J. G. and Carrington, E. (2005). Biophysics, physiological ecology, and climate change: does mechanism matter? *Annu. Rev. Physiol.* 67, 177–201.

Hetz, S. K. and Bradley, T. J. (2005). Insects breathe discontinuously to avoid oxygen toxicity. *Nature* 433, 516–519.

Hotaling, S., Finn, D. S., Joseph Giersch, J., Weisrock, D. W. and Jacobsen, D. (2017). Climate change and alpine stream biology : progress , challenges , and opportunities for the future. *Biol. Rev.* 92, 2024–2045.

Hynes, H. B. N. (1970). *The Ecology of Running Waters*. Liverpool: Liverpool University Press.

IPCC (2018). Summary for Policymakers. In *Global Warming of 1.5ºC. An IPCC Special Report on the impacts of global warming of 1.5ºC above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change,* (ed. Masson-Delmotte, V.), Zhai, P.), Pörtner, H.), Roberts, D.), Skea, J.), Shukla, P.), Pirani, A.), Moufouma-Okia, W.), Péan, C.), Pidcock, R.), et al.), p. Geneva, Switzerland: World Meteorological Organization.

Jacobsen, D. and Dangles, O. (2017). *Ecology of High Altitude Waters*. Oxford: Oxford University Press.

Jacobsen, D., Rostgaard, S. and Vásconez, J. J. (2003). Are macroinvertebrates in high altitude streams affected by oxygen deficiency? *Freshw. Biol.* 48, 2025–2032.

Jutfelt, F., Norin, T., Ern, R., Overgaard, J., Wang, T., McKenzie, D. J., Lefevre, S., Nilsson, G. E., Metcalfe, N. B., Hickey, A. J. R., et al. (2018). Oxygen- and capacity-limited thermal tolerance: Blurring ecology and physiology. *J. Exp. Biol.* 221, 2016–2019.

Kim, K. S., Chou, H., Funk, D. H., Jackson, J. K., Sweeney, B. W. and Buchwalter, D. B. (2017). Physiological responses to short-term thermal stress in mayfly (Neocloeon triangulifer) larvae in relation to upper thermal limits. *J. Exp. Biol.* 220, 2598–2605.

Kovalak, W. P. (1976). Seasonal and diel changes in the positioning of Glossosoma nigrior Banks (Trichoptera: Glossosomatidae) on artificial substrates. *Can. J. Zool.* 54, 1585–1594.

Lancaster, J. and Downes, Barbara, J. (2013). *Aquatic Entomology*. Oxford: Oxford University Press.

Macadam, C. R. and Stockan, J. A. (2015). More than just fish food: Ecosystem services provided by freshwater insects. *Ecol. Entomol.* 40, 113–123.

Malison, R. L., Frakes, J. I., Andreas, A. L., Keller, P. R., Hamant, E., Shah, A. A. and Woods, H. A. (2022). Plasticity of salmonfly (Pteronarcys californica) respiratory phenotypes in response to changes in temperature and oxygen. *J. Exp. Biol.* 225, 1–11.

Merritt, R. W., Cummins, K. W. and Berg, M. B. (2008). *An introduction to the aquatic insects of North America. Kendall Hunt, 1996.* Kendall.

Nehring, R. B., Heinold, B. and Pomeranz, J. (2011). Colorado River Aquatic Resources Investigations Federal Aid Project F-237R-18. *Color. Div. Wildl.*

Pinder, A. W. and Feder, M. E. (1990). Effect of boundary layers on cutaneous gas exchange. *J. Exp. Biol.* 154, 67–80.

Pörtner, H. O. and Knust, R. (2007). Climate Change Affects Marine Fishes Through the Oxygen Limitation of Thermal Tolerance. *Science (80-.).* 315, 95–97.

Rezende, E. L., Castañeda, L. E. and Santos, M. (2014). Tolerance landscapes in thermal ecology. *Funct. Ecol.* 28, 799–809.

Rubalcaba, J. G., Verberk, W. C. E. P., Jan Hendriks, A., Saris, B. and Arthur Woods, H. (2020). Oxygen limitation may affect the temperature and size dependence of metabolism in aquatic ectotherms. *Proc. Natl. Acad. Sci. U. S. A.* 117, 31963–31968.

Shi, N. N., Tsai, C.-C., Camino, F., Bernard, G. D., Yu, N. and Wehner, R. (2015). Keeping cool: Enhanced optical reflection and radiative heat dissipation in Saharan silver ants. *Science (80-.).* 349, 298–301.

Sinclair, B. J., Marshall, K. E., Sewell, M. A., Levesque, D. L., Willett, C. S., Slotsbo, S., Dong, Y., Harley, C. D. G., Marshall, D. J., Helmuth, B. S., et al. (2016). Can we predict ectotherm responses to climate change using thermal performance curves and body temperatures? *Ecol. Lett.* 19, 1372–1385.

Strayer, D. L. (2006). Challenges for freshwater invertebrate conservation. *J. North Am. Benthol. Soc.* 25, 271–287.

Todgham, A. E. and Stillman, J. H. (2013). Physiological Responses to Shifts in Multiple Environmental Stressors: Relevance in a Changing World. *Integr. Comp. Biol.* 53, 539–544.

Verberk, W. C. E. P. and Atkinson, D. (2013). Why polar gigantism and Palaeozoic gigantism are not equivalent: Effects of oxygen and temperature on the body size of ectotherms. *Funct. Ecol.* 27, 1275–1285.

Verberk, W. C. E. P. and Bilton, D. T. (2013). Respiratory control in aquatic insects dictates their vulnerability to global warming. *Biol. Lett.* 9, 0–3.

Verberk, W. C. E. P., Bilton, D. T., Calosi, P. and Spicer, J. I. (2011). Oxygen supply in aquatic ectotherms: Partial pressure and solubility together explain biodiversity and size patterns. *Ecology* 92, 1565–1572.

Verberk, W. C. E. P., Durance, I., Vaughan, I. P. and Ormerod, S. J. (2016). Field and laboratory studies reveal interacting effects of stream oxygenation and warming on aquatic ectotherms. *Glob. Chang. Biol.* 22, 1769–1778.

Verberk, W. C. E. P., Overgaard, J., Ern, R., Bayley, M., Wang, T., Boardman, L. and Terblanche, J. S. (2016a). Does oxygen limit thermal tolerance in arthropods? A critical review of current evidence. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 192, 64–78.

Walters, D. M., Wesner, J. S., Zuellig, R. E., Kowalski, D. A. and Kondratieff, M. C. (2018). Holy flux: spatial and temporal variation in massive pulses of emerging insect biomass from western U.S. rivers. *Ecology* 99, 238–240.

Wiley, M. J. and Kohler, S. L. (1980). Positioning changes of mayfly nymphs due to behavioral regulation of oxygen consumption. *Can. J. Zool.* 58, 618–622.

Woods, H. A. (1999). Egg-mass size and cell size: Effects of temperature on oxygen distribution. *Am. Zool.* 39, 244–252.

Zorov, D. B., Juhaszova, M. and Sollott, S. J. (2014). Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiol. Rev.* 94, 909–950.

Chapter 1

Remarkably low tissue oxygen levels in aquatic insects reflect a balance between toxicity and asphyxiation

Tissue oxygen levels in insects reflect a balance between toxicity and asphyxiation

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Abstract

Animals 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 to low 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 animals with either limited access to environmental oxygen (i.e., aquatic animals, with low internal levels facilitating diffusive oxygen uptake, reducing asphyxiation), consistently low metabolic rates (i.e., inactive animals, requiring limited internal oxygen stores), or long life-stage durations (reducing the toxicity from ROS production). We tested these predictions by measuring partial pressures of oxygen (PO_2) in tissues from aquatic and terrestrial insects. We measured juvenile and adult stages from 15 species comprising nine insect orders. Tissue PO_2 varied greatly (from 0 to18.8 kPa) and this variation was significantly related to differences in habitat, life stage, activity level, and life stage duration. Aquatic juveniles sustained remarkably low PO_2 (mean = 0.88 kPa), which appears to reflect active regulation, as levels remained low in living but not dead juvenile stoneflies exposed to hyperoxia. Tissue PO_2 was positively related to activity levels of insect life stages across all species and was highest in stages with short durations. These results suggest that internal PO_2 is an evolutionarily labile trait that reflects the balance between oxygen supply and demand within the context of the 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 from most other molecules. 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. 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 just enough is supplied to meet the oxygen demand of the mitochondria. Thus, PO_2 tend to decrease across the steps of the oxygen transport cascade from ambient (often ~21 kPa) via a convective distribution system (e.g., blood, hemolymph, or tracheal system) before the final diffusion-based step into the mitochondria. Low PO_2 can reduce rates of ROS production (Bovaris & Chance 1973; Costa et al. 1993; Hetz & Bradley 2005; Starkov et al. 2008), but simultaneously increase the PO_2 gradient, which elevates rates of diffusive oxygen transport.

Data on mammals and insects show that tissue PO_2 is indeed 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 & White 2011a; 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). Hetz & Bradley (2005), 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 values around 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 & Downes 2013; Verberk & 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, aquatic juveniles typically are longer lived, and in the case of most aquatic insects much longer lived, than either the adults they become or terrestrial juveniles of other species. Avoiding damage from ROS should thus be a high priority for aquatic juveniles. Alternatively, we also hypothesized that more active species with higher metabolic demand for oxygen may maintain *higher* levels of tissue PO_2 to provide larger reservoirs of oxygen for immediate use during locomotion.

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 aquatic and five terrestrial – aquatic orders: Ephemeroptera, Plecoptera, Trichoptera, Odonata, Diptera; terrestrial orders: Coleoptera, Phasmatodea, Orthoptera, Diptera, Lepidoptera.

Because obtaining juveniles and adults of the same species was often difficult, more than one species was occasionally used from each order, for a total of six species (Table S1). 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 open-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 (Table S1). 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 airstone 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, adult 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 (Merritt et al. 2008; Borror & DeLong 2004). 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. Peer-reviewed sources could not be used in every case because of limited published data on some species. For some species, no stage duration data were available, in which case stage durations of closely related species were substituted (Table S1). In addition, activity levels of 1 or 2 from were assigned to each species based on our knowledge of their lifestyles, 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) (Table S1).

Because differences in activity levels may be associated with variation in metabolism, metabolic rates were estimated for each species and stage. Metabolic rate data were retrieved from peer-reviewed sources and published, online datasets or directly from authors for data used in published papers, but which were not readily accessible (see S1 for references). 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 (Table S1). 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 were measured at different experimental temperatures, the R function, predict, was used to estimate mass specific metabolic rates at standardized temperature of 20°C from a linear model (R function: lm) of log10(metabolic rate) ~ log10(mean mass) + temperature for each species and stage.

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) 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 meters 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) 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) or room air (19 kPa). Readings for both the electrodes and optodes were highly stable, and we performed the calibration procedure every day that measurements were taken.

PO_2 was measured inside the thorax and abdomen of individuals 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). PO_2 was recorded based on the values shown on the acquisition system or computer program as soon as the reading stabilized for ~ 2 seconds. 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.

In a separate experiment, aquatic juveniles of the stonefly, *Pteronarcys californica*, were acclimated 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 for ~ 2 seconds, 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. 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 & 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 3 days at room temperature. After 3 days of 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 seconds.

Statistical analysis

We first calculated the Spearman correlation coefficient among each covariate (stage, habitat, stage duration, activity level, and body mas) and the internal PO_2 of insects using the R function, cor (R Core Team 2022). Strong correlations (correlation coefficient > 0.4) were found between focal explanatory variables – habitat, life stage – and mass as well as between life stage duration and activity level, respectively (Fig. S1). If colinear covariates were included in the same model, their effects were modelled additively to avoid biasing the results. Additionally, colinear covariates were substituted for one another to help identify the best-fit model.

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 a 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). Random effects methods for zero-inflated, continuous data are rarely published in statistical packages, and their outputs often cannot be used to compute metrics commonly used in model selection, such as R^2 or AIC (but see R package: censReg; Henningsen 2010). To assess the performance of our models, we therefore analyzed residuals and then checked the robustness of our linear mixed-effects models by reanalyzing our models using methods designed for zero-inflated, semi-continuous data (function: censReg; package: censReg).

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 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 mass. However, because they were non-significant both as main effects and in interaction with stage and habitat, we dropped them from further analysis. The remaining covariates could not be included in a single model without causing the fitting procedure to fail, so we tested six more simplified models, which, in total, included all possible combinations of covariates (Table S2). R^2 and AIC were calculated and used for model selection via the R functions, r.squaredGLMM (package: MuMIn) (Barton 2009) and aictab (package: AICcmodavg) (Mazerolle 2017).

The six models together point to a complex set of relationships among PO_2 and the underlying predictors. Nevertheless, several larger patterns stand out. In particular, habitat always significantly interacted with stage, activity, and stage duration. However, when stage, activity level, and stage duration were included as main effects only, they were significant in some of the models but not others (Table S3). R^2 values were similar between models 1, 3, 4, and 6, with models 1 and 4 yielding the highest R^2 of 0.63. AIC values differed more strongly among models, with models 1 and 6 yielding the lowest and best supported AIC score of 98.09 and 93.88, respectively. Although R^2 and AIC did not agree on the single best model (Table S2), model 1 ($\log10(PO_2) \sim$ stage × habitat + activity level) was strongly supported by both selection criteria and was used as our final model. Residuals were calculated for model 1 using the R function, residuals (R Core Team 2022), and visual analysis suggested that they were approximately normally distributed (Fig. S2).

Because model 1 did not include stage duration, however, 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 stage duration had a significant effect on tissue PO_2, model 7 had a considerably lower R^2 (0.35) and higher AIC (182.21) than the models with interactions above.

Nonetheless, model 7 suggests that patterns of internal PO_2 in insects can be explained by a single, alternative explanatory variable, and was thus used as our second, final model. Note that each instance in which stage duration was significant, it was a negative relationship similar to the second final model.

In a supplemental analysis, we analyzed the data using censored regression (i.e., Tobit) models with mixed effects (function: censReg) with the package censReg, which is designed to handle continuous, non-parametric data (Henningsen 2010). Here, species was also included as a random effect, and measurement location and mass were initially added as covariates. As in the linear mixed effects models, measurement location and mass were dropped because they did not have significant effects on tissue PO_2 when analyzed in isolation or when added to models with an interaction between stage and habitat. Thus, the same combinations of covariates were analyzed in seven alternative models, as above. Supplementary models yielded similar results to the linear mixed effects models, with significant interactions between habitat and stage, activity level, and stage duration found in most models and with stage, activity level, and stage duration frequently having significant main effects (Table S4). Based on these results, we consider the outputs from the linear mixed effects models, above, to be valid and used them to interpret our findings.

In an additional analysis, we analyzed the effect of mass-specific metabolic rate on 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 (Table S5; Fig. S3).

To analyze the effect of acclimation treatment (i.e., normoxia vs. hyperoxia) on internal PO_2 of live salmonfly (*Pteronarcys californica*) nymphs, we used linear mixed effects models (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 log10. 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 acclimation, and later checked the results using censored regression (Tobit) models with mixed effects, as above (function: censReg; 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 (Table S6).

To analyze the effect of exposure treatment (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). Tissue PO_2 of live insects was significantly explained by interactions between life stage and habitat (final model: $P < 0.0001$) (Fig. 1A) (Table 1). This interaction was driven by the remarkably low internal PO_2 values in aquatic insects (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). Internal PO_2 was also significantly affected by activity level (final model 1: $P < 0.0001$) and the duration of life stages (final model 2: $P < 0.0001$) (Table 1), with juvenile aquatic insects generally displaying the lowest activity levels (mean = 1.1) (Fig. 1B) and the highest stage durations (> 300 days) (Fig. 1C) and adult aquatic insects displaying the highest activity levels (mean = 2.0) and the shortest stage durations (mean = 10 days).

Table 1: Linear mixed effects models of the effects of stage, habitat, activity level and stage duration on tissue PO_2 of live insects.

Final linear mixed effects model 1					
Predictor	Estimate	Std. Error	Df	T-value	P-value
Stage	-0.423	0.076	68.025	-5.596	< 0.001
Habitat	-0.174	0.070	23.228	-2.486	0.021
Activity level	0.541	0.060	24.466	9.025	< 0.001
Stage*Habitat	0.503	0.079	189.453	6.400	< 0.001
Final linear mixed effects model 2					
Predictor	Estimate	Std. Error	Df	T-value	P-value
Stage duration	-0.001	< 0.001	245.400	-12.189	< 0.001

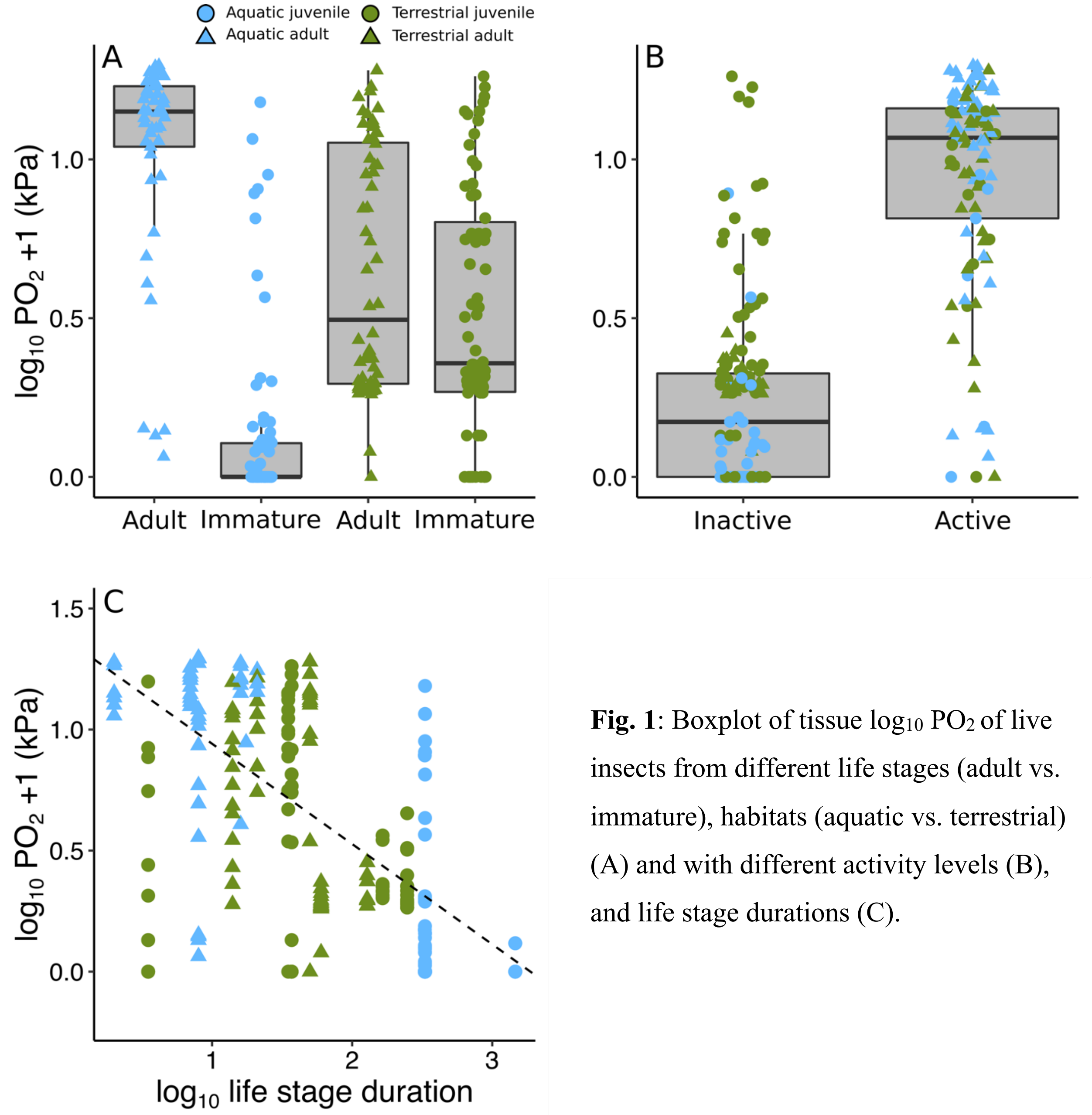

Fig. 1: Boxplot of tissue $\log_{10}$ PO_2 of live insects from different life stages (adult vs. immature), habitats (aquatic vs. terrestrial) (A) and with different activity levels (B), and life stage durations (C).

In addition, internal PO_2 of salmonfly nymphs was not significantly affected by PO_2 of water (19 kPa vs. 60 kPa) during a 24 h acclimation to hyperoxia ($P = 0.89$) (Fig. 2A) (Table 2). 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 2).

Table 2: 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.

Live salmonflies in water					
Predictor	Estimate	Std. Error	Df	T-value	P-value
Treatment	-0.005	0.038	23.000	-0.141	0.889
Dead salmonflies in ethanol					
Predictor	Estimate	Std. Error	Df	T-value	P-value
Treatment	34.052	3.902	9.271	8.726	< 0.001

Discussion

We observed an 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 & 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 high internal oxygen levels (~ 18 kPa) in ambient normoxia and significantly higher levels in hyperoxia (~ 32 kPa), further supporting our conclusion that live aquatic juveniles engage in active regulation of internal PO_2.

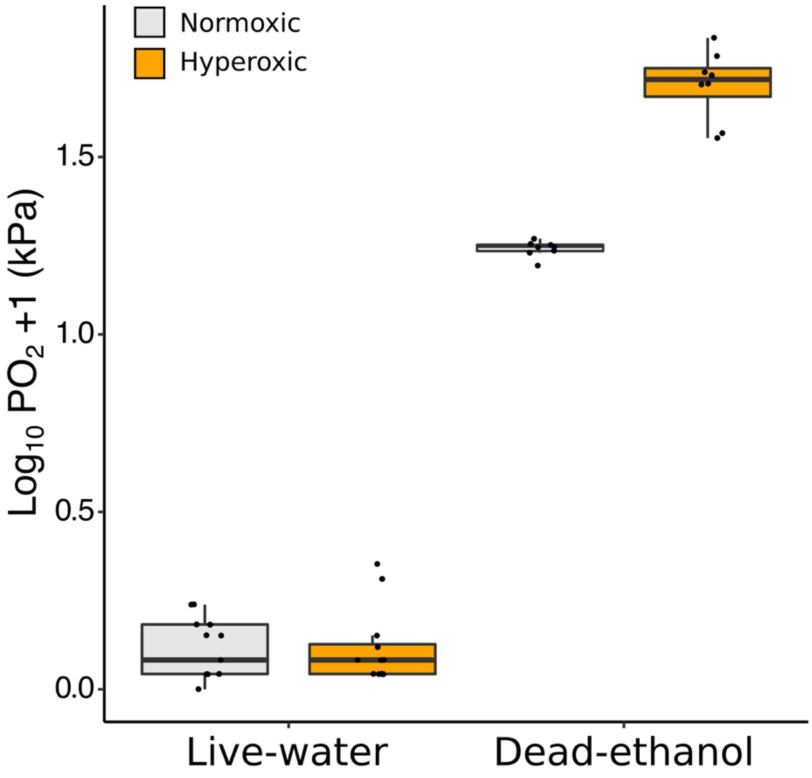

Fig. 2: Boxplots of tissue PO_2 of both live and dead juvenile salmonflies. Measurements on live individuals made after 24hr exposure to normoxia (19 kPa) and hyperoxia 60 (kPA). Measurements on dead individuals in ethanol at normoxia (19kPa) and 24hr exposure to hyperoxia (60kPa).

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-tissue 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. 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 & Atkinson 2013; Woods & 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; Birrell et al. 2022; Frakes et al. 2021). Our finding of consistently low PO_2 in juveniles across five orders of aquatic insects, nevertheless, suggests that holding PO_2 to low levels is a widely utilized, and perhaps universal, mechanism for increasing oxygen uptake among aquatic insects. This innovation is likely key 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 (Fig. S3).

Aquatic lifestyles, however, are also correlated with several other factors, each of which may also drive the evolution of low tissue PO_2 in non-mutually exclusive ways. First, across all stages and species, 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 approximately the same, 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 & 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 bursts of locomotion – e.g., winged flight – and thus, hold tissue PO_2 at lower levels. Living life in the slow lane could help prevent accumulation of ROS (see below) but also increase energy savings (Hetz & Bradley 2005; Speers-Roesch 2018). Indeed, energy savings could be 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 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.

A second alternative hypothesis invokes damage from reactive oxygen species (ROS) as a driver of low tissue PO_2. ROS, produced in mitochondria as a by-product of cellular respiration (Murphy et al. 2009), are a major driver of cellular damage, disease, and aging (Boveris et al. 1973; Costa et al. 1993, Starkov et al. 2008, Zorov et al. 2014). Low ROS production could thus allow longer-lived species to avoid cumulative, long-term effects of ROS (Shields et al. 2021). Indeed, insects with longer stage durations had significantly lower tissue PO_2, a pattern that was partly driven by aquatic juveniles, which had both the longest life stages and the lowest PO_2. Although the relationship between ROS production and PO_2 is not straightforward (Zorov et al. 2014), aquatic juveniles may nevertheless benefit from holding PO_2 at low levels if extramitochondrial metabolic pathways evolve to accommodate hypoxic conditions, leading to overall lower ROS production. Indeed, aquatic insects employ a host of antioxidant systems (Felton & Summers 1995; Xie et al. 2009, Sanz et al. 2017), and maintaining low PO_2 could thus be an additional method of avoiding damage from ROS. However, downregulating internal PO_2 to limit ROS production would likely be counterproductive for active, short-lived insects, like terrestrial from aquatic juveniles, as reducing internal PO_2 could pose more of a risk to fitness by impeding activity levels necessary to reproduce than short-term damage from ROS.

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 five 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, and how their mitochondria function under low internal oxygen levels, provide fruitful direction for follow up research. 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.

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References

Barton, K. (2009). MuMIn: multi-model inference. R package version 1. 0. 0.

Bates, D., Mächler, M., Bolker, B.M., and Walker, S.C. (2015). Fitting linear mixed-effects models using lme4. J. Stat. Softw. *67*. 10.18637/jss.v067.i01.

Boardman, L., Terblanche, J.S., Hetz, S.K., Marais, E., and Chown, S.L. (2012). Reactive oxygen species production and discontinuous gas exchange in insects. Proc. R. Soc. B Biol. Sci. *279*, 893–901. 10.1098/rspb.2011.1243.

Borror, D.J., and DeLong, D.M. (1971). An introduction to the study of insects. (Holt, Rinehart & Winston).

Boveris, A., and Chance, B. (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem. J. *134*, 707–716. 10.1042/bj1340707.

Brieger, K., Schiavone, S., Miller, F.J., and Krause, K.H. (2012). Reactive oxygen species: From health to disease. Swiss Med. Wkly. *142*. 10.4414/smw.2012.13659.

Buchwalter, D.B., Resh, V.H., Lamberti, G.A., and Verberk, W.C.E.P. (2020) Chapter 4: Aquatic insect respiration. In An Introduction to the Aquatic Insects of North America, Merrit, R.W., Cummins, K.W. and Berg, M.B., eds. (Kendall Hunt Publishing).

Chown, S.L., Gibbs, A.G., Hetz, S.K., Klok, C.J., Lighton, J.R.B., and Marais, E. (2006). Discontinuous gas exchange in insects: A clarification of hypotheses and approaches. Physiol. Biochem. Zool. *79*, 333–343. 10.1086/499992.

Costa, L.E., Llesuy, S., and Boveris, A. (1993). Active oxygen species in the liver of rats submitted to chronic hypobaric hypoxia. Am. J. Physiol. - Cell Physiol. *264*. 10.1152/ajpcell.1993.264.6.c1395.

Davalli, P., Mitic, T., Caporali, A., Lauriola, A., and D’Arca, D. (2016). ROS, Cell Senescence, and Novel Molecular Mechanisms in Aging and Age-Related Diseases. Oxid. Med. Cell. Longev. *2016*, 1–18. 10.1155/2016/3565127.

Denny, M.W. (1993). Air and water: the biology and physics of life’s media (Princeton University Press).

Felton, G.W., and Summers, C.B. (1995). Antioxidant systems in insects. Arch. Insect Biochem. Physiol. *29*, 187–197. 10.1002/arch.940290208.

Frakes, J.I., Birrell, J.H., Shah, A.A., and Woods, H.A. (2021). Flow increases tolerance of heat and hypoxia of an aquatic insect. Biol. Lett. *17*. 10.1098/rsbl.2021.0004.

Harrison, J.F., Woods, H.A., and Roberts, S.P. (2012). *Ecological and environmental physiology of insects*. (Oxford University Press).

Henningsen, A. (2010). Estimating Censored Regression Models in R using the censReg Package. R Packag. vignettes Collect. *5*, 12.

Hetz, S.K., and Bradley, T.J. (2005). Insects breathe discontinuously to avoid oxygen toxicity. Nature *433*, 516–519. 10.1038/nature03106.

Hynes, H.B.N. (1970). The Ecology of Running Waters (Liverpool University Press).

Kirkton, S.D. (2007). Effects of insect body size on tracheal structure and function. In Hypoxia and the circulation. Advances in experimental medicine and biology, Roach, R.C., Wagner, P.D., Hackett, P.H., eds. (Springer, Berlin), pp. 221–228.

Knoll, A.H. (1992). The early evolution of eukaryotes: A geological perspective. Science. *256*, 622–627. 10.1126/science.1585174.

Komai, Y. (1998). Augmented respiration in a flying insect. J. Exp. Biol. *201*, 2359–2366. 10.1242/jeb.201.16.2359.

Kulisz, A., Chen, N., Chandel, N.S., Shao, Z., and Schumacker, P.T. (2002). Mitochondrial ROS initiate phosphorylation of p38 MAP kinase during hypoxia in cardiomyocytes. Am. J. Physiol. - Lung Cell. Mol. Physiol. *282*, 1324–1329. 10.1152/ajplung.00326.2001.

Lancaster, J., and Downes, Barbara, J. (2013). Aquatic Entomology (Oxford University Press).

Lehmann, P., Javal, M., and Terblanche, J.S. (2019). Oxygen limitation is not the cause of death during lethal heat exposure in an insect. Biol. Lett. *15*, 20180701. 10.1098/rsbl.2018.0701.

Matthews, P.G.D., and White, C.R. (2011a). Regulation of gas exchange and haemolymph pH in the cockroach Nauphoeta cinerea. J. Exp. Biol. *214*, 3062–3073. 10.1242/jeb.053991.

Matthews, P.G.D., and White, C.R. (2011b). Discontinuous gas exchange in insects: Is it all in their heads? Am. Nat. *177*, 130–134. 10.1086/657619.

Mazerolle, M.J., and Mazerolle, M.M.J. (2017). Package ‘AICcmodavg’. *R package*, *281*.

Merritt, R.W., Cummins, K.W., and Berg, M.B. (2008). An introduction to the aquatic insects of North America. Kendall Hunt, 1996. (Kendall).

Murphy, M.P. (2009). How mitochondria produce reactive oxygen species. Biochem. J. *417*, 1–13. 10.1042/BJ20081386.

Poole, D.C., Pittman, R.N., Musch, T.I., and Østergaard, L. (2020). August Krogh's theory of muscle microvascular control and oxygen delivery: a paradigm shift based on new data. J. Physiol. *598*, 4473–4507. 10.1113/JP279223.

Ristow, M., and Schmeisser, K. (2014). Mitohormesis: Promoting health and lifespan by increased levels of reactive oxygen species (ROS). Dose-Response *12*, 288–341. 10.2203/dose-response.13-035.

Rowe, T.T.C., Gutbrod, M.S., and Matthews, P.G.D. (2022). Discontinuous gas exchange in Madagascar hissing cockroaches is not a consequence of hysteresis around a fixed PCO2 threshold. J. Exp. Biol. *225*. 10.1242/jeb.242860.

Sanz, A., López-Rodríguez, M.J., García-Mesa, S., Trenzado, C.E., Ferrer, R.M., and Tierno De Figueroa, J.M. (2017). Are antioxidant capacity and oxidative damage related to biological and autecological characteristics in aquatic insects? J. Limnol. *76*, 170–181. 10.4081/jlimnol.2016.1581.

Shchukarev, S.A., and Tolmacheva, T.A. (1968). Solubility of oxygen in ethanol – water mixtures. J. Struct. Chem. *9*, 16–21.

Shields, H.J., Traa, A., and Van Raamsdonk, J.M. (2021). Beneficial and Detrimental Effects of Reactive Oxygen Species on Lifespan: A Comprehensive Review of Comparative and Experimental Studies. Front. Cell Dev. Biol. *9*, 1–27. 10.3389/fcell.2021.628157.

Starkov, A.A. (2008). The role of mitochondria in reactive oxygen species metabolism and signaling. Ann. N. Y. Acad. Sci. *1147*, 37–52. 10.1196/annals.1427.015.

Speers-Roesch, B., Norin, T., and Driedzic, W.R. (2018). The benefit of being still: energy savings during winter dormancy in fish come from inactivity and the cold, not from metabolic rate depression. Proc. R. Soc. B Biol. Sci. *285*, 20181593. 10.1098/rspb.2018.1593.

Vanderkooi, J.M., Erecinska, M., and Silver, I.A. (1991). Oxygen in mammalian tissue: Methods of measurement and affinities of various reactions. Am. J. Physiol. - Cell Physiol. *260*. 10.1152/ajpcell.1991.260.6.c1131.

Verberk, W.C.E.P., Bilton, D.T., Calosi, P., and Spicer, J.I. (2011). Oxygen supply in aquatic ectotherms: Partial pressure and solubility together explain biodiversity and size patterns. Ecology *92*, 1565–1572. 10.1890/10-2369.1.

Verberk, W.C.E.P., and Atkinson, D. (2013). Why polar gigantism and Palaeozoic gigantism are not equivalent: Effects of oxygen and temperature on the body size of ectotherms. Funct. Ecol. *27*, 1275–1285. 10.1111/1365-2435.12152.

Verberk, W.C.E.P., and Bilton, D.T. (2013). Respiratory control in aquatic insects dictates their vulnerability to global warming. Biol. Lett. *9*, 0–3. 10.1098/rsbl.2013.0473.

Wittenberg, B.A., and Wittenberg, J.B. (1989). Transport of oxygen in muscle. Annual Review of Physiology, *51*(1), 857-878.

Wittenberg, B.A., and Wittenberg, J.B. (1989). Transport of Oxygen in Muscle. Annu. Rev. Physiol. *51*, 857–878. 10.1146/annurev.ph.51.030189.004233.

Woods, H. A., and Moran, A.L. (2020). Reconsidering the oxygen-temperature hypothesis of polar gigantism: Successes, failures, and nuance. Integr. Comp. Biol. *60*, 1438–1453. 10.1093/icb/icaa088.

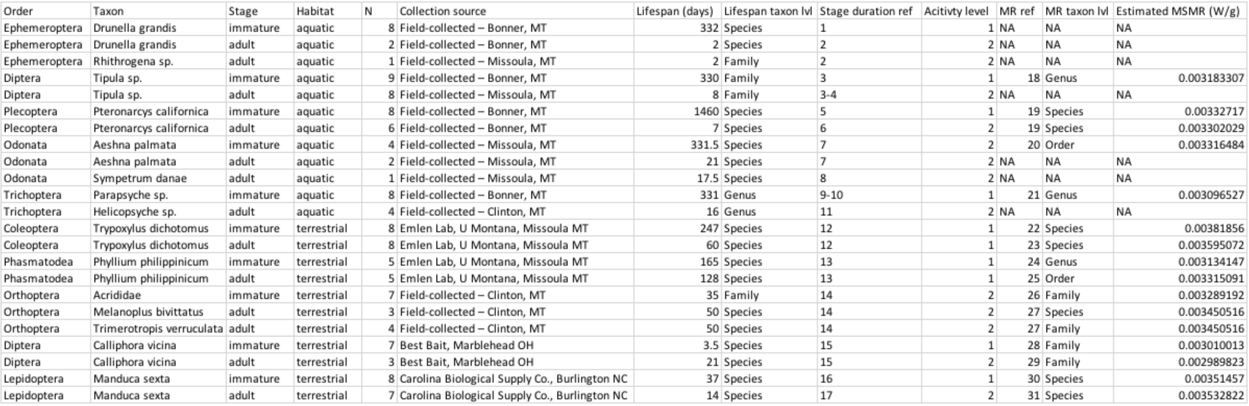
Xie, L., Flippin, J.L., Deighton, N., Funk, D.H., Dickey, D.A., and Buchwalter, D.B. (2009). Mercury(II) bioaccumulation and antioxidant physiology in four aquatic insects. Environ. Sci. Technol. *43*, 934–940. 10.1021/es802323r.

Zhang, Q., Yan, Q., Yang, H., and Wei, W. (2019). Oxygen sensing and adaptability won the 2019 Nobel Prize in Physiology or medicine. Genes Dis. *6*, 328–332. 10.1016/j.gendis.2019.10.006.

Zorov, D.B., Juhaszova, M., and Sollott, S.J. (2014). Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiol. Rev. *94*, 909–950. 10.1152/physrev.00026.2013.

Supplemental information

Table S1: Table of orders and species at each stage and habitat for which internal PO_2 was measured. Number of replicates and sources for each species is listed along with sources and data on mean internal PO_2, stage duration, activity level, and mass-specific metabolic rate for each species and stage.



Order	Taxon	Stage	Habitat	N	Collection source	Lifespan (days)	Lifespan taxon lvl	Stage duration ref	Acitivty level	MR ref	MR taxon lvl	Estimated MSMR (W/g)
Ephemeroptera	Drunella grandis	immature	aquatic	8	Field-collected – Bonner, MT	332	Species	1	1	NA	NA	NA
Ephemeroptera	Drunella grandis	adult	aquatic	2	Field-collected – Bonner, MT	2	Species	2	2	NA	NA	NA
Ephemeroptera	Rhithrogena sp.	adult	aquatic	1	Field-collected – Missoula, MT	2	Family	2	2	NA	NA	NA
Diptera	Tipula sp.	immature	aquatic	9	Field-collected – Bonner, MT	330	Family	3	1	18	Genus	0.003183307
Diptera	Tipula sp.	adult	aquatic	8	Field-collected – Missoula, MT	8	Family	3-4	2	NA	NA	NA
Plecoptera	Pteronarcys californica	immature	aquatic	8	Field-collected – Bonner, MT	1460	Species	5	1	19	Species	0.00332717
Plecoptera	Pteronarcys californica	adult	aquatic	6	Field-collected – Bonner, MT	7	Species	6	2	19	Species	0.003302029
Odonata	Aeshna palmata	immature	aquatic	4	Field-collected – Missoula, MT	331.5	Species	7	2	20	Order	0.003316484
Odonata	Aeshna palmata	adult	aquatic	2	Field-collected – Missoula, MT	21	Species	7	2	NA	NA	NA
Odonata	Sympetrum danae	adult	aquatic	1	Field-collected – Missoula, MT	17.5	Species	8	2	NA	NA	NA
Trichoptera	Parapsyche sp.	immature	aquatic	8	Field-collected – Bonner, MT	331	Genus	9-10	1	21	Genus	0.003096527
Trichoptera	Helicopsyche sp.	adult	aquatic	4	Field-collected – Clinton, MT	16	Genus	11	2	NA	NA	NA
Coleoptera	Trypoxylus dichotomus	immature	terrestrial	8	Emlen Lab, U Montana, Missoula MT	247	Species	12	1	22	Species	0.00381856
Coleoptera	Trypoxylus dichotomus	adult	terrestrial	8	Emlen Lab, U Montana, Missoula MT	60	Species	12	1	23	Species	0.003595072
Phasmatodea	Phyllium philippinicum	immature	terrestrial	5	Emlen Lab, U Montana, Missoula MT	165	Species	13	1	24	Genus	0.003134147
Phasmatodea	Phyllium philippinicum	adult	terrestrial	5	Emlen Lab, U Montana, Missoula MT	128	Species	13	1	25	Order	0.003315091
Orthoptera	Acrididae	immature	terrestrial	7	Field-collected – Clinton, MT	35	Family	14	2	26	Family	0.003289192
Orthoptera	Melanoplus bivittatus	adult	terrestrial	3	Field-collected – Clinton, MT	50	Species	14	2	27	Species	0.003450516
Orthoptera	Trimerotropis verruculata	adult	terrestrial	4	Field-collected – Clinton, MT	50	Species	14	2	27	Family	0.003450516
Diptera	Calliphora vicina	immature	terrestrial	7	Best Bait, Marblehead OH	3.5	Species	15	1	28	Family	0.003010013
Diptera	Calliphora vicina	adult	terrestrial	3	Best Bait, Marblehead OH	21	Species	15	2	29	Family	0.002989823
Lepidoptera	Manduca sexta	immature	terrestrial	8	Carolina Biological Supply Co., Burlington NC	37	Species	16	1	30	Species	0.00351457
Lepidoptera	Manduca sexta	adult	terrestrial	7	Carolina Biological Supply Co., Burlington NC	14	Species	17	2	31	Species	0.003532822

Table S1 References:

1. Rader, R. B., & Ward, J. V. (1989). Influence of impoundments on mayfly diets, life histories, and production. Journal of the North American Benthological Society, 8(1), 64-73.
2. NatureServe. 2022. NatureServe Network Biodiversity Location Data accessed through NatureServe Explorer [Google Chrome]. NatureServe, Arlington, Virginia. Available https://explorer.natureserve.org/Taxon/ELEMENT_GLOBAL.2.118672/Drunella_grandis (Accessed: February 22, 2022).
3. Young, C. W., Yule, C. M., & Yong, H. S. (2004). Insecta: Diptera, Tipulidae. Freshwater invertebrates of the Malaysian region, 775-785. https://web.archive.org/web/20170709155220/http://iz.carnegiemnh.org/cranefly/introduction.htm
4. Pritchard, G., & Hall, H. A. (1971). An introduction to the biology of craneflies in a series of abandoned beaver ponds, with an account of the life cycle of Tipula sacra Alexander (Diptera; Tipulidae). Canadian Journal of Zoology, 49(4), 467-482.
5. Townsend, G.D., Pritchard, G., 1998. Larval growth and development of the stonefly Pteronarcys californica (Insecta: Plecoptera) in the Crowsnest River, Alberta. Can. J. Zool. 76, 2274–2280.
6. J. H. Birrell, personal communication, February 22, 2022
7. Sanders, H. 2012. "Aeshna umbrosa" (On-line), Animal Diversity Web. Accessed December 18, 2022 at https://animaldiversity.org/accounts/Aeshna_umbrosa/
8. Black darter dragonfly facts and information. Trees for Life. (2021, March 5). Retrieved February 12, 2022, from https://treesforlife.org.uk/into-the-forest/trees-plants-animals/insects-2/black-darter/
9. 伊藤富子. (1999). Life History of a Net-spinning Caddisfly, Parapsyche shikotsuensis in a Headwater Stream of Hokkaido, Northern Japan. 陸水学雑誌, 60(2), 159-175

10. Benke, A. C., & Wallace, J. B. (1980). Trophic basis of production among net-spinning caddisflies in a southern Appalachian stream. Ecology, 61(1), 108-118.
11. Jackson, J. K. (1988). Diel emergence, swarming and longevity of selected adult aquatic insects from a Sonoran Desert stream. American Midland Naturalist, 344-352.
12. Jonathan Lai & Ko Hsin-ping (2008). For the love of rhinoceros and stag beetles (2nd edition).
13. R. Boisseau, personal communication, February 22, 2022
14. iNaturalist community. Observations of [Melanoplus bivittatus, Trimerotropis verruculata] from [Montana], [United States] observed between [Aug-Nov 2018-2021]. Exported from https://www.inaturalist.org on [February 22, 2022].
15. Wikimedia Foundation. (2022, May 24). Calliphora vicina. Wikipedia. Retrieved June 12, 2022, from https://en.wikipedia.org/wiki/Calliphora_vicina#:~:text=vicina's%20life%20cycle%20lasts%20approximately,cycle%20takes%20a%20little%20longer.
16. Care guide: Hornworms. Carolina. (n.d.). Retrieved December 17, 2022, from https://www.carolina.com/teacher-resources/Interactive/care-guide-hornworms/tr10510.tr
17. H. A. Woods, personal communication, February 23, 2022
18. Vannote, R. L., & Sweeney, B. W. (1985). Larval feeding and growth rate of the stream cranefly Tipula abdominalis in gradients of temperature and nutrition. Proceedings of the Academy of Natural Sciences of Philadelphia, 119-128.
19. Knight, A.W., Gaufin, A.R., 1966. Oxygen consumption of several species of stoneflies (Plecoptera). J. Insect Physiol. 12, 347–355. https://doi.org/10.1016/0022-1910(66)90149-14
20. Petitpren, M. F., & Knight, A. W. (1970). Oxygen consumption of the dragonfly, Anax junius. Journal of insect physiology, 16(3), 449-459.
21. Lowe, W. H., & Hauer, F. R. (1999). Ecology of two large, net-spinning caddisfly species in a mountain stream: distribution, abundance, and metabolic response to a thermal gradient. Canadian Journal of Zoology, 77(10), 1637-1644.
22. Cooley, N. L., Emlen, D. J., & Woods, H. A. (2016). Self-heating by large insect larvae?. Journal of Thermal Biology, 62, 76-83.
23. O'Brien, D. M., Boisseau, R. P., Duell, M., McCullough, E., Powell, E. C., Somjee, U., ... & Emlen, D. J. (2019). Muscle mass drives cost in sexually selected arthropod weapons. Proceedings of the Royal Society B, 286(1905), 20191063.
24. Maino, J. L., & Kearney, M. R. (2014). Ontogenetic and interspecific metabolic scaling in insects. The American Naturalist, 184(6), 695-701.
25. Roark, A.M., Bjorndal, K.A., 2009. Metabolic rate depression is induced by caloric restriction and correlates with rate of development and lifespan in a parthenogenetic insect. Exp. Gerontol. 44, 413–419.
26. Snelling, E.P., Seymour, R.S., Matthews, P.G.D., Runciman, S., White, C.R., 2011. Scaling of resting and maximum hopping metabolic rate throughout the life cycle of the locust Locusta migratoria. J. Exp. Biol. 214, 3218–3224.
27. Harrison, J. F., Phillips, J. E., & Gleeson, T. T. (1991). Activity physiology of the two-striped grasshopper, Melanoplus bivittatus: gas exchange, hemolymph acid-base status, lactate production, and the effect of temperature. Physiological Zoology, 64(2), 451-472.
28. Berrigan, D., & Lighton, J. R. B. (1993). Bioenergetic and kinematic consequences of limblessness in larval Diptera. Journal of Experimental Biology, 179(1), 245-259.
29. Berrigan, D., & Lighton, J. R. (1994). Energetics of pedestrian locomotion in adult male blowflies, Protophormia terraenovae (Diptera: Calliphoridae). Physiological zoology, 67(5), 1140-1153.

30. Greenlee, K. J., & Harrison, J. F. (2005). Respiratory changes throughout ontogeny in the tobacco hornworm caterpillar, Manduca sexta. Journal of Experimental Biology, 208(7), 1385-1392.
31. Woods Jr, W. A., & Stevenson, R. D. (1996). Time and energy costs of copulation for the sphinx moth, Manduca sexta. Physiological zoology, 69(3), 682-700.

Table S2: Model predictors with significant effects and model selection outputs for linear mixed models. Significant effects and final models used are shown in bold. For R^2 and AIC, higher and lower numbers indicate better fit, respectively.

Model predictors and model selection for linear mixed models			
Model	Covariates	R2	AIC
1	stage **x** habitat + **activity level**	0.63	98.09
2	stage **x** habitat + log10(stage duration)	0.41	138.29
3	activity level **x** habitat + stage	0.62	108.11
4	activity level **x** habitat + log10(**stage duration**)	0.63	105.81
5	log10(stage duration) **x** habitat + **stage**	0.42	113.51
6	log10(stage duration) **x** habitat + **activity level**	0.58	93.88
7	log10(**stage duration**)	0.34	182.21

Table S3: Linear mixed effects models for observational PO_2 measurements of live insects

Linear Mixed Effects Model 1					
Predictor	Estimate	Std. Error	Df	T-value	P-value
Stage	-0.423	0.076	68.025	-5.596	< 0.001
Habitat	-0.174	0.070	23.228	-2.486	0.021
Activity level	0.541	0.060	24.466	9.025	< 0.001
Stage*Habitat	0.503	0.079	189.453	6.400	< 0.001

Linear Mixed Effects Model 2					
Predictor	Estimate	Std. Error	Df	T-value	P-value
Stage	-0.910	0.165	162.297	-5.530	< 0.001
Habitat	-0.351	0.151	19.144	-2.327	0.031
log10(Stage duration)	0.011	0.082	155.332	0.131	0.896
Stage*Habitat	0.761	0.160	193.079	4.757	< 0.001

Linear Mixed Effects Model 3					
Predictor	Estimate	Std. Error	Df	T-value	P-value
Activity level	0.926	0.074	60.143	12.542	< 0.001
Habitat	0.793	0.135	31.680	5.872	< 0.001
Stage	-0.011	0.053	61.068	-0.213	0.832

Activity level*Habitat	-0.455	0.084	78.963	-5.422	< 0.001
Linear Mixed Effects Model 4					
Predictor	Estimate	Std. Error	Df	T-value	P-value
Activity level	0.811	0.094	18.780	8.646	< 0.001
Habitat	0.602	0.175	13.531	3.439	0.004
log10(Stage duration)	-0.074	0.045	20.559	-1.637	0.117
Activity level*Habitat	-0.342	0.107	18.511	-3.212	0.005
Linear Mixed Effects Model 5					
Predictor	Estimate	Std. Error	Df	T-value	P-value
log10(Stage duration)	-0.377	0.041	243.444	-9.253	< 0.001
Habitat	-1.203	0.229	37.614	-5.248	< 0.001
Stage	-0.200	0.055	243.611	-3.628	< 0.001
log10(Stage duration)*Habitat	0.727	0.099	217.307	7.355	< 0.001
Linear Mixed Effects Model 6					
Predictor	Estimate	Std. Error	Df	T-value	P-value
log10(Stage duration)	-0.282	0.041	170.137	-6.943	< 0.001
Habitat	-0.675	0.170	28.398	-3.984	< 0.001
Activity level	0.427	0.062	88.440	6.903	< 0.001
log10(Stage duration)*Habitat	0.430	0.088	49.710	4.895	< 0.001
Linear Mixed Effects Model 7					
Predictor	Estimate	Std. Error	Df	T-value	P-value
log10(Stage duration)	-0.414	0.031	246.941	-13.160	< 0.001

Table S4: Outputs for censored regression (CensReg) models for the effects of habitat, stage, activity level, and stage duration on tissue PO_2 of live insects.

CensReg Model 1				
Predictor	Estimate	Std. Error	T-value	P-value
Habitat	-9.586	3.519	-2.724	0.006
Stage	-5.153	2.465	-2.091	0.037
Activity level	6.199	3.512	1.765	0.078
Stage*Habitat	10.559	1.345	7.851	< 0.001
CensReg Model 2				
Predictor	Estimate	Std. Error	T-value	P-value
Stage	-14.443	1.847	-7.819	< 0.001
Habitat	-7.155	3.485	-2.053	0.040
log10(Stage duration)	0.299	0.664	0.451	0.652
Stage*Habitat	12.573	2.521	4.987	< 0.001

CensReg Model 3				
Predictor	Estimate	Std. Error	T-value	P-value
Activity level	15.101	1.281	11.788	< 0.001
Habitat	18.278	2.883	6.340	< 0.001
Stage	-1.052	0.762	-1.380	0.168
Activity level*Habitat	-11.369	1.109	-10.252	< 0.001
CensReg Model 4				
Predictor	Estimate	Std. Error	T-value	P-value
Activity level	12.385	1.237	10.015	< 0.001
Habitat	11.913	3.180	3.746	< 0.001
log10(Stage duration)	-1.886	0.649	-2.907	0.004
Activity level*Habitat	-7.085	1.229	-5.766	< 0.001
CensReg Model 5				
Predictor	Estimate	Std. Error	T-value	P-value
log10(Stage duration)	-6.715	0.917	-7.323	< 0.001
Habitat	-22.472	14.970	-1.501	0.133
Stage	-1.895	0.884	-2.144	0.032
log10(Stage duration)*Habitat	11.564	9.868	1.172	0.241
CensReg Model 6				
Predictor	Estimate	Std. Error	T-value	P-value
log10(Stage duration)	-5.371	1.003	-5.353	< 0.001
Habitat	-8.670	9.236	-0.939	0.348
Activity level	5.690	0.688	8.267	< 0.001
log10(Stage duration)*Habitat	5.234	4.795	1.092	0.275
CensReg Model 7				
Predictor	Estimate	Std. Error	T-value	P-value
Stage duration	-6.691	0.315	-21.220	< 0.001

Table S5: Linear mixed effects models on the effect of mass-specific metabolic rate (MSMR) on tissue PO2 in live insects.

Linear Mixed Effects Model 1					
Predictor	Estimate	Std. Error	Df	T-value	P-value
MSMR	0.762	2.737	33.420	0.278	0.782
Linear Mixed Effects Model 2					
Predictor	Estimate	Std. Error	Df	T-value	P-value
Habitat	-0.672	0.220	13.741	-3.052	0.009

Stage	-1.156	0.104	192.234	-11.163	< 0.001
MSMR	1.568	2.381	53.634	0.659	0.513
Stage*Habitat	1.007	0.117	191.837	8.625	< 0.001

Table S6: Censored regression (CensReg) models for the effect of treatment (hyperoxia vs. normoxia), measurement method, and mass on tissue PO2 in live salmonflies.

CensReg Model				
Predictor	Estimate	Std. Error	T-value	P-value
Treatment	0.060	0.181	0.330	0.741
Method	0.17371	0.17784	0.977	0.329
Mass	0.06573	0.47119	0.139	0.889

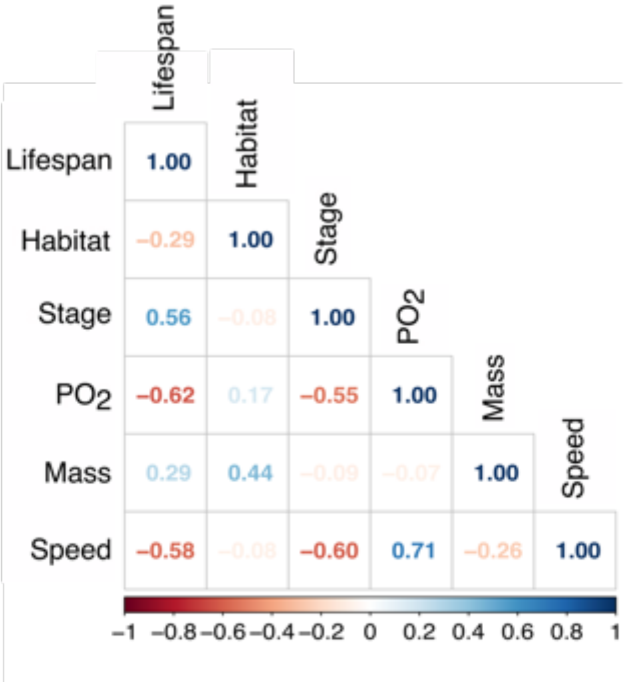


Fig. S1: Correlation matrix showing spearman correlation coefficients between predictors from observational PO$_2$ measurements on live insects. Opacity of the numbers represent the strength of the correlation. Predictors with coefficients < -0.4 and > 0.4, respectively, were considered strongly colinear and if, included in the same model during analysis their effects were modelled additively to avoid biasing the results.

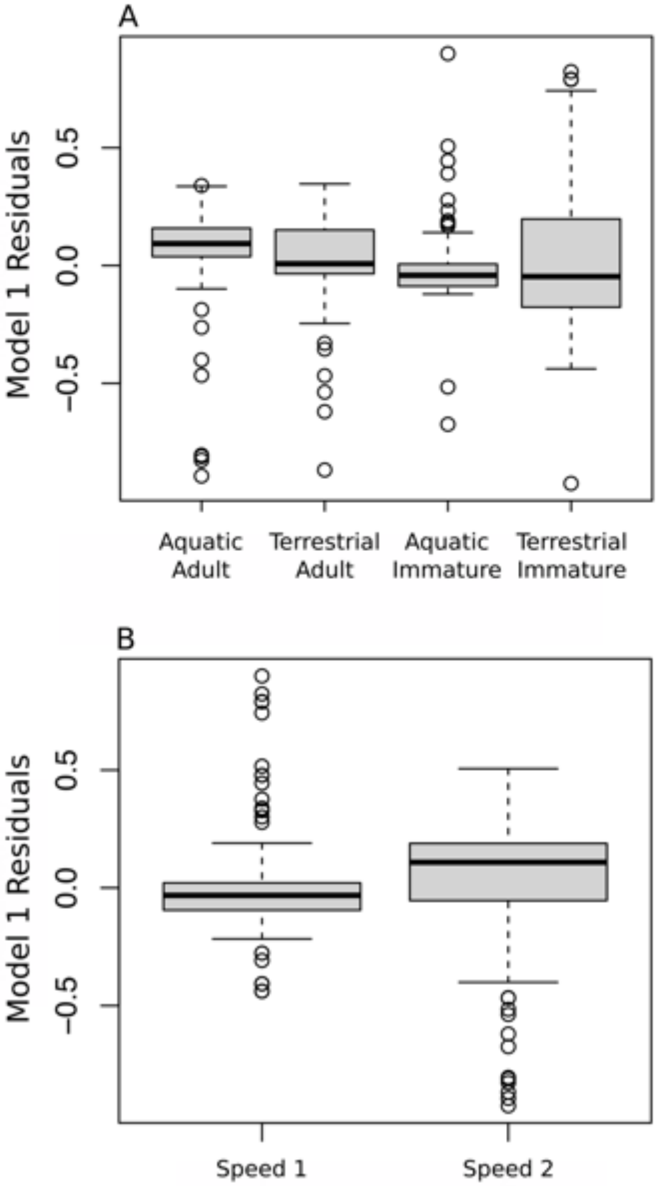


Fig. S2: Boxplots of residuals of final model 1 for stage and habitat (A) and activity level (B).

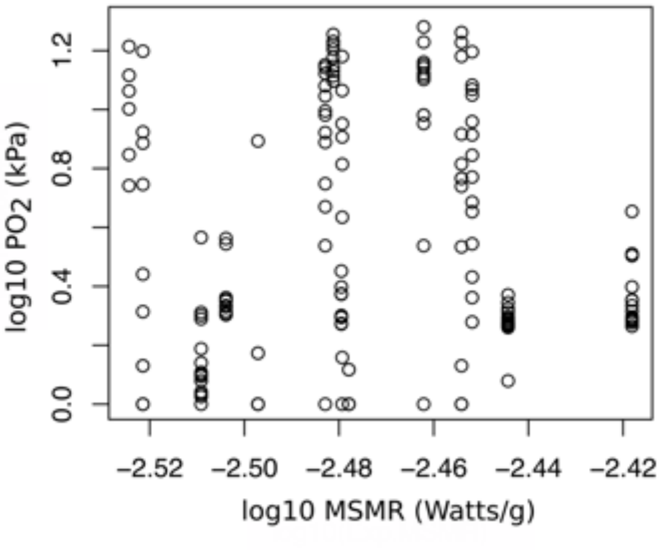


Fig. S3: Scatterplot of mass-specific metabolic rate (MSMR) and log10 PO_2 of insect tissues.

Chapter 2

Insects in high-elevation streams: life in extreme environments imperiled by climate change

Insects in high-elevation streams: life in extreme environments imperiled by climate change

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Abstract

Climate change is altering conditions in high-elevation streams worldwide, with largely unknown effects on resident communities of aquatic insects. Here, we review the challenges of climate change for high-elevation aquatic insects and how they may respond, focusing on current gaps in knowledge. Understanding current effects and predicting future impacts will depend on progress in three areas. First, we need better descriptions of the multivariate physical challenges and interactions among challenges in high-elevation streams, which include low but rising temperatures, low and decreasing levels of oxygen supply relative to demand, high and rising exposure to ultraviolet radiation, low ionic strength, and variable but shifting flow regimes. These factors are often studied in isolation even though they covary in nature and interact in space and time. Second, we need a better mechanistic understanding of how physical conditions in streams drive the performance of individual insects. Environment-performance links are mediated by physiology and behavior, which are poorly known in high-elevation taxa. Third, we need to define the scope and importance of potential responses across levels of biological organization. Short-term responses are defined by the tolerances of individuals, their capacities

to perform adequately across a range of conditions, and behaviors used to exploit local, fine-scale variation in abiotic factors. Longer-term responses to climate change, however, may include individual plasticity and evolution of populations. Whether high-elevation aquatic insects can mitigate climatic risks via these pathways is largely unknown.

Introduction

High-elevation streams are some of the most extreme ecosystems on Earth, yet they harbor extensive aquatic insect biodiversity and support a high degree of endemism (Hotaling et al., 2017). High-elevation streams occur from > 2000 m (at higher latitudes) to > 4000 m (lower latitudes) and represent nearly 5% of the world's waterways (Fig. 1). They are typically fed by multiple meltwater sources, can be covered by snow and ice for most of the year, and are often fragmented and isolated, with cold, turbulent, fast-flowing water, low ionic strength, low oxygen availability, and high levels of UV radiation (when not covered by snow) (Jacobsen & Dangles, 2017). High-elevation stream conditions are also highly variable in space and time, depending on source, drainage geology, elevation, aspect, latitude, and time of year. Collectively, however, high-elevation streams are experiencing some of the most rapid climate-driven changes on Earth and appear to be warming faster than aquatic systems elsewhere in the world (IPCC, 2018; Pepin et al., 2015; Wang et al., 2016). Rising temperatures, in turn, are leading to lower average snowfall and spring snowpack (Marty & Blanchet, 2012; Mote et al., 2005), shrinkage or loss of glaciers and permanent snowfields (Beniston et al., 2018; Zemp et al., 2015), and subsequent shifts in flow regimes and water chemistry (Huss & Hock, 2018; IPCC, 2019). More broadly, understanding the challenges faced by high-elevation stream insects is paramount for preserving biodiversity and for protecting the ecosystem services provided by high-elevation biomes, which include providing clean, predictable flows for lower-elevation ecosystems and the human populations that depend on them and sustaining high quality areas for tourism, recreation, and cultural practices (Klein et al., 2019; Locatelli et al., 2017; Viviroli et al., 2007).

Insects (including mayflies, stoneflies, caddisflies, beetles, and true flies) are the dominant fauna in high-elevation streams (Jacobsen & Dangles, 2017). Such broad taxonomic diversity indicates that insects have repeatedly invaded high-elevation waters. How have high-elevation stream insects responded to anthropogenic climate change? How will they respond in the future? In general, the outlook is bleak (Brown et al., 2007; Giersch et al., 2017; Hotaling et al., 2017; Lencioni, 2018). Aquatic insects are thought to be among the most sensitive aquatic taxa to environmental change and are commonly used as bioindicators of water quality (DeWalt et al., 2005). Extirpations of some high-elevation stream taxa have already occurred (Lencioni, 2018), and two species are now listed as threatened in the United States (U.S. Fish & Wildlife Service, 2019). Even when populations persist, they may contain fewer individuals, reflecting stress from novel combinations of physical conditions or negative biotic interactions with newly arrived lowland species (Lencioni, 2018). In the future, additional shifts in species ranges and community composition will likely occur, as well as declines in habitat heterogeneity and beta (difference among elevations) and gamma (regional) biodiversity (Brown et al., 2007; Hotaling et al., 2017; Jacobsen et al., 2012; but see Muhlfeld et al., 2020). The predictions above, however, rest on limited climatic and biological knowledge. Despite relatively detailed analyses of temperature, other impacts of climate change on high-elevation communities are poorly known. Likewise, aside from research on a few focal taxa, the behavioral, physiological, and evolutionary mechanisms underlying potential responses to climate change have been understudied. Insect responses to climate change will ultimately stem from both abiotic *and* biotic interactions. However, the first priority is to understand the influence of the physical environment on insects, before including the complexity of biotic interactions.

Our ability to understand and predict current and future effects of climate change will, therefore, hinge on addressing the following gaps in knowledge. First, in high-elevation streams, multiple physical characteristics are changing simultaneously in time and space, including temperature, flow regime, water chemistry, and exposure to ultraviolet radiation (Jacobsen & Dangles, 2017). Thus, understanding insect responses will require theory and experiments that explicitly incorporate *multivariate* challenges and that examine populations distributed across varying environmental conditions (Todgham & Stillman, 2013). Second, insect performance and fitness depend on *interactions* among physical factors. Interactions arise because core life processes – e.g., balancing rates of oxygen supply and demand – integrate multiple aspects of the physical environment such as dissolved oxygen, temperature, and flow velocity (Jacobsen, 2000; Verberk et al., 2011; Woods, 1999). Interactions also arise because insect responses are constrained by tradeoffs, e.g., metabolic energy devoted to coping with UV damage becomes unavailable for mitigating thermal stress. Predicting the outcomes of such interactions requires understanding the behavioral and physiological mechanisms that aquatic insects use to respond to local temporal and spatial variation (Helmuth et al., 2005). Third, insect responses span multiple time scales and levels of organization. For example, in the short term, individual insects may mitigate difficult conditions via behavioral, physiological, or morphological plasticity. Over longer time scales, the evolution of new behavioral or physiological capacities may allow insects to live in novel environments. Thus, predicting the fates of populations requires understanding their immediate responses to climatic challenges and the complex interactions that will drive evolutionary change.

We build on related reviews (e.g., Elser et al., in press; Hotaling et al., 2017; Jacobsen & Dangles, 2017; McGregor et al., 1995; Milner & Petts, 1994; Ward, 1994) by synthesizing the physiological challenges beyond temperature extremes that insects face in high elevation streams, from both singular and interactive perspectives. We explore the knowledge gaps identified above by first describing the physical and physiological challenges of life in high-elevation streams and how they are being altered by climate change. Next, we highlight key physiological and behavioral pathways by which physical challenges interact with one another and potential behavioral, plastic, and evolutionary responses by individuals, populations, and species. Because aquatic insects have complex life cycles that typically involve aquatic and terrestrial stages, responses to climate change will depend on changes to aquatic as well as nearby terrestrial conditions (Kingsolver et al., 2011). However, because aquatic insects spend the majority of their lives developing in streams, we focus primarily on aquatic juvenile stages – nymphs and larvae.

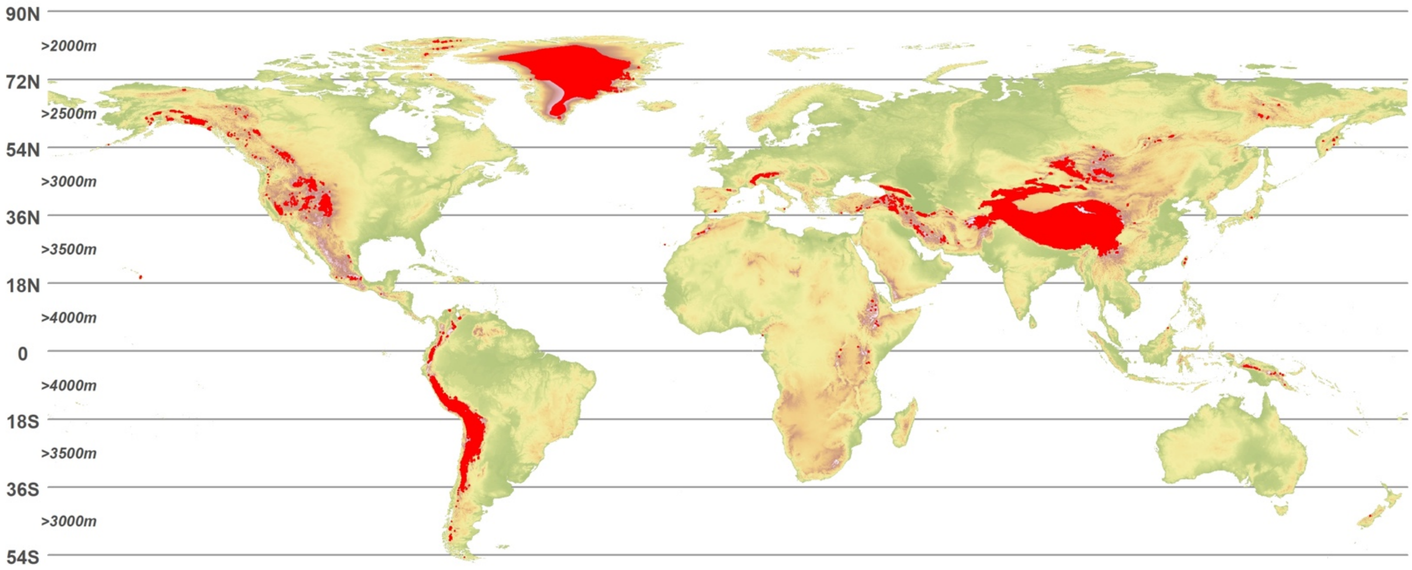


Fig.1. The definition of 'high-elevation' is somewhat subjective (Jacobsen & Dangles 2017) but is characterized, overall, by a combination of factors including cold temperatures, low oxygen availability, strong contributions from melting ice or snow, and lack of surrounding woody vegetation. In general, these conditions and our definition of 'high-elevation' correspond to elevations above 4000 m at low-latitudes, declining to above 2000 m at high-latitudes. By this definition, approximately 3.88% of Earth's surface (excluding Antarctica) and 4.85% of streams reside at high elevations. High-elevation regions are shown in red. Map and calculations were made by analyzing stream (HydroRIVERS, World Wildlife Fund), elevation (Global Land One-km Base Elevation Project, NOAA) and latitude-longitude (World Latitude and Longitude service pack, ESRI) datasets, using GIS software (Arc GIS 10.6).

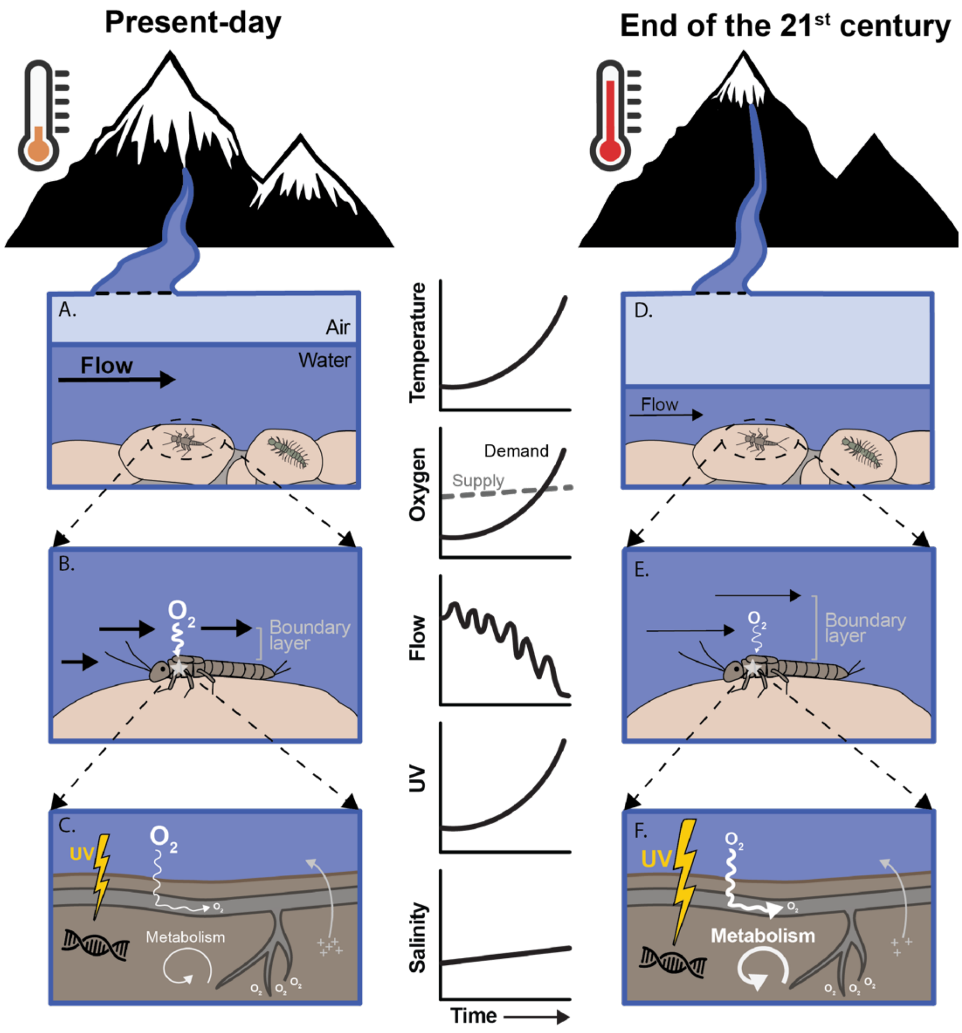


Fig. 2. Physical and physiological challenges faced by high-elevation stream insects in the present day (left) and at the end of the 21st century (right). The scenario depicted here is for organisms that will not undergo uphill migrations and for streams with strong glacial meltwater influences. Graphs represent predicted trends in temperature, oxygen, flow, UV, and salinity. **(A):** Aquatic insects currently inhabiting high-elevation streams typically experience relatively deep, cold, fast-flowing water due to high meltwater input. **(B):** This results in high oxygen solubility and thin boundary layers around insect bodies. But low diffusion coefficients reduce the net supply of oxygen (i.e., rate of oxygen diffusion) to insects **(C):** However, metabolic rates are also low at cold temperatures, and oxygen demand remains low. Current levels of UV radiation, although harmful, are partially blocked by deep water, glacial till, or snow cover. Insects are also challenged by low salinity as they must expend energy to maintain ionic homeostasis, especially during floods. By the end of the 21st Century, we predict a number of changes to high-elevation streams. **(D):** Increased air temperatures will result in weaker meltwater contributions, shallower streams, warmer water temperatures, and lower flows, with multiple effects on the factors that influence rates of oxygen supply. **(E):** Lower flows will thicken boundary layers and higher temperatures will decrease oxygen solubility. **(F):** High temperatures will also increase diffusion coefficients and decrease water viscosity (partially offsetting the effect of low flows on boundary layers), leading to higher levels of oxygen supply. Higher rates of supply, however, are unlikely to meet insects' now much higher metabolic demand. Decreased snow cover, glacial till, and water depth will subject insects to increased damage from UV radiation, though these may be offset at moderate elevations by rising treeline. Lower streamflow may also increase salinity, reducing costs of osmoregulation.

Challenges to life in high-elevation streams

For high-elevation aquatic taxa, life is presumably structured by the challenges of physically and chemically extreme environments. Below, we focus on five key challenges – low temperature, low oxygen availability, variable flow regimes, high levels of ultraviolet radiation, and low ionic strength – as well as their interactions. We also discuss how climate change is altering these challenges and the resulting consequences for aquatic insects (Fig. 2). Determining which changes will threaten insects most will require identifying how physiological traits vary across stages of development (e.g., eggs, larvae, adults) and how insects respond to both acute and prolonged exposure to novel conditions. Below, we use the terms "summer" and "winter" to refer to seasonal conditions specific to temperate streams.

Low water temperature. Temperature strongly influences biological processes and has thus dominated studies on the effects of climate change on small ectotherms (Clarke, 1998; Kingsolver & Huey, 2008). High-elevation streams generally present insects with very low temperatures. However, thermal regimes can vary widely and warm temperatures (>10 °C) can readily occur in summer (Fig. 3). Rapidly increasing air temperatures coupled with receding permanent snowfields are predicted to result in shorter winters and to extend the duration of extreme heat events (IPCC, 2018, 2019). How aquatic taxa cope with prolonged cold in the present and how they will respond to more frequent heat stress in the future requires further research. Here, we discuss our current understanding of thermal tolerance in high-elevation aquatic insects.

In many localities, high-elevation insects can encounter prolonged periods of injurious or lethally cold temperatures, especially in winter (Wissinger et al., 2016). At temperatures below freezing, insects can enter a reversible paralysis ('chill coma') at their critical thermal minimum (CT_{MIN}), where they become susceptible to injury (Denlinger & Lee, 2010; MacMillan & Sinclair, 2011). Internal ice formation is the clearest threat to insect survival, but prolonged exposure to near freezing temperatures can also be problematic (Bale, 1993; Lencioni, 2004). The capacity for survival at low temperatures is referred to as 'cold hardiness,' and insects can be grouped into three categories based on their responses (from most to least susceptible): chill-susceptible, freeze-avoidant, and freeze-tolerant (Bale, 1993; Sinclair et al., 2015) with each strategy supported by distinct physiological mechanisms (Lencioni, 2004). For high-elevation stream insects, however, these mechanisms remain poorly known, in part due to the practical challenges of studying cold physiology in habitats that are difficult to access and typically under deep snow for prolonged periods. In summer, meltwater stoneflies (*Lednia tumana*) do not survive being encapsulated in ice, suggesting that under the right circumstances, mid-summer cold snaps could be lethal to stoneflies (Hotaling et al., in press). If these traits are plastic, insects may become seasonally tolerant of winter cold and ice like stoneflies residing in the Arctic (Walters et al., 2009, 2011).

Although high-elevation streams are dominated by cold temperatures, they can warm significantly in summer (>10 °C) during periods of higher air temperatures and intense solar radiation (Hotaling et al., 2020; Jacobsen et al., 2010). Stream temperatures are already warming worldwide (Mohseni & Stefan, 1999; Pilgrim et al., 1998). Average winter temperatures are increasing by ~0.7 °C per decade in forest and moorland streams in the United Kingdom (Durance & Ormerod, 2007) and summer stream temperatures are increasing by ~0.2 °C per decade in the northwestern United States (Isaak et al., 2012). Rates of warming are likely even greater at high elevations (e.g., Bradley et al., 2006; Cannone et al., 2008). For example, on the Tibetan plateau, air temperatures are increasing by ~0.5 °C per decade at 2,800 m but by ~0.7 °C per decade at 4,600 m (Pepin et al., 2015). By 2100, stream temperatures are predicted to rise by 3 °C and 1.8 °C in the Cascade and Rocky Mountains of western North America, respectively (Hill et al., 2014). In some regions, rates of warming may be even more intense, as mean summer stream temperatures in the European Alps are rising at 2.5 °C per decade (Niedrist & Füreder, 2020). Climate change scenarios also predict greater variance in temperature and more frequent occurrence of temperature extremes, with pervasive effects on organisms (Dillon et al., 2016; Hansen et al., 2012; IPCC, 2018; Jacobsen & Dangles, 2017; Shah, Gill, et al., 2017).

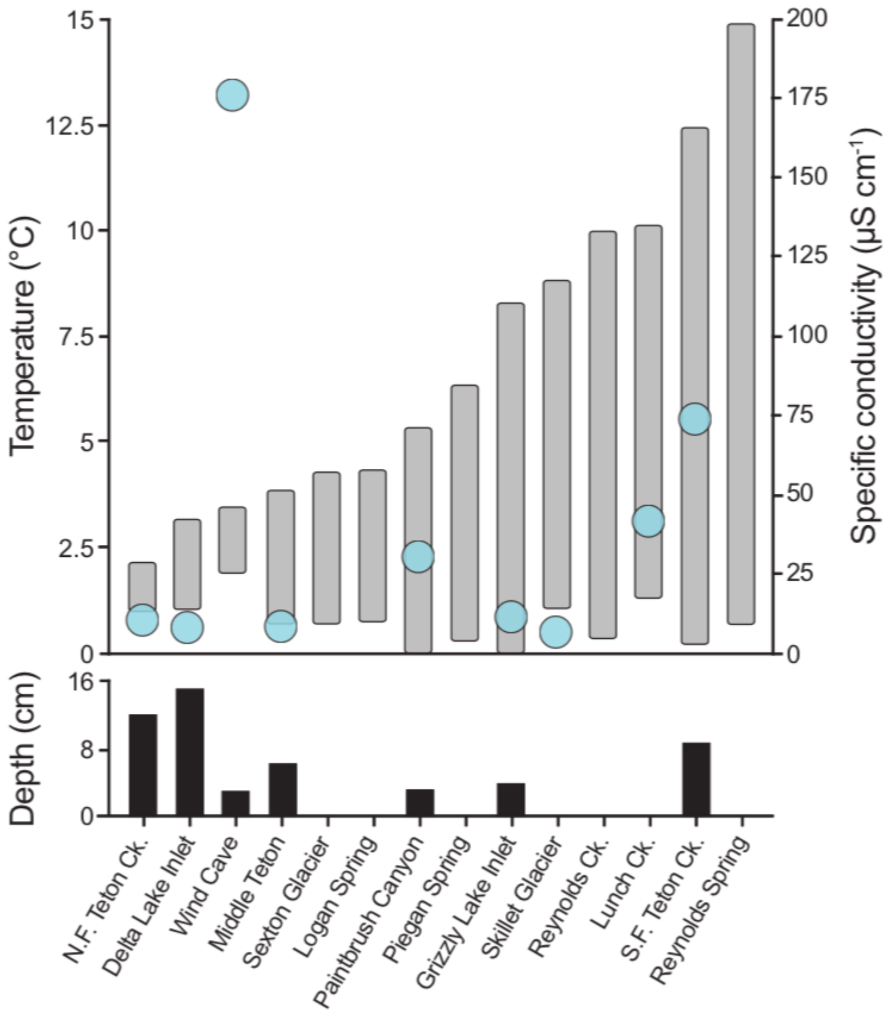


Fig. 3. Water temperature (annual ranges indicated by grey bars), conductivity (single measurements, blue circles), and depth (single measurements, black bars) are generally low for high-elevation streams but are highly variable among streams. Water temperature ranges and conductivity levels from low-elevation mountain streams also vary, but are generally higher: 0-22 °C, 229 µS cm^{-1}; 0-18 °C, 166 µS cm^{-1}; 0-18 °C, 44 µS cm^{-1} (Blackfoot, Bitterroot, Flathead Rivers, Montana, USA, respectively). High-elevation stream data were collected from Glacier National Park, Montana, and Teton National Park, Wyoming, USA (Hotaling, Foley, et al., 2019; Hotaling, Shah, et al., 2019; Tronstad et al., 2019; unpublished data). Low-elevation data were retrieved from the U.S. Geological Survey, National Water Information System (2020).

The vulnerability of high-elevation stream insects to climate change may depend largely on whether they can tolerate warmer temperatures. The upper thermal limits of aquatic taxa, however, are poorly known, especially compared to terrestrial insects (reviewed by Chown et al., 2015). Compared to temperate taxa, even less is known about taxa in the tropics (Chown et al., 2015), where aquatic insect biodiversity is high (Gill et al., 2016; Jacobsen et al., 1997; Polato et al., 2018) and species may be living closer to their upper thermal limits (Deutsch et al., 2008). In a comparison of critical thermal maximum (CT_{MAX}) of aquatic insects in the Rocky Mountains (temperate) and Andes (tropical), temperate species exhibited higher CT_{MAX} than their equatorial relatives (Shah, Gill, et al., 2017). This pattern may be explained by the much higher summer maxima in temperate than in tropical alpine streams. Similar patterns are found at local scales. For example, populations of an alpine stonefly experiencing low summer maximum temperatures (due to constant input from ice melt) had lower CTMAX values than nearby populations from warmer streams (Hotaling et al., 2020). Species that exhibit a lower CTMAX are likely to be more vulnerable to warming, especially if they lack the capacity to disperse widely or exhibit plasticity in thermal tolerance traits (Shah, Funk, et al., 2017; Shah, Gill, et al., 2017).

As with most taxa, studies of aquatic insect thermal tolerance tend to focus on acute, extreme warming events (e.g., CT_{MAX}). But high-elevation insects also face prolonged exposure to warming, which can affect development rates and emergence timing (Harper & Peckarsky, 2006), alter life histories (e.g., shift populations from semi- to univoltine) (Braune et al., 2008), and depress fertility (Walsh et al., 2019). Decrements in performance or even death may also occur from prolonged temperature-induced oxygen shortages (Verberk et al., 2016). Understanding the effects of chronic, sublethal warm temperatures remains one of the most important avenues of future research.

Functional hypoxia arising from interactions between oxygen and temperature. For aerobic animals, including aquatic insects, ensuring that oxygen supply matches metabolic demand is a fundamental challenge (Jacobsen, 2020). Whether oxygen supply and demand are equivalent depends on both physical conditions (level of oxygen, flow velocity, turbulence, viscosity, and temperature) and organismal traits (body size, positioning in flow, and metabolic density). Stream warming will have large, systemic effects on oxygen supply and demand. Warmer water generally stimulates metabolic demand for oxygen more than it increases supply from the environment and can lead to functional hypoxia (inadequate oxygen supply to tissues) (Harrison et al., 2018; Verberk et al., 2011; Woods, 1999; but see Rostgaard and Jacobsen, 2005 and Jacobsen, 2020). We examine this problem by considering how elevation and temperature jointly affect (i) stream oxygen levels, (ii) oxygen *transport* to aquatic insects, and (iii) the ratio of oxygen supply to demand. Given the central, integrating roles of aerobic metabolism in insect performance and life history, we provide additional detail in this section compared to the others.

Whether high-elevation streams are perceived as oxygen-rich or oxygen-poor depends in part on how oxygen levels are expressed – as partial pressures (PO_2) or concentrations (Verberk et al., 2011). Atmospheric PO_2 declines with elevation (e.g., 21.2 kPa at sea level; 14.6 kPa at 3000 m at 10 °C), and because high-elevation streams typically have fast flows, with substantial turbulence and mixing, PO_2 in the water will match local atmospheric values. Expressed as PO_2, oxygen levels thus fall with elevation. At lower temperatures, however, oxygen solubility in water rises, which increases oxygen concentrations for a given PO_2. At high elevations, the temperature effect on solubility largely offsets decreasing local PO_2. Jacobsen (2000), for example, showed that oxygen concentrations were virtually constant at 8 mg/L in 46 small streams distributed across a 4000-m elevational gradient in the Andes.

For aquatic insects, however, oxygen level in the stream (expressed as partial pressure or concentration) is only one of several factors that determine rates of oxygen supply, which is the *rate* (flux, J) at which oxygen moves from the water into the insect by diffusion. Counterintuitively, oxygen supply declines in cold water – even though low temperatures increase oxygen solubility – because low temperatures also depress diffusion coefficients of oxygen and increase the thickness of boundary layers by making water more viscous (Fig. 2) (Denny, 1993). These factors are usefully summarized in a simplified version of Fick's first law of diffusion:

$$\text{Equation 1: } \boxed{J = AD_{O2}\alpha_{O2}\Delta PO_{O2}/L}$$

where A is the area of the respiratory surface (or whole body surface if oxygen is taken up everywhere), L is the diffusion length (related to the thicknesses of the adherent boundary layer of water and the insect cuticle), D_{O2} and α_{O2} are the diffusion coefficient and solubility of O_2 in water, respectively, and ΔP_{O2} is the difference between P_{O2} in the environment and inside the insect. From Eq. 1, Verberk et al. (2011) derived the oxygen supply index (OSI), which predicts how oxygen supply changes with both temperature and elevation (Fig. 4). The OSI predicts that oxygen supply decreases with increasing elevation and decreasing temperature. For example, the predicted OSI at 4000 m is only 32% of its value at sea level (see also Jacobsen, 2000). Thus, regardless of the metric used, partial pressure or concentration of oxygen, the potential rate of oxygen supply declines with elevation.

Finally, rate of oxygen supply itself matters only in relation to *metabolic demand for oxygen*; supply and demand must balance over time scales longer than a few minutes, and mismatches can lead to the buildup of anaerobic metabolites (Verberk et al., 2013). In principle, demand for oxygen by high-elevation insects should be low, because they are often cold, and metabolism generally declines more steeply with temperature than supply (Verberk et al., 2011; Woods, 1999). Rostgaard and Jacobsen (2005), however, showed that aquatic insects from several orders had metabolic rates that declined only modestly up to almost 4000 m in the Andes.

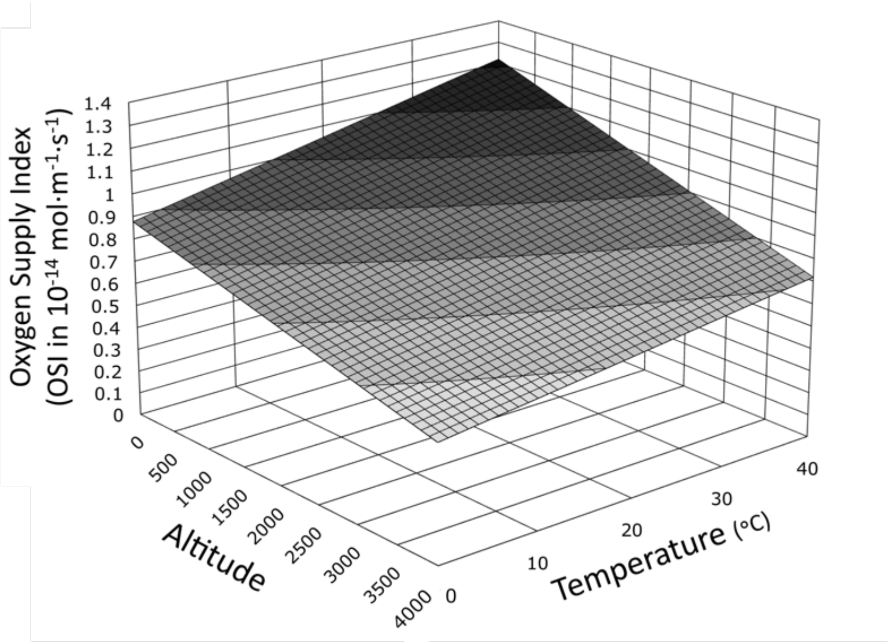


Fig. 4. In high-elevation streams, the oxygen supply index (OSI) is low because partial pressures of oxygen are low and because cold temperatures depress diffusion coefficients and increase the thicknesses of boundary layers (and these effects more than offset cold-driven increases in oxygen solubility). Thus, oxygen is less available to aquatic insects in high-elevation streams than it is in warmer, low-elevation streams. Stream warming will raise the OSI, but this effect likely will not be enough to offset rising metabolic demand for oxygen. Figure reproduced with permission from Verberk et al. (2011).

At high elevations, aquatic insects may increase oxygen supply by using specialized adaptations, which modify the parameters in Eq. 1. Oxygen uptake, for example, is enhanced by increasing respiratory surface area ($\boxed{A}$) relative to body mass, and this approach is used by species that have tracheal gills, thin-walled outgrowths of the tracheal system used for gas exchange (Wiggins, 2004). There is, however, no consistent support for this strategy among high-elevation aquatic insects: relative gill sizes of caddisfly larvae along a 4000 m elevation gradient in Ecuador did not increase significantly with elevation (Jacobsen, 2000), nor was there any difference between mean body size of stream macroinvertebrate families from below 1000 m and above 3500 m in Ecuador (Jacobsen et al., 2003). Oxygen uptake can also be enhanced by reducing diffusion lengths ($\boxed{L}$) through the cuticle or by minimizing the thickness of adherent boundary layers of water. Thin boundary layers can be achieved by an animal choosing positions in higher velocity flows (Resh & Rosenberg, 1984) or by disrupting them transiently using body or gill movements (Lancaster & Downes, Barbara, 2013; Rostgaard & Jacobsen, 2005). Finally, oxygen uptake can be increased by lowering the internal PO_2, such that the gradient ($\boxed{\Delta PO_2}$) is maximized (see Lane et al., 2017), or by expressing the oxygen-binding respiratory protein hemocyanin (Amore et al., 2009; Hagner-Holler et al., 2004). Hemocyanin is expressed, for example, in high-elevation stoneflies and may be upregulated at high temperatures (Hotaling et al., 2020).

The roles that oxygen will play in organismal responses to climate change will depend on understanding the complex set of physical and physiological factors bearing on oxygen supply and demand. Shifts in these factors will be manifest primarily through warming, which may threaten high-elevation aquatic insects by decreasing the ratio of oxygen supply to demand, especially during summertime spikes in daytime temperatures. If oxygen supply consistently falls short of demand, the organism faces functional hypoxia (Harrison et al., 2018). Even if not immediately lethal, functional hypoxia may still suppress the capacity for core functions such as homeostasis, activity, and growth, with negative effects on fitness (Pörtner & Knust, 2007). Indeed, sub-lethal effects of oxygen shortage in high-elevation streams have been proposed to drive community composition of macroinvertebrates (Jacobsen, 2008; Jacobsen et al., 2003; Verberk et al., 2011) and may prevent species from tracking their thermal niche by moving to higher elevations during climate warming (Jacobsen, 2020). Developmental changes in body size may also influence the relative effects of oxygen-temperature interactions. In particular, smaller individuals will have higher surface-to-volume ratios, whereas larger individuals may be able to ventilate themselves more easily by moving into higher flows or by using body movements to break up boundary layers. Unlike terrestrial insects (Greenlee & Harrison, 2005; Kirkton et al., 2005), we know little about the relative respiratory performance of aquatic individuals within and across instars.

Variable flow regimes. Flow distributes nutrients, homogenizes temperatures over small spatial scales, and influences oxygen supply to aquatic insects. Flow is also complex in both space and time, especially in mountain streams. Within stream substrates, where most insects live, flow fields reflect interactions between fast, turbulent water in the main channel (reaching 1.5 m s^{-1} or greater) and slower, meandering flows in interstitial spaces (<5 cm s^{-1} (Comiti et al., 2007; White, 1990). Slower, more laminar flows result in thicker boundary layers around surfaces (e.g., rocks, insects), which in turn reduce oxygen supply to insects (Vogel, 1994). Because flow velocities are highly variable over small spatial scales, individual insects have enormous potential to alter local flow regimes through their movement and may be able to offset thermally induced oxygen limitation or avoid scouring from floods (Hynes, 1970). Floods can occur daily and seasonally at high elevations (Jacobsen & Dangles, 2017) and disturb aquatic communities by dislodging individuals, scouring substrates, and decreasing food availability (Allan & Castillo, 2007).

As glaciers and snowfields melt at higher rates, flows are predicted to increase in the short term and alter the frequency and severity of flood and drought events (Gobiet et al., 2014; Milner et al., 2017). Although some high-elevation aquatic insects are able to recover after such disturbances (Poff et al., 2018), the higher magnitude and frequency of future floods may negatively affect insect populations and their food sources (Jacobsen et al., 2014). Additionally, increased glacial melting will lead to prolonged periods of higher turbidity and lower water temperatures, which may drive aquatic fauna downstream (Jacobsen et al., 2012, 2014). As glaciers and snowfields decline, however, decreasing runoff may allow some organisms to colonize locations closer to stream sources (Jacobsen et al., 2014). Thus, multidirectional shifts in elevation/longitudinal species ranges may reflect different phases of glacial shrinkage (Jacobsen et al., 2014). Eventually, meltwater sources in some regions will disappear altogether and the ensuing shift in streamflow (e.g., perennial to seasonal) will likely result in habitat-loss and extirpations of local taxa (Jacobsen et al., 2012).

High levels of ultraviolet radiation. Organisms living in high-elevation streams experience extremely high levels of damaging UV radiation. Incident UV-B radiation (320 nm) increases by 11% for every 1,000 m of elevation (Blumthaler et al., 1997). Terrestrially derived dissolved organic matter (DOM) strongly and selectively absorbs the most damaging UV-B radiation and decreases at higher elevations due to low inputs arising from sparse streamside terrestrial vegetation (Clements et al., 2008; Jacobsen & Dangles, 2017; Moser et al., 2019). Turbidity from glacial flour in meltwater can moderate UV exposure, but it does not selectively absorb UV like DOM (Rose et al., 2014). This can lead to very high UV exposure as glacial influences recede, exposing insects to as much as 70% or more of incoming solar UV radiation (Fig. 5; Clements et al., 2008; Williamson et al., 1996, 2001). Low temperatures in high-elevation streams also reduce the ability of invertebrates to repair UV-damaged DNA by slowing down the activity of key enzymes (photolyases) for photo-repair (MacFadyen et al., 2004).

High levels of UV can depress local macroinvertebrate densities. Experimental exclusion of solar UV radiation from high-elevation streams typically increases the abundance of stream insects, by as much as 54% (Clements et al., 2008; Kelly et al., 2003; Kiffney et al., 1997). Aquatic insects at higher latitudes are shielded from harmful UV radiation during winter, when streams are covered by snow and ice. This cover can last up to, or even beyond, the summer solstice when UV radiation is highest. However, climate warming is exposing benthic habitats to higher levels of UV radiation for longer periods, by shortening the duration of snow and ice cover, decreasing stream depths, and reducing glacial till (Benson et al., 2012; IPCC, 2019). Some aquatic insects may be able to reduce the impact of higher UV radiation via photoprotective pigments such as melanin (Loayza-Muro et al., 2013). Other species are able to detect and avoid UV radiation by selecting sheltered microhabitats (Johansson & Nyström, 2004).

Over longer timescales, however, climate change may result in lower UV exposure at moderately high elevations. Succession of terrestrial vegetation following deglaciation (Cannone et al., 2008) will increase concentrations of terrestrially derived DOM (Williamson et al., 2001). Further, treelines are expected to rise 100-640 m by 2100 (Grace et al., 2002), which, in combination with high DOM concentrations, should reduce UV exposure to levels similar to lower elevations (Frost et al., 2005).

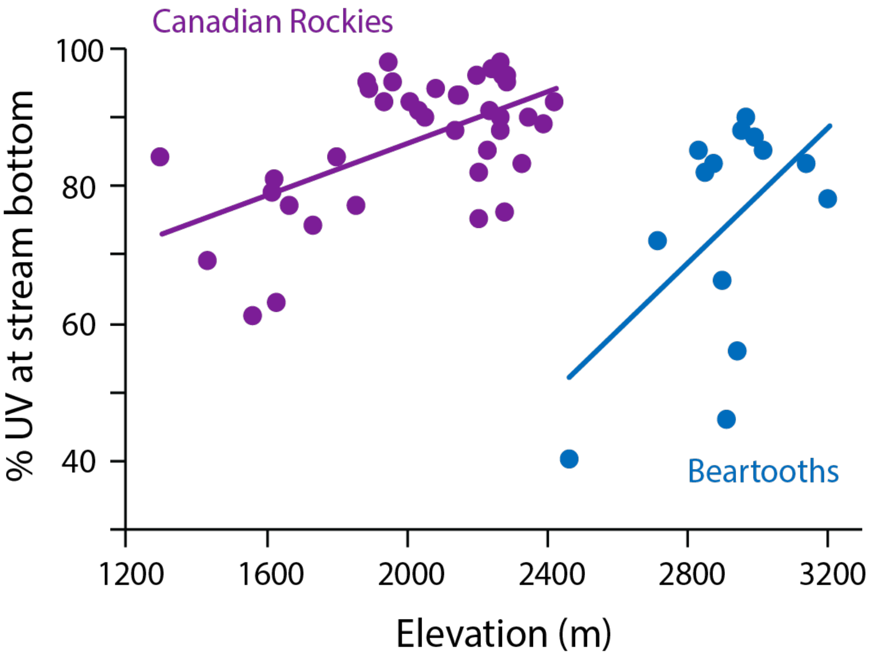


Fig. 5. Estimated percent of subsurface incident 320 nm UV radiation reaching the bottom of a 10 cm deep stream across an elevation gradient in the Canadian Rocky Mountains and the Beartooth Mountains of Montana, USA. Estimates are based on UV transparency measurements from the surfaces of lakes from which streams flow at the given elevation. Canadian Rockies data from Olson et al. (2018); Beartooth Mountains data from Williamson, unpublished.

Low ionic strength. Ion concentrations in high-elevation streams are generally low but can be highly variable (Fig. 3). Ionic concentrations depend on the relative contributions of hydrological sources, streamflow, and bedrock type, especially igneous versus calcareous (Stottlemyer & Troendle, 1992). Headwaters often have reduced ionic content compared to lower reaches, with specific conductivities below 20 µS cm^{-1}, but sometimes as low as 2 µS cm^{-1} (close to distilled water; Fig. 3) (Hotaling et al., 2019, 2020; Tronstad et al., in press). Low ionic concentrations are common in systems that drain metamorphic rocks or that are fed primarily by glacier or snowmelt (reviewed in Jacobsen & Dangles, 2017). Maintaining ion homeostasis in dilute water is an underappreciated challenge that may influence high-elevation aquatic insects.

The challenge of maintaining ion homeostasis stems from strong gradients between tissues and water (Dowse et al., 2017; Kefford, 2019). Inward fluxes of water are driven by large osmotic gradients, generally 200 – 400 mOsm/L, across the cuticle, respiratory surfaces, and gut. Large ionic gradients in the opposite direction drive losses of salts, most critically sodium and chloride, even though most insects have mechanisms for recapturing ions from urine and feces (Phillips et al., 1987). Ionic losses are exacerbated by having to produce large quantities of urine to offset osmotic uptake of water (Buchwalter et al., 2002; Kapoor, 1979). To cope, many aquatic insects have evolved chloride cells, chloride epithelia, or anal papillae to capture ions from dilute water (Komnick, 1977; Scheibener et al., 2016) and at least some stonefly nymphs can osmoregulate even in distilled water (Kapoor, 1979). However, iono- and osmoregulation can be energy and oxygen-intensive (Buchwalter et al., 2002; Kapoor, 1978; Orr & Buchwalter, 2020).

Climate change may exacerbate osmoregulatory challenges. In high-elevation streams, ion concentrations are typically highest when flows are low. During periods of heavy runoff, like spring and early summer, the ionic strength of alpine streams can be diluted by a factor of 2 or 3 (Caine & Thurman, 1990). As glaciers and snow fields melt at higher rates, flows during runoff are expected to increase in the short term (Milner et al., 2017), which may expose insects to more severe and prolonged periods of low ionic strength. In the long-term, however, stream ionic concentrations may rebound in response to lower flows as glaciers disappear.

Interactions among abiotic challenges. Although the challenges described above are physically and physiologically distinct, they are likely to interact strongly (Fig. 6). Below, we discuss four such interactions that may challenge high-elevation aquatic insects during climate change.

The first is an interaction between water temperature and UV radiation (Fig. 6A). Climate change will subject high-elevation aquatic insects to greater levels of damaging UV radiation due to decreased snow cover, water depth, and levels of glacial flour (IPCC, 2019). Because the action of UV-repair enzymes is slowed by low temperatures, higher levels of UV damage may be especially difficult to sustain in cold, high-elevation streams (MacFadyen et al., 2004). In this context, rising stream temperatures may thus help aquatic insects.

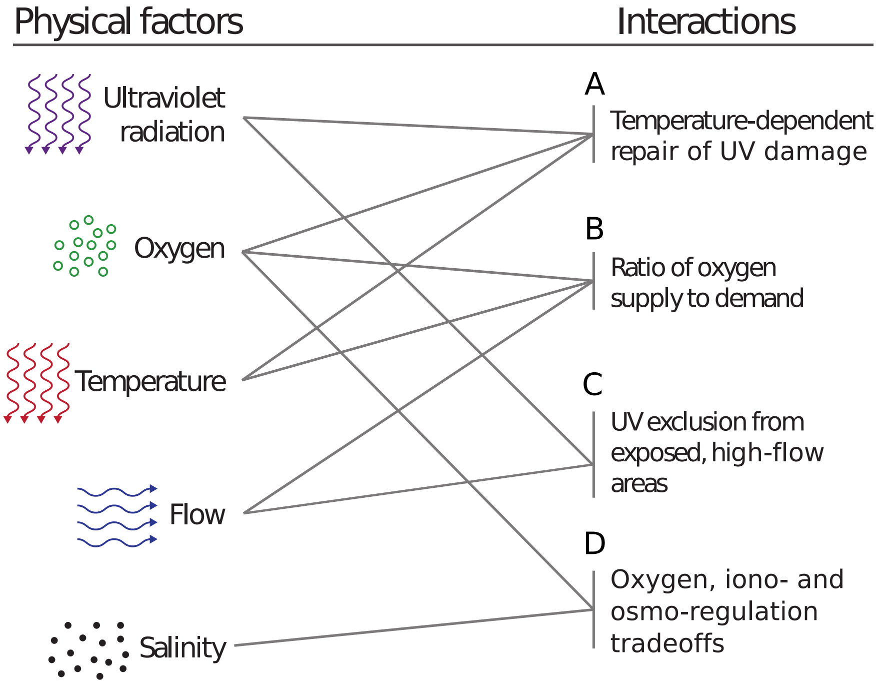


Fig. 6. Many of the physiological challenges experienced by high-elevation stream insects result from complex interactions among abiotic factors.

Second, we expect a three-way interaction among temperature, oxygen, and flow, which jointly determine the ratio of oxygen supply to demand in aquatic insects (Fig. 6B). In summer, the co-occurrence of higher temperatures and lower flows will likely be acutely problematic: Higher temperatures will stimulate demand for oxygen, while lower flows will make it difficult to obtain adequate supply across thicker boundary layers. This proposed mechanism is consistent with studies showing that oxygen limitation can influence upper thermal limits in aquatic insects (Pörtner, 2001; Verberk et al., 2013, 2016; Verberk & Calosi, 2012).

Third, we expect warming streams to drive interactions among UV, thermal, and respiratory challenges (Fig. 6C). In particular, higher levels of UV radiation may force aquatic insects into more sheltered locations (Johansson & Nyström, 2004) with lower flows, magnifying the oxygen stress arising from higher stream temperatures. More generally, higher UV will restrict the range of behavioral options available to individuals moving in response to other high-elevation challenges. However, negative effects of UV damage may be offset by faster-working UV repair enzymes, as explained above.

The fourth is an interaction between low ionic strength and oxygen homeostasis (Fig. 6D). In freshwater, ion capture is energy-intensive, requiring specialized cells with abundant mitochondria. In freshwater fishes, ion homeostasis can account for 20% of an organism's energy budget (Soengas et al., 2007). These figures may be even higher in aquatic insects given that they exhibit higher ratios of surface area to volume (Buchwalter et al., 2002; but see Edwards, 1982). In some insects (e.g., stoneflies), chloride cells occur on the same tracheal gill filaments involved in oxygen uptake, suggesting tradeoffs between osmo/ionoregulatory and respiratory functions (Kapoor, 1978; Kapoor & Zachariah, 1973; Komnick, 1977). As streams warm, rates of active ion transport will likely increase, raising the metabolic cost of ionoregulation (Orr & Buchwalter, 2020). These interactions may magnify respiratory challenges arising from lower oxygen supply at higher elevations and may prevent uphill shifts of local populations into reaches with lower conductivity.

Insect responses

Whether high-elevation aquatic insects will persist under climate change depends largely on how they respond to the environmental challenges described above. Below, we discuss three fundamental classes of response – behavior, plasticity, and evolution. In almost all cases, we conclude that too little is known about high-elevation insects to make confident predictions, highlighting the pressing need for more research on these taxa.

Behavior. By moving within streams, aquatic organisms can alter their local environments. Such movements may allow individuals to escape, or at least mitigate, deteriorating climatic conditions. Because so few behavioral studies have focused on high-elevation aquatic taxa, the possibilities outlined below draw mostly from low-elevation studies.

To mitigate the effects of oxygen shortages at high elevations, individuals may rely on more frequent respiratory movements. When oxygen availability is reduced due to high temperature or low flow, many aquatic insects undulate their bodies (Gallon et al., 2008; Genkai-Kato et al., 2000; Williams et al., 1987). This disrupts boundary layers around respiratory surfaces and increases oxygen supply (Eriksen, 1963). Such movements may have enabled some caddisfly lineages to radiate into lentic habitats (Wiggins & Wichard, 1989) and may allow high-elevation insects to mitigate low-oxygen stress, at the cost of additional energy expended.

High-elevation insects may also offset changing physical conditions by repositioning themselves locally within the stream substrate. In mountain streams, water velocity is highly heterogeneous over small spatial scales and affects insect micro-distributions (Brooks et al., 2005; Mérigoux & Dolédec, 2004). By adjusting their position on substrate, aquatic insects can alter flows around their bodies (Genkai-Kato et al., 2005; Kovalak, 1979; Wiley & Kohler, 1980). This allows them to escape damaging flow conditions during floods (Townsend et al., 1997) and may relieve oxygen limitation arising from increased temperatures, low dissolved oxygen, or low flows (Genkai-Kato et al., 2005). Additionally, during warm events, insects may be able to exploit cool patches of upwelling groundwater (e.g., Ebersole et al., 2001) or local inputs from icy seeps and springs (Hotaling et al., 2017, 2019; Tronstad et al., in press). Hyporheic habitats may also provide refuge (Wood et al., 2010) from warm, summer conditions and from UV radiation. However, flow and dissolved oxygen typically decrease with depth, which may limit the utility of vertical migrations for solving problems of oxygen supply and demand (Williams & Hynes, 1974).

High-elevation aquatic insects may also exploit temperature gradients on larger spatial scales. First, individuals, and possibly entire communities, may migrate upslope into cooler temperatures, if there is appropriate habitat still higher up (Giersch et al., 2015; Milner et al., 2008; Sheldon, 2012). Second, aquatic insects could disperse across drainages to more suitable habitat as winged adults. This may not be a viable option for many high-elevation species given that topographies are complex and flight abilities of stream insects are often poor (Finn et al., 2006; Giersch et al., 2017). However, a high degree of genetic connectivity among some alpine stoneflies has been observed (Dussex et al., 2016; Hotaling et al., 2018).

Physiological plasticity. Although plasticity is used by many organisms to cope with environmental variation (Gotthard et al., 1995; Levins, 1968), whether it can be used to mitigate the effects of climate change in aquatic systems remains largely unknown, especially at high elevations.

Many studies have found plasticity in upper thermal tolerance traits, (e.g., Calosi et al., 2008; Dallas & Rivers-Moore, 2012; Heiman & Knight, 1972; Shah, Funk, et al., 2017), but fewer have focused on plasticity in other traits (e.g., the temperature-sensitivity of metabolic rates, Buffington, 1969). In line with the expectation that species evolving in more variable environments should have greater acclimation capacities (Ghalambor et al., 2006; Janzen, 1967), mayflies from alpine streams showed greater acclimation of CTMAX in temperate compared to tropical streams (Shah, Funk, et al., 2017). This pattern, however, was reversed in stoneflies, indicating that plasticity can vary among co-occurring taxa (Shah, Funk, et al., 2017). To date, most studies on thermal acclimation of insects have focused on short-term exposure to stressful temperatures. However, the level and type (reversible or irreversible) of plasticity an organism exhibits is related to the length of exposure to the new environment (Angilletta Jr., 2009; Piersma & Drent, 2003). Because climate change will result in longer term changes to stream thermal regimes, experiments addressing long-term effects are urgently needed (Rezende et al., 2014).

Rising temperatures reduce the ratio of oxygen supply to demand, and therefore, plasticity in traits related to oxygen transport may be necessary for aquatic insect survival. Terrestrial insects show significant plasticity in tracheal morphology in response to experimentally altered oxygen levels; insects developing in hypoxia have larger primary tracheal tubes and greater elaboration of the finest, tracheolar branches (Harrison et al., 2006; Loudon, 1989). However, few studies have explored the plasticity of tracheal systems in aquatic insects, focusing instead on the plasticity of external gill structures. Wichard (1974) showed that the number of gill filaments of limnephilid caddisflies varied inversely with the oxygen level in experimental tanks. Similarly, both wild and captive caddisflies show increased numbers of gill tufts and gill filaments when exposed to warm conditions (Badcock et al., 1987). However, these findings require functional testing to understand their role in organismal performance.

Whether aquatic insects show plasticity in response to abiotic factors beyond temperature is less well known. For example, although climate change is altering flow regimes, no study has explicitly examined plasticity in morphology or overall body shape as a function of flow. In one instance, stonefly body shape was shown to be correlated with varying flow regimes but it is unclear whether these differences arose via plasticity or local adaptation (Edwards & Moore, 2017). Flow-induced plasticity in body shape may be common in aquatic insects given how common it is for other aquatic taxa, especially fish (e.g., Haas et al., 2015). Additionally, changing ionic strengths in high-elevation streams may drive plasticity in chloride cell morphology and associated metabolic costs. Aquatic insects reared in water with reduced ionic content show greater mitochondrial densities and more infolding of plasma membranes associated with ion pumping epithelial cells (Komnick, 1977). The metabolic consequences of changes in ionoregulatory capacity may be large and interact with other aspects of oxygen physiology, but how these influence aquatic insect survival is unknown. Lastly, no studies have investigated the potential for plasticity in UV tolerance in aquatic insects. However, plasticity in the pigmentation of other aquatic invertebrates exists (Rangel et al., 2005) and may be possible in high-elevation insects.

Evolution. Can high-elevation stream insects evolve rapidly enough to keep pace with climate change? Evolution on contemporary timescales has been reviewed elsewhere (e.g., Hendry et al., 2018; Stockwell et al., 2003), and many documented examples now exist (e.g., Grant et al., 2017; Lescak et al., 2015; Reznick et al., 1997). However, the adaptive potential of high-elevation taxa *per se* is unknown and will depend on a complex set of factors.

Evolutionary change proceeds most rapidly when standing genetic variation relevant to new conditions already exists in a population (Barrett & Schluter, 2008). One approach to understanding evolutionary potential of high-elevation aquatic insects thus would be to identify key ecologically relevant phenotypes – e.g., thermal tolerance limits, oxygen handling capacity, or UV resistance – and to assess genetic variation at loci underlying them. To date, these kinds of studies have not been done for any high-elevation taxa. Another approach would be to assess levels of genetic diversity broadly across genomes within and among populations, with higher genetic diversity suggesting greater adaptive potential (i.e., more standing genetic variation on which selection can act).

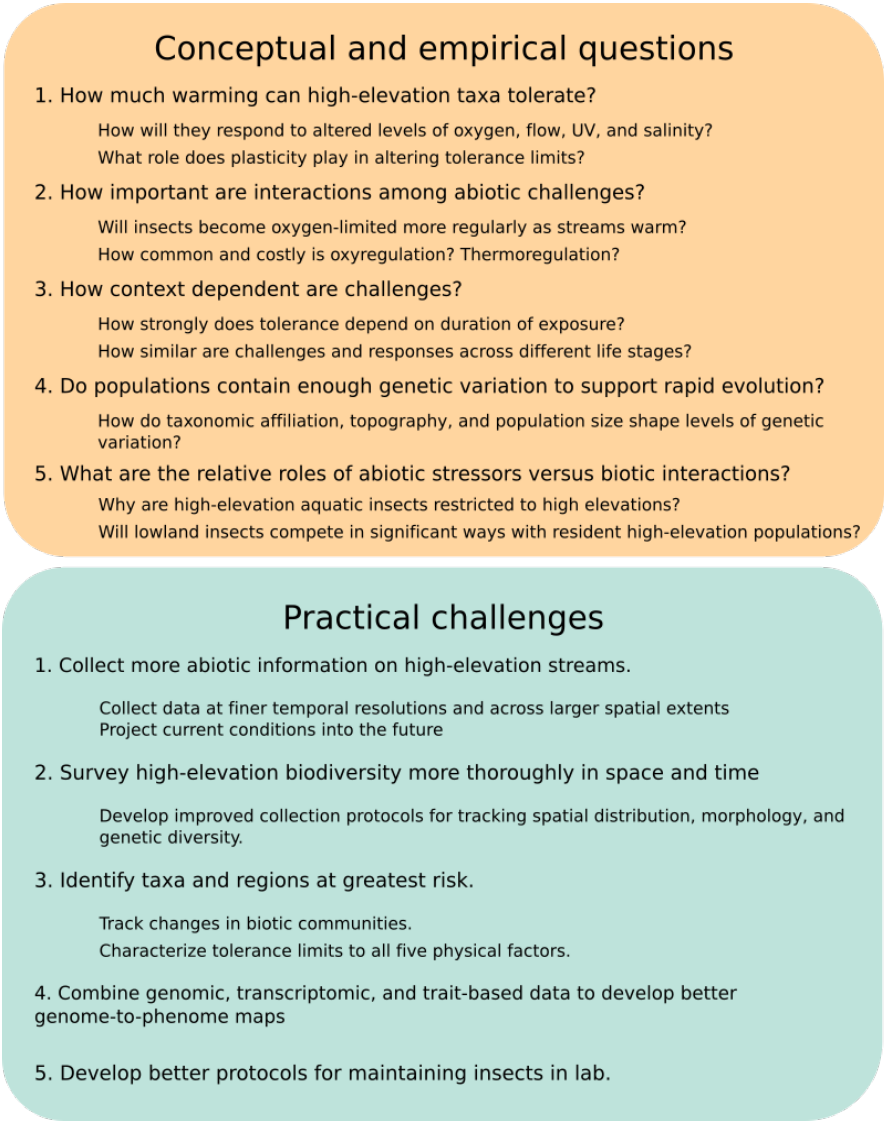
There are two general expectations about patterns of genetic diversity in high-elevation stream insects. The first is that individual populations should contain low levels of genetic diversity because they tend to be small, isolated, and perhaps of recent origin (i.e., greater influence of genetic drift, less gene flow among populations, less time to accrue genetic diversity) (e.g., Finn et al., 2006). Empirical support for this pattern has been mixed. For instance, among montane mayflies in the Andes, higher elevation populations are less genetically diverse and show reduced gene flow versus populations 1,400 m downslope (Polato et al., 2017). Finn et al. (2013), by contrast, showed that mayfly (*Baetis alpinus*) populations inhabiting more glacially influenced streams contained greater mitochondrial genetic diversity. Climate change itself may further erode the evolutionary potential of high-elevation aquatic insects. Given the possibility for substantial isolation by distance in headwaters (Finn & Poff, 2011), declining population sizes or local extirpation could exacerbate isolation, reduce gene flow, and increase the relative influence of genetic drift versus other evolutionary forces (e.g., gene flow) (Finn et al., 2013). Consistent with this idea, warming has been posited to explain the loss of genetic diversity in a high-elevation stonefly (Jordan et al., 2016).

At the same time, barriers to dispersal paired with weak capacities for active dispersal may lead to substantial genetic differentiation among populations in space and possibly local adaptation (Kawecki & Ebert, 2004). Here too, the data are mixed. Dussex et al. (2016) tested for differentiation within co-distributed species of mountain stoneflies that differ in wingedness, and found genetic structuring within the wingless species (*Zelandoperla fenestrata*), indicating reduced gene flow among populations, but not within the winged species (*Z. decorata*). More recently, Hotaling et al. (2018) identified a link between timing of glacial retreat and the evolutionary history of *L. tumana*, with genome-wide evidence supporting a history of divergence with gene flow since the last glacial maximum (~20,000 years ago). These findings run counter to general expectations of isolation with limited gene flow and suggest that gene flow potential and effective population sizes of high-elevation stream insects may be larger than previously thought. However, populations can also be differentiated enough that substantial genetic variation exists at landscape scales (e.g., Finn et al., 2006; Monaghan et al., 2001; Polato et al., 2018). It is thus possible, and perhaps likely, that general conclusions are inappropriate and that patterns of differentiation and overall levels of genetic diversity vary strongly among taxa (e.g., Monaghan et al., 2002). At present, no targeted studies of local adaptation have been conducted for high-elevation stream insects. However, the results of a single, multi-taxon reciprocal transplant study showed that lower elevation taxa can survive harsher, headwater conditions for a short period of time (2 weeks; Madsen et al., 2015).

Conclusions and outlook

One possibility is that high-elevation stream insects will endure in the face of climate change. Indeed, some species can tolerate at least short-term exposure to high temperatures (> 20 °C) (Hotaling et al., 2020),and cool microclimates may provide refugia from rapidly changing conditions (Tronstad et al., in press). In fact, there is some evidence that annual snow and ice melt, which should continue into the next century, may help maintain community structure over larger spatial scales even after complete deglaciation (Muhlfeld et al., 2020). Due to the variable nature of high-elevation streams, particularly in temperate regions, resident taxa may also exhibit impressive capacities for plasticity and adaptation, which may shield them from increasingly stressful conditions.

More likely, however, is that high-elevation insects will be severely threatened by future climate change (Hannah et al., 2007). Although conserving these ecosystems is a high priority (Hotaling et al., 2017), it will be difficult because slowing climate change will require global rather than local action (Khamis et al., 2014). We propose that the most productive course of action is, first, to understand the challenges that threaten high-elevation taxa and the mechanisms by which they might respond. This may allow us to more clearly identify threats and vulnerable species so that resources can be allocated where they are needed most. However, our current knowledge is severely limited, as reflected by our common conclusion in this review that, "little is known." We therefore provide a prioritized list of key conceptual questions and practical challenges to guide future research and fill important knowledge gaps (Fig. 7).



Conceptual and empirical questions

1. How much warming can high-elevation taxa tolerate?
    - How will they respond to altered levels of oxygen, flow, UV, and salinity?
    - What role does plasticity play in altering tolerance limits?
2. How important are interactions among abiotic challenges?
    - Will insects become oxygen-limited more regularly as streams warm?
    - How common and costly is oxyregulation? Thermoregulation?
3. How context dependent are challenges?
    - How strongly does tolerance depend on duration of exposure?
    - How similar are challenges and responses across different life stages?
4. Do populations contain enough genetic variation to support rapid evolution?
    - How do taxonomic affiliation, topography, and population size shape levels of genetic variation?
5. What are the relative roles of abiotic stressors versus biotic interactions?
    - Why are high-elevation aquatic insects restricted to high elevations?
    - Will lowland insects compete in significant ways with resident high-elevation populations?

Practical challenges

1. Collect more abiotic information on high-elevation streams.
    - Collect data at finer temporal resolutions and across larger spatial extents
    - Project current conditions into the future
2. Survey high-elevation biodiversity more thoroughly in space and time
    - Develop improved collection protocols for tracking spatial distribution, morphology, and genetic diversity.
3. Identify taxa and regions at greatest risk.
    - Track changes in biotic communities.
    - Characterize tolerance limits to all five physical factors.
4. Combine genomic, transcriptomic, and trait-based data to develop better genome-to-phenome maps
5. Develop better protocols for maintaining insects in lab.

Fig. 7. A prioritized list of key conceptual questions to answer and practical challenges to solve. Answering the conceptual questions will provide a firmer theoretical foundation for connecting physical and physiological challenges of climate change to the expected responses of individuals and populations. Solving the practical challenges will provide better long-term data on spatial and temporal variation in conditions and will provide new tools and information for carrying out more comprehensive experiments. Ultimately, these aims will aid in forecasting species responses to climate change and conserving stream insect diversity at high elevations.

We especially stress the need for more work on high-elevation stream insects in the tropics, which have been studied far less and may be more threatened than those in temperate regions (Jacobsen & Dangles, 2017; Polato et al., 2018). Traditional thinking suggests that temperate populations are most at risk because air temperatures are projected to warm most rapidly at high latitudes (IPCC 2018). However, winter may provide some respite from high temperatures by recharging meltwater sources with new snowfall. This may slow the rates at which temperate glaciers recede and streams warm. Tropical and subtropical streams do not exhibit as much seasonal variation in temperature, however, and will likely warm more consistently and rapidly (Boulton et al., 2008). Current data from the Andes in South America and the Himalayas in South Asia show rapid glacial retreat and warming (Shrestha & Aryal, 2011; Vuille et al., 2008). Because tropical stream insects tend to have narrow thermal tolerances (Shah, Gill, et al., 2017) and a reduced capacity to acclimate (Shah, Funk, et al., 2017), they may be less physiologically equipped to mitigate such challenges, making them especially susceptible to climate change (Polato et al., 2018).

Finally, we reiterate that although our focus is on abiotic factors, biotic interactions will also be crucial to understanding responses to climate change. For instance, it is likely that cold headwaters prevent invasion by lower elevation predators and competitors (Isaak et al., 2015). The fates of high-elevation insects may, therefore, depend on whether and how abiotic conditions set the upper elevation limits of *lower elevation* insects, how temperature, oxygen, flow, UV, and salinity influence predation and competition, and whether altered conditions under climate change will allow for widespread uphill migrations of lowland taxa (Dunson & Travis, 1991; Schmitz & Barton, 2014; Sheldon et al., 2011). For some species, another possible consequence of the upward migration of lower elevation populations is hybridization with high-elevation populations (Shah et al., 2020). Hybridization could introduce new ‘lowland’ alleles into high-elevation populations, creating the potential for genetic rescue (Hamilton & Miller, 2016), but may also accelerate population declines if maladaptive alleles swamp out local adaptation of high-elevation populations (Kirkpatrick & Barton, 1997).

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References

Allan, D. J., & Castillo, M. M. (2007). *Stream ecology: structure and function of running waters*. Springer Science & Business Media.

Amore, V., Belardinelli, M., Guerra, L., Buonocore, F., Fausto, A. M., Ubero-Pascal, N., & Fochetti, R. (2009). Do all stoneflies nymphs have respiratory proteins? Further data on the presence of hemocyanin in the larval stages of plecoptera species. *Insect Molecular Biology*, *18*(2), 203–211. https://doi.org/10.1111/j.1365-2583.2008.00859.x

Angilletta Jr., M. J. (2009). Looking for answers to questions about heat stress: researchers are getting warmer. *Functional Ecology*, *23*(2), 231–232. https://doi.org/10.1111/j.1365-2435.2009.01548.x

Badcock, R. M., Bales, M. T., & Harrison, J. D. (1987). Observations on gill number and respiratory adaptation in caddis larvae. In M. Bournaud & H. Tachet (Eds.), *Proceedings of the Fifth International Symposium on Trichoptera* (pp. 175–178). Dr. W. Junk Publishers.

Bale, J. S. (1993). Classes of insect cold hardiness. *Functional Ecology*, *7*(6), 751–753.

Barrett, R. D. H., & Schluter, D. (2008). Adaptation from standing genetic variation. *Trends in Ecology and Evolution*, *23*(1), 38–44. https://doi.org/10.1016/j.tree.2007.09.008

Beniston, M., Farinotti, D., Stoffel, M., Andreassen, L. M., Coppola, E., Eckert, N., Fantini, A., Giacona, F., Hauck, C., Huss, M., Huwald, H., Lehning, M., López-Moreno, J. I., Magnusson, J., Marty, C., Morán-Tejéda, E., Morin, S., Naaim, M., Provenzale, A., … Vincent, C. (2018). The European mountain cryosphere: A review of its current state, trends, and future challenges. *Cryosphere*, *12*(2), 759–794. https://doi.org/10.5194/tc-12-759-2018

Benson, B. J., Magnuson, J. J., Jensen, O. P., Card, V. M., Hodgkins, G., Korhonen, J., Livingstone, D. M., Stewart, K. M., Weyhenmeyer, G. A., & Granin, N. G. (2012). Extreme events, trends, and variability in Northern Hemisphere lake-ice phenology (1855-2005). *Climatic Change*, *112*(2), 299–323. https://doi.org/10.1007/s10584-011-0212-8

Blumthaler, M., Ambach, W., & Ellinger, R. (1997). Increase in solar UV radiation with altitude. *Journal of Photochemistry and Photobiology B: Biology*. https://doi.org/10.1016/S1011-1344(96)00018-8

Boulton, A. J., Boyero, L., Covich, A. P., Dobson, M., Lake, S., & Pearson, R. (2008). Are tropical streams ecologically different from temperate streams? In D. Dudgeon (Ed.), *Tropical Stream Ecology* (pp. 257–284). Academic Press. https://doi.org/10.1016/B978-012088449-0.50011-X

Bradley, R. S., Vuille, M., Diaz, H. F., & Vergara, W. (2006). Threats to water supplies in the tropical andes. In *Science* (Vol. 312, Issue 5781, pp. 1755–1756). https://doi.org/10.1126/science.1128087

Braune, E., Richter, O., Söndgerath, D., & Suhling, F. (2008). Voltinism flexibility of a riverine dragonfly along thermal gradients. *Global Change Biology*, *14*(3), 470–482. https://doi.org/10.1111/j.1365-2486.2007.01525.x

Brooks, A. J., Haeusler, T., Reinfelds, I., & Williams, S. (2005). Hydraulic microhabitats and the distribution of macroinvertebrate assemblages in riffles. *Freshwater Biology*, *50*(2), 331–344. https://doi.org/10.1111/j.1365-2427.2004.01322.x

Brown, L. E., Hannah, D. M., & Milner, A. M. (2007). Vulnerability of alpine stream biodiversity to shrinking glaciers and snowpacks. *Global Change Biology*, *13*(5), 958–966. https://doi.org/10.1111/j.1365-2486.2007.01341.x

Brown, L. E., Khamis, K., Wilkes, M., Blaen, P., Brittain, J. E., Carrivick, J. L., Fell, S., Friberg, N., Füreder, L., Gislason, G. M., Hainie, S., Hannah, D. M., James, W. H. M., Lencioni, V., Olafsson, J. S., Robinson, C. T., Saltveit, S. J., Thompson, C., & Milner, A. M. (2018). Functional diversity and community assembly of river invertebrates show globally consistent responses to decreasing glacier cover. *Nature Ecology and Evolution*, *2*(2), 325–333. https://doi.org/10.1038/s41559-017-0426-x

Buchwalter, D. B., Jenkins, J. J., & Curtis, L. R. (2002). Respiratory strategy is a major determinant of [3H]water and [14C]chlorpyrifos uptake in aquatic insects. *Canadian Journal of Fisheries and Aquatic Sciences*, *59*(8), 1315–1322. https://doi.org/10.1139/f02-107

Buffington, J. D. (1969). Temperature acclimation of respiration in Culex pipiens pipiens (Diptera: Culicidae) and the influence of seasonal selection. *Comparative Biochemistry and Physiology*, *30*(5), 865–878.

Caine, N., & Thurman, E. M. (1990). Temporal and spatial variations in the solute content of an alpine stream, Colorado Front Range. *Geomorphology*, *4*(1), 55–72. https://doi.org/10.1016/0169-555X(90)90026-M

Calosi, P., Bilton, D. T., & Spicer, J. I. (2008). Thermal tolerance, acclimatory capacity and vulnerability to global climate change. *Biology Letters*, *4*(1), 99–102. https://doi.org/10.1098/rsbl.2007.0408

Cannone, N., Diolaiuti, G., Guglielmin, M., & Smiraglia, C. (2008). Accelerating climate change impacts on alpine glacier forefield ecosystems in the European Alps. *Ecological Applications*, *18*(3), 637–648. https://doi.org/10.1890/07-1188.1

Chown, S. L., Duffy, G. A., & Sørensen, J. G. (2015). Upper thermal tolerance in aquatic insects. *Current Opinion in Insect Science*, *11*, 78–83. https://doi.org/10.1016/j.cois.2015.09.012

Clarke, A. (1998). Temperature and energetics: an introduction to cold ocean physiology. In H. Pörtner & R. Playle (Eds.), *Cold Ocean Physiology* (pp. 3–30). Cambridge University Press. https://doi.org/10.1017/CBO9780511661723.002

Clements, W. H., Brooks, M. L., Kashian, D. R., & Zuellig, R. E. (2008). Changes in dissolved organic material determine exposure of stream benthic communities to UV-B radiation and heavy metals: Implications for climate change. *Global Change Biology*, *14*(9), 2201–2214. https://doi.org/10.1111/j.1365-2486.2008.01632.x

Comiti, F., Mao, L., Wilcox, A., Wohl, E. E., & Lenzi, M. A. (2007). Field-derived relationships for flow velocity and resistance in high-gradient streams. *Journal of Hydrology*, *340*(1–2), 48–62. https://doi.org/10.1016/j.jhydrol.2007.03.021

Dallas, H. F., & Rivers-Moore, N. A. (2012). Critical Thermal Maxima of aquatic macroinvertebrates: Towards identifying bioindicators of thermal alteration. *Hydrobiologia*, *679*(1), 61–76. https://doi.org/10.1007/s10750-011-0856-4

Denlinger, D. L., & Lee, R. E. (2010). Low Temperature Biology of Insects. In D. L. Denlinger & R. E. J. Lee (Eds.), *Low Temperature Biology of Insects*. Cambridge University Press. https://doi.org/10.1017/CBO9780511675997

Denny, M. W. (1993). *Air and water: the biology and physics of life's media*. Princeton University Press.

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C., & Martin, P. R. (2008). Impacts of climate warming on terrestrial ectotherms across latitude. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(18), 6668–6672. https://doi.org/10.1073/pnas.0709472105

DeWalt, R. E., Favret, C., & Webb, D. W. (2005). Just How Imperiled Are Aquatic Insects? A Case Study of Stoneflies (Plecoptera) in Illinois. *Annals of the Entomological Society of*

America, *98*(6), 941–950. https://doi.org/10.1603/0013-8746(2005)098[0941:JHIAAI]2.0.CO;2

Dillon, M. E., Woods, H. A., Wang, G., Fey, S. B., Vasseur, D. A., Telemeco, R. S., Marshall, K., & Pincebourde, S. (2016). Life in the frequency domain: The biological impacts of changes in climate variability at multiple time scales. *Integrative and Comparative Biology*, *56*(1), 14–30. https://doi.org/10.1093/icb/icw024

Dowse, R., Palmer, C. G., Hills, K., Torpy, F., & Kefford, B. J. (2017). The mayfly nymph Austrophlebioides pusillus harker defies common osmoregulatory assumptions. *Royal Society Open Science*, *4*(1), 1–9. https://doi.org/10.1098/rsos.160520

Durance, I., & Ormerod, S. J. (2007). Climate change effects on upland stream macroinvertebrates over a 25-year period. *Global Change Biology*, *13*(5), 942–957. https://doi.org/10.1111/j.1365-2486.2007.01340.x

Dussex, N., Chuah, A., & Waters, J. M. (2016). Genome-wide SNPs reveal fine-scale differentiation among wingless alpine stonefly populations and introgression between winged and wingless forms. *Evolution*, *70*(1), 38–47. https://doi.org/10.1111/evo.12826

Ebersole, J. L., Liss, W. J., & Frissell, C. A. (2001). Relationship between stream temperature, thermal refugia and rainbow trout Oncorhynchus mykiss abundance in arid-land streams in the northwestern United States. *Ecology of Freshwater Fish*, *10*(1), 1–10. https://doi.org/10.1034/j.1600-0633.2001.100101.x

Edwards, D. D., & Moore, P. A. (2017). Body-shape variation of Acroneuria lycorias (Plecoptera:Perlidae) nymphs across magnitude and frequency stream flows. *Freshwater Science*, *36*(3), 571–584. https://doi.org/10.1086/693011

Edwards, H. A. (1982). Aedes Aegypti: Energetics of Osmoregulation. *Journal of Experimental Biology*, *101*(1), 135–141.

Elser, J. J., Wu, C., González, A. L., Shain, D. H., Smith, H. J., Sommaruga, R., Williamson, C. E., Brahney, J., Hotaling, S., Vanderwall, J., Yu, J., Aizen, V., Aizen, E., Battin, T. J., Camassa, R., Feng, X., Jiang, H., Lu, L., Qu, J. J., … Saros, J. E. (n.d.). *Key rules of life and the fading cryosphere: impacts in alpine lakes and streams*.

Eriksen, C. H. (1963). Respiratory regulation in Ephemera simulans Walker and Hexagenia limbata (Serville) (Ephemeroptera). *Journal of Experimental Biology*, *40*, 455–467.

Finn, D. S., Khamis, K., & Milner, A. M. (2013). Loss of small glaciers will diminish beta diversity in Pyrenean streams at two levels of biological organization. *Global Ecology and Biogeography*, *22*(1), 40–51. https://doi.org/10.1111/j.1466-8238.2012.00766.x

Finn, D. S., & Poff, N. L. (2011). Examining spatial concordance of genetic and species diversity patterns to evaluate the role of dispersal limitation in structuring headwater metacommunities. *Journal of the North American Benthological Society*, *30*(1), 273–283. https://doi.org/10.1899/10-035.1

Finn, D. S., Theobald, D. M., Black IV, W. C., & Poff, N. L. (2006). Spatial population genetic structure and limited dispersal in a rocky mountain alpine stream insect. *Molecular Ecology*, *15*(12), 3553–3566. https://doi.org/10.1111/j.1365-294X.2006.03034.x

Frost, P. C., Larson, J. H., Kinsman, L. E., Lamberti, G. A., & Bridgham, S. D. (2005). Attenuation of ultraviolet radiation in streams of northern Michigan. *Journal of the North American Benthological Society*, *24*(2), 246–255. https://doi.org/10.1899/04-127.1

Gallon, C., Hare, L., & Tessier, A. (2008). Surviving in anoxic surroundings: how burrowing aquatic insects create an oxic microhabitat. *Journal of the North American Benthological Society*, *27*(3), 570–580. https://doi.org/10.1899/07

Genkai-Kato, M., Mitsuhashi, H., Kohmatsu, Y., Miyasaka, H., Nozaki, K., & Nakanishi, M. (2005). A seasonal change in the distribution of a stream-dwelling stonefly nymph reflects oxygen supply and water flow. *Ecological Research*, *20*(2), 223–226. https://doi.org/10.1007/s11284-004-0029-2

Genkai-Kato, M., Nozaki, K., Mitsuhashi, H., Kohmatsu, Y., Miyasaka, H., & Nakanishi, M. (2000). Push-up response of stonefly larvae in low-oxygen conditions. *Ecological Research*, *15*(2), 175–179. https://doi.org/10.1046/j.1440-1703.2000.00336.x

Ghalambor, C. K., Huey, R. B., Martin, P. R., Tewksbury, J. J., & Wang, G. (2006). Are mountain passes higher in the tropics? janzen's hypothesis revisited. *Integrative and Comparative Biology*, *46*(1), 5–17. https://doi.org/doi.org/10.1093/icb/icj003

Giersch, J. J., Hotaling, S., Kovach, R. P., Jones, L. A., & Muhlfeld, C. C. (2017). Climate-induced glacier and snow loss imperils alpine stream insects. *Global Change Biology*, *23*(7), 2577–2589. https://doi.org/10.1111/gcb.13565

Giersch, J. J., Jordan, S., Luikart, G., Jones, L. A., Hauer, F. R., & Muhlfeld, C. C. (2015). Climate-induced range contraction of a rare alpine aquatic invertebrate. *Freshwater Science*, *34*(1), 53–65. https://doi.org/10.1086/679490

Gill, B. A., Kondratieff, B. C., Casner, K. L., Encalada, A. C., Flecker, A. S., Gannon, D. G., Ghalambor, C. K., Guayasamin, J. M., Poff, N. L., Simmons, M. P., Thomas, S. A., Zamudio, K. R., & Funk, W. C. (2016). Cryptic species diversity reveals biogeographic support for the 'mountain passes are higher in the tropics' hypothesis. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1832), 20160553. https://doi.org/10.1098/rspb.2016.0553

Gobiet, A., Kotlarski, S., Beniston, M., Heinrich, G., Rajczak, J., & Stoffel, M. (2014). 21st century climate change in the European Alps-A review. *Science of the Total Environment*, *493*, 1138–1151. https://doi.org/10.1016/j.scitotenv.2013.07.050

Gotthard, K., Nylin, S., & Nylin, S. (1995). Adaptive Plasticity and Plasticity as an Adaptation: A Selective Review of Plasticity in Animal Morphology and Life History. *Oikos*, *74*(1), 3. https://doi.org/10.2307/3545669

Grace, J., Berninger, F., & Nagy, L. (2002). Impacts of climate change on the tree line. *Annals of Botany*, *90*(4), 537–544. https://doi.org/10.1093/aob/mcf222

Grant, P. R., Rosemary Grant, B., Huey, R. B., Johnson, M. T. J., Knoll, A. H., & Schmitt, J. (2017). Evolution caused by extreme events. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *372*(1723), 1–10. https://doi.org/10.1098/rstb.2016.0146

Greenlee, K. J., & Harrison, J. F. (2005). Respiratory changes throughout ontogeny in the tobacco hornworm caterpillar, Manduca sexta. *Journal of Experimental Biology*, *208*(7), 1385–1392. https://doi.org/10.1242/jeb.01521

Haas, T. C., Heins, D. C., & Blum, M. J. (2015). Predictors of body shape among populations of a stream fish (Cyprinella venusta, Cypriniformes: Cyprinidae). *Biological Journal of the Linnean Society*, *115*(4), 842–858. https://doi.org/10.1111/bij.12539

Hagner-Holler, S., Schoen, A., Erker, W., Marden, J. H., Rupprecht, R., Decker, H., & Burmester, T. (2004). A respiratory hemocyanin from an insect. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(3), 871–874. https://doi.org/10.1073/pnas.0305872101

Hamilton, J. A., & Miller, J. M. (2016). Adaptive introgression as a resource for management and genetic conservation in a changing climate. *Conservation Biology*, *30*(1), 33–41. https://doi.org/10.1111/cobi.12574

Hannah, D. M., Brown, L. E., Milner, A. M., Gurnell, A. M., McGregor, G. R., Petts, G. E., Smith, B. P. G., & Snook, D. L. (2007). Integrating climate–hydrology–ecology for alpine river systems. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *17*(6), 636–656. https://doi.org/10.1002/aqc.800

Hansen, J., Ruedy, R., Sato, M., & Lo, K. (2012). Global temperature in 2011, trends, and prospects. *URL: Http://Www. Gci. Org. Uk/ …*, *2*(November 2011), 2–10. https://doi.org/10.1029/2010RG000345

Harper, M. P., & Peckarsky, B. L. (2006). Emergence cues of a mayfly in a high-altitude stream ecosystem: Potential response to climate change. *Ecological Applications*, *16*(2), 612–621. https://doi.org/10.1890/1051-0761(2006)016[0612:ECOAMI]2.0.CO;2

Harrison, J. F., Frazier, M. R., Henry, J. R., Kaiser, A., Klok, C. J., & Rascón, B. (2006). Responses of terrestrial insects to hypoxia or hyperoxia. *Respiratory Physiology & Neurobiology*, *154*(1–2), 4–17.

Harrison, J. F., Greenlee, K. J., & Verberk, W. C. E. P. (2018). Functional Hypoxia in Insects: Definition, Assessment, and Consequences for Physiology, Ecology, and Evolution. *Annual Review of Entomology*, *63*(1), 303–325. https://doi.org/10.1146/annurev-ento-020117-043145

Heiman, D. R., & Knight, A. W. (1972). Upper-lethal-temperature relations of the nymphs of the stonefly, paragnetina media. *Hydrobiologia*, *39*(4), 479–493. https://doi.org/10.1007/BF00046741

Helmuth, B., Kingsolver, J. G., & Carrington, E. (2005). Biophysics, physiological ecology, and climate change: does mechanism matter? *Annual Review of Physiology*, *67*(1), 177–201. https://doi.org/10.1146/annurev.physiol.67.040403.105027

Hendry, A. P., Schoen, D. J., Wolak, M. E., & Reid, J. M. (2018). The Contemporary Evolution of Fitness. *Annual Review of Ecology, Evolution, and Systematics*, *49*(1), 457–476. https://doi.org/10.1146/annurev-ecolsys-110617-062358

Hill, R. A., Hawkins, C. P., & Jin, J. (2014). Predicting thermal vulnerability of stream and river ecosystems to climate change. *Climatic Change*, *125*(3–4), 399–412. https://doi.org/10.1007/s10584-014-1174-4

Hotaling, S., Finn, D. S., Joseph Giersch, J., Weisrock, D. W., & Jacobsen, D. (2017). Climate change and alpine stream biology : progress , challenges , and opportunities for the future. *Biological Reviews*, *92*(4), 2024–2045. https://doi.org/10.1111/brv.12319

Hotaling, S., Foley, M. E., Zeglin, L. H., Finn, D. S., Tronstad, L. M., Giersch, J. J., Muhlfeld, C. C., & Weisrock, D. W. (2019). Microbial assemblages reflect environmental heterogeneity in alpine streams. *Global Change Biology*, *25*(8), 2576–2590. https://doi.org/10.1111/gcb.14683

Hotaling, S., Muhlfeld, C. C., Giersch, J. J., Ali, O. A., Jordan, S., Miller, M. R., Luikart, G., & Weisrock, D. W. (2018). Demographic modelling reveals a history of divergence with gene flow for a glacially tied stonefly in a changing post-Pleistocene landscape. *Journal of Biogeography*, *45*(2), 304–317. https://doi.org/10.1111/jbi.13125

Hotaling, S., Shah, A. A., Dillon, M. E., Giersch, J. J., Tronstad, L. M., Finn, D. S., & Kelley, J. L. (n.d.). Cold physiology of mountain stoneflies (Plecoptera: Nemouridae): insights from the high Rocky Mountains. *Western North American Naturalist*.

Hotaling, S., Shah, A. A., McGowan, K. L., Tronstad, L. M., Giersch, J. J., Finn, D. S., Woods, H. A., Dillon, M. E., & Kelley, J. L. (2020). Mountain stoneflies may tolerate warming streams: evidence from organismal physiology and gene expression. *Global Change Biology*. https://doi.org/10.1111/gcb.15294

Huss, M., & Hock, R. (2018). Global-scale hydrological response to future glacier mass loss. *Nature Climate Change*, *8*(2), 135–140. https://doi.org/10.1038/s41558-017-0049-x

Hynes, H. B. N. (1970). *The Ecology of Running Waters*. Liverpool University Press.

IPCC. (2018). Summary for Policymakers. In V. Masson-Delmotte, P. Zhai, H. Pörtner, D. Roberts, J. Skea, P. Shukla, A. Pirani, W. Moufouma-Okia, C. Péan, R. Pidcock, S. Connors, J. B. R. Matthews, Y. Chen, X. Zhou, M. I. Gomis, E. Lonnoy, Maycock, M. Tignor, & T. Waterfield (Eds.), *Global Warming of 1.5^{o}C. An IPCC Special Report on the impacts of global warming of 1.5^{o}C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change,*. World Meteorological Organization.

IPCC. (2019). Technical Summary. In H.-O. Pörtner, D. C. Roberts, V. Masson-Delmotte, P. Zhai, E. Poloczanska, K. Mintenbeck, M. Tignor, A. Alegría, M. Nicolai, A. Okem, J. Petzold, B. Rama, & N. M. Weyer (Eds.), *IPCC Special Report on the Ocean and Cryosphere in a Changing Climate*. World Meteorological Organization.

Isaak, D. J., Wollrab, S., Horan, D. L., & Chandler, G. (2012). Climate change effects on stream and river temperatures across the northwest U.S. from 1980–2009 and implications for salmonid fishes. *Climatic Change*, *113*(2), 499–524. https://doi.org/10.1007/s10584-011-0326-z

Isaak, D. J., Young, M. K., Nagel, D. E., Horan, D. L., & Groce, M. C. (2015). The cold-water climate shield: delineating refugia for preserving salmonid fishes through the 21st century. *Global Change Biology*, *21*(7), 2540–2553. https://doi.org/10.1111/gcb.12879

Jacobsen, D. (2000). Gill size of trichopteran larvae and oxygen supply in streams along a 4000-m gradient of altitude. *Journal of the North American Benthological Society*, *19*(2), 329–343. https://doi.org/10.2307/1468075

Jacobsen, D. (2008). Low oxygen pressure as a driving factor for the altitudinal decline in taxon richness of stream macroinvertebrates. *Oecologia*, *154*(4), 795–807. https://doi.org/10.1007/s00442-007-0877-x

Jacobsen, D. (2020). The dilemma of altitudinal shifts: caught between high temperature and low oxygen. *Frontiers in Ecology and the Environment*.

Jacobsen, D., Cauvy-Fraunie, S., Andino, P., Espinosa, R., Cueva, D., & Dangles, O. (2014). Runoff and the longitudinal distribution of macroinvertebrates in a glacier-fed stream: Implications for the effects of global warming. *Freshwater Biology*, *59*(10), 2038–2050. https://doi.org/10.1111/fwb.12405

Jacobsen, D., & Dangles, O. (2017). *Ecology of High Altitude Waters*. Oxford University Press. https://doi.org/DOI:10.1093/oso/9780198736868.001.0001

Jacobsen, D., Dangles, O., Andino, P., Espinosa, R., Hamerlík, L., & Cadier, E. (2010). Longitudinal zonation of macroinvertebrates in an Ecuadorian glacier-fed stream: do tropical glacial systems fit the temperate model? *Freshwater Biology*, *55*(6), 1234–1248. https://doi.org/10.1111/j.1365-2427.2009.02348.x

Jacobsen, D., Milner, A. M., Brown, L. E., & Dangles, O. (2012). Biodiversity under threat in glacier-fed river systems. *Nature Climate Change*, *2*(5), 361–364. https://doi.org/10.1038/nclimate1435

Jacobsen, D., Rostgaard, S., & Vásconez, J. J. (2003). Are macroinvertebrates in high altitude streams affected by oxygen deficiency? *Freshwater Biology*, *48*(11), 2025–2032. https://doi.org/10.1046/j.1365-2427.2003.01140.x

Jacobsen, D., Schultz, R., & Encalada, A. (1997). Structure and diversity of stream invertebrate assemblages : the influence of temperature with altitude and latitude. *Freshwater Biology*, *38*(2), 247–261. https://doi.org/10.1046/j.1365-2427.1997.00210.x

Janzen, D. H. (1967). Why Mountain Passes are Higher in the Tropics. *The American Naturalist*, *101*(919), 233–249. https://doi.org/10.1086/282487

Johansson, J., & Nyström, P. (2004). Effects of ambient UV-radiation on the behaviour of mayfly larvae of the genus Deleatidium from trout bearing and fishless streams in New Zealand. *Archiv Fur Hydrobiologie*, *161*(3), 403–415. https://doi.org/10.1127/0003-9136/2004/0161-0403

Jordan, S., Giersch, J. J., Muhlfeld, C. C., Hotaling, S., Fanning, L., Tappenbeck, T. H., & Luikart, G. (2016). Loss of genetic diversity and increased subdivision in an endemic alpine stonefly threatened by climate change. *PLoS ONE*, *11*(7), 1–12. https://doi.org/10.1371/journal.pone.0159931

Kapoor, N. N. (1978). Effect of salinity on the osmoregulatory cells in the tracheal gills of the stonefly nymph, Paragnetina media (Plecoptera: Perlidae). *Canadian Journal of Zoology*, *56*(12), 2608–2613. https://doi.org/10.1139/z78-350

Kapoor, N. N. (1979). Osmotic regulation and salinity tolerance of the stonefly nymph, Paragnetina media. *Journal of Insect Physiology*, *25*(1), 17–20. https://doi.org/10.1016/0022-1910(79)90031-3

Kapoor, N. N., & Zachariah, K. (1973). Presence of specialized cellular complexes in the tracheal gills of stonefly nymph, Paragnetina media (Walker). *Experientia*, *29*(7), 848–850. https://doi.org/10.1007/BF01946324

Kawecki, T. J., & Ebert, D. (2004). Conceptual issues in local adaptation. *Ecology Letters*, *7*(12), 1225–1241. https://doi.org/10.1111/j.1461-0248.2004.00684.x

Kefford, B. J. (2019). Why are mayflies (Ephemeroptera) lost following small increases in salinity? Three conceptual osmophysiological hypotheses. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *374*(1764). https://doi.org/10.1098/rstb.2018.0021

Kelly, D. J., Bothwell, M. L., & Schindler, D. W. (2003). Effects of solar ultraviolet radiation on stream benthic communities: An intersite comparison. *Ecology*, *84*(10), 2724–2740. https://doi.org/10.1890/02-0658

Khamis, K., Hannah, D. M., Clarvis, M. H., Brown, L. E., Castella, E., & Milner, A. M. (2014). Alpine aquatic ecosystem conservation policy in a changing climate. *Environmental Science & Policy*, *43*, 39–55. https://doi.org/10.1016/j.envsci.2013.10.004

Kiffney, P. M., Clements, W. H., & Cady, T. A. (1997). Influence of Ultraviolet Radiation on the Colonization Dynamics of a Rocky Mountain Stream Benthic Community. *Journal of the North American Benthological Society*, *16*(3), 520–530. https://doi.org/10.2307/1468141

Kingsolver, J. G., Arthur Woods, H., Buckley, L. B., Potter, K. A., MacLean, H. J., & Higgins, J. K. (2011). Complex life cycles and the responses of insects to climate change. *Integrative and Comparative Biology*, *51*(5), 719–732. https://doi.org/10.1093/icb/icr015

Kingsolver, J. G., & Huey, R. B. (2008). Size, temperature, and fitness: Three rules. *Evolutionary Ecology Research*, *10*(2), 251–268.

Kirkpatrick, M., & Barton, N. H. (1997). Evolution of a Species' Range. *The American Naturalist*, *150*(1), 1–23. https://doi.org/10.1086/286054

Kirkton, S. D., Niska, J. A., & Harrison, J. F. (2005). Ontogenetic effects on aerobic and anaerobic metabolism during jumping in the American locust, Schistocerca americana. *Journal of Experimental Biology*, *208*(15), 3003–3012. https://doi.org/10.1242/jeb.01747

Klein, J. A., Tucker, C. M., Nolin, A. W., Hopping, K. A., Reid, R. S., Steger, C., Grêt-Regamey, A., Lavorel, S., Müller, B., Yeh, E. T., Boone, R. B., Bourgeron, P., Butsic, V., Castellanos, E., Chen, X., Dong, S. K., Greenwood, G., Keiler, M., Marchant, R., … Yager, K. (2019). Catalyzing Transformations to Sustainability in the World's Mountains. *Earth's Future*, *15*(3), 547–557. https://doi.org/10.1029/2018EF001024

Komnick, H. (1977). Chloride Cells and Chloride Epithelia of Aquatic Insects. In *International Review of Cytology* (Vol. 49, pp. 285–329). Academic Press. https://doi.org/10.1016/S0074-7696(08)61951-8

Kovalak, W. P. (1979). Day-night changes in stream benthos density in relation to current velocity. In *Archive of Hydrobiologia* (Vol. 87, Issue 1, pp. 1–18).

Lancaster, J., & Downes, Barbara, J. (2013). *Aquatic Entomology*. Oxford University Press.

Lane, S. J., Shishido, C. M., Moran, A. L., Tobalske, B. W., Arango, C. P., & Woods, H. A. (2017). Upper limits to body size imposed by respiratory–structural trade-offs in Antarctic pycnogonids. *Proceedings of the Royal Society B: Biological Sciences*, *284*(20171779). https://doi.org/10.1098/rspb.2017.1779

Lencioni, V. (2004). Survival strategies of freshwater insects in cold environments. *Journal of Limnology*, *63*(1), 45–55. https://doi.org/10.4081/jlimnol.2004.s1.45

Lencioni, V. (2018). Glacial influence and stream macroinvertebrate biodiversity under climate change: Lessons from the Southern Alps. *Science of The Total Environment*, *622–623*, 563–575. https://doi.org/10.1016/j.scitotenv.2017.11.266

Lescak, E. A., Bassham, S. L., Catchen, J., Gelmond, O., Sherbick, M. L., von Hippel, F. A., & Cresko, W. A. (2015). Evolution of stickleback in 50 years on earthquake-uplifted islands. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(52), E7204–E7212. https://doi.org/10.1073/pnas.1512020112

Levins, R. (1968). *Evolution in changing environments: some theoretical explorations* (2nd ed.). Princeton University Press.

Loayza-Muro, R. A., Marticorena-Ruíz, J. K., Palomino, E. J., Merritt, C., Breeuwer, J. A. J., Kuperus, P., Kraak, M. H. S., & Admiraal, W. (2013). Ultraviolet-B-driven pigmentation and genetic diversity of benthic macroinvertebrates from high-altitude Andean streams. *Freshwater Biology*, *58*(8), 1710–1719. https://doi.org/10.1111/fwb.12161

Locatelli, B., Lavorel, S., Sloan, S., Tappeiner, U., & Geneletti, D. (2017). Characteristic trajectories of ecosystem services in mountains. *Frontiers in Ecology and the Environment*, *15*(3), 150–159. https://doi.org/10.1002/fee.1470

Loudon, C. (1989). Tracheal hyptertrophy in mealworms: design and plasticity in oxygen supply systems. *Journal of Experimental Biology*, *147*, 217–235.

MacFadyen, E. J., Williamson, C. E., Grad, G., Lowery, M., Jeffrey, W. H., & Mitchell, D. L. (2004). Molecular response to climate change: Temperature dependence of UV-induced DNA damage and repair in the freshwater crustacean Daphnia pulicaria. *Global Change Biology*, *10*(4), 408–416. https://doi.org/10.1111/j.1529-8817.2003.00750.x

MacMillan, H. A., & Sinclair, B. J. (2011). Mechanisms underlying insect chill-coma. *Journal of Insect Physiology*, *57*(1), 12–20. https://doi.org/10.1016/j.jinsphys.2010.10.004

Madsen, P. B., Morabowen, A., Andino, P., Espinosa, R., Cauvy-Fraunié, S., Dangles, O., & Jacobsen, D. (2015). Altitudinal distribution limits of aquatic macroinvertebrates: an experimental test in a tropical alpine stream. *Ecological Entomology*, *40*(5), 629–638. https://doi.org/10.1111/een.12232

Marty, C., & Blanchet, J. (2012). Long-term changes in annual maximum snow depth and snowfall in Switzerland based on extreme value statistics. *Climatic Change*, *111*(3–4), 705–721. https://doi.org/10.1007/s10584-011-0159-9

McGregor, G., Petts, G. E., Gurnell, A. M., & Milner, A. M. (1995). Sensitivity of alpine stream ecosystems to climate change and human impacts. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *5*(3), 233–247. https://doi.org/10.1002/aqc.3270050306

Mérigoux, S., & Dolédec, S. (2004). Hydraulic requirements of stream communities: a case study on invertebrates. *Freshwater Biology*, *49*(5), 600–613. https://doi.org/10.1111/j.1365-2427.2004.01214.x

Milner, A. M., Khamis, K., Battin, T. J., Brittain, J. E., Barrand, N. E., Füreder, L., Cauvy-Fraunié, S., Gíslason, G. M., Jacobsen, D., Hannah, D. M., Hodson, A. J., Hood, E., Lencioni, V., Ólafsson, J. S., Robinson, C. T., Tranter, M., & Brown, L. E. (2017). Glacier shrinkage driving global changes in downstream systems. *Proceedings of the National Academy of Sciences*, *114*(37), 9770–9778. https://doi.org/10.1073/pnas.1619807114

Milner, A. M., & Petts, G. E. (1994). Glacial rivers: physical habitat and ecology. *Freshwater Biology*, *32*(2), 295–307. https://doi.org/10.1111/j.1365-2427.1994.tb01127.x

Milner, A. M., Robertson, A. L., Monaghan, K. A., Veal, A. J., & Flory, E. A. (2008). Colonization and development of an Alaskan stream community over 28 years. *Frontiers in Ecology and the Environment*, *6*(8), 413–419. https://doi.org/10.1890/060149

Mohseni, O., & Stefan, H. G. (1999). Stream temperature/air temperature relationship: a physical interpretation. *Journal of Hydrology*, *218*(3–4), 128–141. https://doi.org/10.1016/S0022-1694(99)00034-7

Monaghan, M. T., Spaak, P., Robinson, C. T., & Ward, J. V. (2001). Genetic differentiation of Baetis alpinus Pictet (Ephemeroptera: Baetidae) in fragmented alpine streams. *Heredity*, *86*(4), 395–403. https://doi.org/10.1046/j.1365-2540.2001.00843.x

Monaghan, M. T., Spaak, P., Robinson, C. T., & Ward, J. V. (2002). Population genetic structure of 3 alpine stream insects: Influences of gene flow, demographics, and habitat fragmentation. *Journal of the North American Benthological Society*, *21*(1), 114–131. https://doi.org/10.2307/1468304

Moser, K. A., Baron, J. S., Brahney, J., Oleksy, I. A., Saros, J. E., Hundey, E. J., Sadro, S. A., Kopáček, J., Sommaruga, R., Kainz, M. J., Strecker, A. L., Chandra, S., Walters, D. M., Preston, D. L., Michelutti, N., Lepori, F., Spaulding, S. A., Christianson, K. R., Melack, J. M., & Smol, J. P. (2019). Mountain lakes: Eyes on global environmental change. *Global and Planetary Change*, *178*, 77–95. https://doi.org/10.1016/j.gloplacha.2019.04.001

Mote, P. W., Hamlet, A. F., Clark, M. P., & Lettenmaier, D. P. (2005). Declining mountain snowpack in western north America. *Bulletin of the American Meteorological Society*, *86*(1), 39–49. https://doi.org/10.1175/BAMS-86-1-39

Muhlfeld, C. C., Cline, T. J., Giersch, J. J., Peitzsch, E., Florentine, C., Jacobsen, D., & Hotaling, S. (2020). Specialized meltwater biodiversity persists despite widespread deglaciation. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(22). https://doi.org/10.1073/pnas.2001697117

Niedrist, G. H., & Füreder, L. (2020). Real-time warming of alpine streams: (re)defining invertebrates' temperature preferences. *River Research and Applications*, *February*, 1–11. https://doi.org/10.1002/rra.3638

Olson, M. H., Fischer, J. M., Williamson, C. E., Overholt, E. P., & Theodore, N. (2018). Landscape-scale regulators of water transparency in mountain lakes: implications of projected glacial loss. *Canadian Journal of Fisheries and Aquatic Sciences*, *75*(7), 1169–1176.

Orr, S. E., & Buchwalter, D. B. (2020). It's all about the fluxes: Temperature influences ion transport and toxicity in aquatic insect. *Aquatic Toxicology*, *221*, 105405.

Pepin, N., Bradley, R. S., Diaz, H. F., Baraer, M., Caceres, E. B., Forsythe, N., Fowler, H., Greenwood, G., Hashmi, M. Z., Liu, X. D., Miller, J. R., Ning, L., Ohmura, A., Palazzi, E., Rangwala, I., Schöner, W., Severskiy, I., Shahgedanova, M., Wang, M. B., … Yang, D. Q. (2015). Elevation-dependent warming in mountain regions of the world. *Nature Climate Change*, *5*(5), 424–430. https://doi.org/10.1038/nclimate2563

Phillips, J. E. E., Hanrahan, J., Chamberlin, M., Thomson, B., Hanrahan, J., & Chamberlin, M. (1987). Mechanisms and Control of Reabsorption in Insect Hindgut. In P. D. Evans & V. B. Wigglesworth (Eds.), *Advances in Insect Physiology* (Vol. 19, pp. 329–422). Academic Press. https://doi.org/10.1016/S0065-2806(08)60103-4

Piersma, T., & Drent, J. (2003). Phenotypic flexibility and the evolution of organismal design. *Trends in Ecology and Evolution*, *18*(5), 228–233. https://doi.org/10.1016/S0169-5347(03)00036-3

Pilgrim, J. M., Fang, X., & Stefan, H. G. (1998). Stream temperature correlations with air temperatures. *Journal Of The American Water Resources Association*, *34*(5), 1109–1121. https://doi.org/10.1111/j.1752-1688.1998.tb04158.x

Poff, N. L., Larson, E. I., Salerno, P. E., Morton, S. G., Kondratieff, B. C., Flecker, A. S., Zamudio, K. R., & Funk, W. C. (2018). Extreme streams: species persistence and genomic change in montane insect populations across a flooding gradient. *Ecology Letters*, *21*(4), 525–535. https://doi.org/10.1111/ele.12918

Polato, N. R., Gill, B. A., Shah, A. A., Gray, M. M., Casner, K. L., Barthelet, A., Messer, P. W., Simmons, M. P., Guayasamin, J. M., Encalada, A. C., Kondratieff, B. C., Flecker, A. S., Thomas, S. A., Ghalambor, C. K., Poff, N. L., Funk, W. C., & Zamudio, K. R. (2018). Narrow thermal tolerance and low dispersal drive higher speciation in tropical mountains. *Proceedings of the National Academy of Sciences*, *115*(49), 12471–12476. https://doi.org/10.1073/pnas.1809326115

Polato, N. R., Gray, M. M., Gill, B. A., Becker, C. G., Casner, K. L., Flecker, A. S., Kondratieff, B. C., Encalada, A. C., Poff, N. L., Funk, W. C., & Zamudio, K. R. (2017). Genetic diversity and gene flow decline with elevation in montane mayflies. *Heredity*, *119*(2), 107–116. https://doi.org/10.1038/hdy.2017.23

Pörtner, H. O. (2001). Climate change and temperature-dependent biogeography: Oxygen limitation of thermal tolerance in animals. *Naturwissenschaften*, *88*(4), 137–146. https://doi.org/10.1007/s001140100216

Pörtner, H. O., & Knust, R. (2007). Climate Change Affects Marine Fishes Through the Oxygen Limitation of Thermal Tolerance. *Science*, *315*(5808), 95–97. https://doi.org/10.1126/science.1135471

Rangel, D. E. N., Braga, G. U. L., Anderson, A. J., & Roberts, D. W. (2005). Influence of growth environment on tolerance to UV-B radiation, germination speed, and morphology of Metarhizium anisopliae var. acridum conidia. *Journal of Invertebrate Pathology*, *90*(1), 55–58. https://doi.org/10.1016/j.jip.2005.05.005

Resh, V. H., & Rosenberg, D. M. (1984). *The ecology of aquatic insects*. Praeger.

Rezende, E. L., Castañeda, L. E., & Santos, M. (2014). Tolerance landscapes in thermal ecology. *Functional Ecology*, *28*(4), 799–809. https://doi.org/10.1111/1365-2435.12268

Reznick, D. N., Shaw, F. H., Rodd, F. H., & Shaw, R. G. (1997). Evaluation of the rate of evolution in natural populations of guppies (Poecilia reticulata). *Science*, *275*(5308), 1934–1937. https://doi.org/10.1126/science.275.5308.1934

Rose, K. C., Hamilton, D. P., Williamson, C. E., McBride, C. G., Fischer, J. M., Olson, M. H., Saros, J. E., Allan, M. G., & Cabrol, N. (2014). Light attenuation characteristics of glacially-fed lakes. *Journal of Geophysical Research: Biogeosciences*, *119*(7), 1446–1457. https://doi.org/10.1002/2014JG002674

Rostgaard, S., & Jacobsen, D. (2005). Respiration rate of stream insects measured in situ along a large altitude range. *Hydrobiologia*, *549*(1), 79–98. https://doi.org/10.1007/s10750-005-4165-7

Scheibener, S. A., Richardi, V. S., & Buchwalter, D. B. (2016). Comparative sodium transport patterns provide clues for understanding salinity and metal responses in aquatic insects. *Aquatic Toxicology*, *171*, 20–29. https://doi.org/10.1016/j.aquatox.2015.12.006

Schmitz, O. J., & Barton, B. T. (2014). Climate change effects on behavioral and physiological ecology of predator-prey interactions: Implications for conservation biological control. *Biological Control*, *75*, 87–96. https://doi.org/10.1016/j.biocontrol.2013.10.001

Shah, A. A., Dillon, M. E., Hotaling, S., & Woods, H. A. (2020). High elevation insect communities face shifting ecological and evolutionary landscapes. *Current Opinion in Insect Science*, *41*, 1–6. https://doi.org/10.1016/j.cois.2020.04.002

Shah, A. A., Funk, W. . C., & Ghalambor, C. K. (2017). Thermal Acclimation Ability Varies in Temperate and Tropical Aquatic Insects from Different Elevations. *Integrative and Comparative Biology*, *57*(5), 977–987. https://doi.org/10.1093/icb/icx101

Shah, A. A., Gill, B. A., Encalada, A. C., Flecker, A. S., Funk, W. C., Guayasamin, J. M., Kondratieff, B. C., Poff, N. L., Thomas, S. A., Zamudio, K. R., & Ghalambor, C. K. (2017). Climate variability predicts thermal limits of aquatic insects across elevation and latitude. *Functional Ecology*, *31*(11), 2118–2127. https://doi.org/10.1111/1365-2435.12906

Sheldon, A. L. (2012). Possible climate-induced shift of stoneflies in a southern Appalachian catchment. *Freshwater Science*, *31*(3), 765–774. https://doi.org/10.1899/11-135.1

Sheldon, K. S., Yang, S., & Tewksbury, J. J. (2011). Climate change and community disassembly: Impacts of warming on tropical and temperate montane community structure. *Ecology Letters*, *14*(12), 1191–1200. https://doi.org/10.1111/j.1461-0248.2011.01689.x

Shrestha, A. B., & Aryal, R. (2011). Climate change in Nepal and its impact on Himalayan glaciers. *Regional Environmental Change*, *11*(Suppl 1), S65–S77. https://doi.org/10.1007/s10113-010-0174-9

Sinclair, B. J., Coello Alvarado, L. E., & Ferguson, L. V. (2015). An invitation to measure insect cold tolerance: Methods, approaches, and workflow. *Journal of Thermal Biology*, *53*, 180–

197. https://doi.org/10.1016/j.jtherbio.2015.11.003

Soengas, J., Sangiao-Alvarellos, S., Laiz-Carrión, R., & Mancera, J. (2007). Energy Metabolism and Osmotic Acclimation in Teleost Fish. In B. Baldisserotto, J. M. Mancera, & B. G. Kapoor (Eds.), *Fish Osmoregulation* (pp. 277–307). Science Publisher.

Stockwell, C. A., Hendry, A. P., & Kinnison, M. T. (2003). Contemporary evolution meets conservation biology. *Trends in Ecology and Evolution*, *18*(2), 94–101. https://doi.org/10.1016/S0169-5347(02)00044-7

Stottlemyer, R., & Troendle, C. A. (1992). Nutrient concentration patterns in streams draining alpine and subalpine catchments, Fraser Experimental Forest, Colorado. *Journal of Hydrology*, *140*(1–4), 179–208. https://doi.org/10.1016/0022-1694(92)90240-V

Todgham, A. E., & Stillman, J. H. (2013). Physiological Responses to Shifts in Multiple Environmental Stressors: Relevance in a Changing World. *Integrative and Comparative Biology*, *53*(4), 539–544. https://doi.org/10.1093/icb/ict086

Townsend, C. R., Dolédec, S., & Scarsbrook, M. R. (1997). Species traits in relation to temporal and spatial heterogeneity in streams: A test of habitat templet theory. *Freshwater Biology*, *37*(2), 367–387. https://doi.org/10.1046/j.1365-2427.1997.00166.x

Tronstad, L. M., Hotaling, S., Giersch, J. J., Wilmot, O. J., & Finn, D. S. Headwaters fed by subterranean ice: potential climate refugia for mountain stream communities? *Western North American Naturalist*. https://doi.org/10.1101/788273

U.S. Fish & Wildlife Service. (2019). Endangered and Threatened Wildlife and Plants: Threatened Species Status for Meltwater Lednian Stonefly and Western Glacier Stonefly 795 with a Section 4(d) Rule. *Federal Register*, *84*, 64210-64227.

U.S. Geological Survey. (2020). *National Water Information System*. USGS Water Data for the Nation. http://waterdata.usgs.gov/nwis/

Verberk, W. C. E. P., Bilton, D. T., Calosi, P., & Spicer, J. I. (2011). Oxygen supply in aquatic ectotherms: Partial pressure and solubility together explain biodiversity and size patterns. *Ecology*, *92*(8), 1565–1572. https://doi.org/10.1890/10-2369.1

Verberk, W. C. E. P., & Calosi, P. (2012). Oxygen limits heat tolerance and drives heat hardening in the aquatic nymphs of the gill breathing damselfly Calopteryx virgo (Linnaeus, 1758). *Journal of Thermal Biology*, *37*(3), 224–229. https://doi.org/10.1016/j.jtherbio.2012.01.004

Verberk, W. C. E. P., Overgaard, J., Ern, R., Bayley, M., Wang, T., Boardman, L., & Terblanche, J. S. (2016). Does oxygen limit thermal tolerance in arthropods? A critical review of current evidence. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *192*, 64–78. https://doi.org/10.1016/j.cbpa.2015.10.020

Verberk, W. C. E. P., Sommer, U., Davidson, R. L., & Viant, M. R. (2013). Anaerobic Metabolism at Thermal Extremes: A Metabolomic Test of the Oxygen Limitation Hypothesis in an Aquatic Insect. *Integrative and Comparative Biology*, *53*(4), 609–619. https://doi.org/10.1093/icb/ict015

Viviroli, D., Dürr, H. H., Messerli, B., Meybeck, M., & Weingartner, R. (2007). Mountains of the world, water towers for humanity: Typology, mapping, and global significance. *Water Resources Research*, *43*(7), 1–13. https://doi.org/10.1029/2006WR005653

Vogel, S. (1994). *Life in moving fluids: The physical biology of flow* (2nd ed.). Princeton University Press.

Vuille, M., Francou, B., Wagnon, P., Juen, I., Kaser, G., Mark, B. G., & Bradley, R. S. (2008). Climate change and tropical Andean glaciers: Past, present and future. *Earth-Science Reviews*, *89*(3–4), 79–96. https://doi.org/10.1016/j.earscirev.2008.04.002

Walsh, B. S., Parratt, S. R., Hoffmann, A. A., Atkinson, D., Snook, R. R., Bretman, A., & Price, T. A. R. (2019). The Impact of Climate Change on Fertility. *Trends in Ecology and Evolution*, *34*(January), 249–259. https://doi.org/10.1016/j.tree.2018.12.002

Walters, K. R., Serianni, A. S., Voituron, Y., Sformo, T., Barnes, B. M., & Duman, J. G. (2011). A thermal hysteresis-producing xylomannan glycolipid antifreeze associated with cold tolerance is found in diverse taxa. *Journal of Comparative Physiology B*, *181*(5), 631–640. https://doi.org/10.1007/s00360-011-0552-8

Walters, K. R., Sformo, T., Barnes, B. M., & Duman, J. G. (2009). Freeze tolerance in an arctic Alaska stonefly. *Journal of Experimental Biology*, *212*(2), 305–312. https://doi.org/10.1242/jeb.020701

Wang, Q., Fan, X., & Wang, M. (2016). Evidence of high-elevation amplification versus Arctic amplification. *Scientific Reports*, *6*(May 2015), 1–8. https://doi.org/10.1038/srep19219

Ward, J. V. (1994). Ecology of alpine streams. *Freshwater Biology*, *32*(2), 277–294. https://doi.org/10.1111/j.1365-2427.1994.tb01126.x

White, D. S. (1990). Biological relationships to convective flow patterns within stream beds.

Hydrobiologia, *196*(2), 149–158. https://doi.org/10.1007/BF00006106

Wichard, W. (1974). Zur morphologischen Anpassung von Tracheenkiemen bei Larven der Limnephilini Kol. (Insecta, Trichoptera). *Oecologia*, *15*(2), 159–167. https://doi.org/10.1007/bf00345743

Wiggins, G. B. (2004). *Caddisflies: the underwater architects*. University of Toronto Press.

Wiggins, G. B., & Wichard, W. (1989). Phylogeny of Pupation in Trichoptera , with Proposals on the Origin and Higher Classification of the Order. *Journal of the North American Benthological Society*, *8*(3), 260–276.

Wiley, M. J., & Kohler, S. L. (1980). Positioning changes of mayfly nymphs due to behavioral regulation of oxygen consumption. *Canadian Journal of Zoology*, *58*(4), 618–622. https://doi.org/10.1139/z80-088

Williams, D. D., & Hynes, H. B. N. (1974). The occurance of benthos deep in substrarum of stream. *Freshwater Biology*, *4*, 233–256.

Williams, D. D., Tavares, A. F., & Bryant, E. (1987). Respiratory Device or Camouflage?: A Case for the Caddisfly. *Oikos*, *50*(1), 42. https://doi.org/10.2307/3565400

Williamson, C. E., Olson, O. G., Lott, S. E., Walker, N. D., Engstrom, D. R., & Hargreaves, B. R. (2001). Ultraviolet radiation and zooplankton community structure following deglaciation in Glacier Bay, Alaska. *Ecology*, *82*(6), 1748–1760. https://doi.org/10.1890/0012-9658(2001)082[1748:URAZCS]2.0.CO;2

Williamson, C. E., Stemberger, R. S., Morris, D. P., Frost, T. M., & Paulsen, S. G. (1996). Ultraviolet radiation in North American lakes: Attenuation estimates from DOC measurements and implications for plankton communities. *Limnology and Oceanography*, *41*(5), 1024–1034. https://doi.org/10.4319/lo.1996.41.5.1024

Wissinger, S. A., Oertli, B., & Rosset, V. (2016). Invertebrate Communities of Alpine Ponds. In D. Batzer & D. Boix (Eds.), *Invertebrates in Freshwater Wetlands* (pp. 55–103). Springer International Publishing. https://doi.org/10.1007/978-3-319-24978-0_3

Wood, P. J., Boulton, A. J., Little, S., & Stubbington, R. (2010). Is the hyporheic zone a refugium for aquatic macroinvertebrates during severe low flow conditions? *Fundamental and Applied Limnology / Archiv Für Hydrobiologie*, *176*(4), 377–390. https://doi.org/10.1127/1863-9135/2010/0176-0377

Woods, H. A. (1999). Egg-mass size and cell size: Effects of temperature on oxygen distribution. *American Zoologist*, *39*(2), 244–252. https://doi.org/10.1093/icb/39.2.244

Zemp, M., Frey, H., Gärtner-Roer, I., Nussbaumer, S. U., Hoelzle, M., Paul, F., Haeberli, W., Denzinger, F., Ahlstrøm, A. P., Anderson, B., Bajracharya, S., Baroni, C., Braun, L. N., Càceres, B. E., Casassa, G., Cobos, G., Dàvila, L. R., Delgado Granados, H., Demuth, M. N., … Vincent, C. (2015). Historically unprecedented global glacier decline in the early 21st century. *Journal of Glaciology*, *61*(228), 745–762. https://doi.org/10.3189/2015JoG15J017

Chapter 3

Going with the flow – how a stream insect, *Pteronarcys californica,* exploits local flows to increase oxygen availability

Going with the flow – how a stream insect, *Pteronarcys californica,* exploits local flows to increase oxygen availability

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Keywords: Aquatic insects, behavior, boundary layer, discharge, microclimates, temperature.

Abstract

For insects, life in water is challenging because oxygen supply is typically low compared to in air. Oxygen limitation may occur when oxygen levels or water flows are low or when warm temperatures stimulate metabolic demand for oxygen. A potential mechanism for mitigating oxygen shortages is behavior – moving to cooler, more oxygenated, or faster flowing microhabitats. Whether stream insects can make meaningful choices, however, depends on: i) how temperature, oxygen, and flow vary at microspatial scales and ii) the ability of insects to sense and exploit that variation. To assess the extent of microspatial variation in conditions, we measured temperature, oxygen saturation, and flow velocity within riffles of two streams in Montana, USA. In the lab, we then examined preferences of nymphs of the stonefly *Pteronarcys californica* to experimental gradients based on field-measured values. Temperature and oxygen level varied only slightly within stream riffles. By contrast, flow velocity was highly heterogeneous, often varying by more than 125 cm s^{-1} within riffles and 44 cm s^{-1} around individual cobbles. Exploiting micro-variation in flow may thus be the most reliable option for altering rates of oxygen transport. In support of this prediction, *P. californica* showed little ability to exploit gradients in temperature and oxygen but readily exploited micro-variation in flow – consistently choosing higher flows when conditions were warm or hypoxic. These behaviors may help stream insects mitigate low-oxygen stress from climate change and other anthropogenic disturbances.

Introduction

Life in water is shaped by the scarcity of oxygen (Hutchinson, 1981; Lancaster & Downes, 2013). Water contains ~ 30 times less oxygen than air (Jones et al., 1972). Water is also dense and viscous, which slows rates of diffusion and makes respiratory ventilation energetically expensive (Denny, 1993; Verberk & Atkinson, 2013). For water-breathing ectotherms like aquatic insects, the challenge is to extract sufficient oxygen to fuel metabolism, despite low levels of environmental oxygen supply (Verberk et al., 2016; Woods & Moran, 2020).

This oxygen problem is complicated further by variation in temperature and water velocity (Woods, 1999; Jacobsen, 2003; Verberk et al., 2011; Frakes et al., 2021; Verberk et al., 2016b). Warmer water modestly increases rates of oxygen supply – oxygen diffuses more rapidly in warmer water which more than offsets declining concentrations – but raises organismal demand even more (Woods, 1999; Woods & Moran, 2008; Verberk et al., 2011; Verberk & Atkinson, 2013). At high temperatures, oxygen demand can exceed the capacity of organisms to obtain sufficient oxygen, resulting in declines in organismal performance and potentially death (Pörtner, 2007; Verberk et al., 2016; Harrison et al., 2018). Higher flows, by contrast, increase rates of oxygen supply by thinning the boundary layers enveloping insect respiratory surfaces (e.g., gills or thin segments of the cuticle) (Hynes, 1970). Water velocity is zero (i.e., no slip condition) at the cuticle-water interface but increases with distance from the body surface until it approaches the velocity of the free-stream flow (Vogel, 1981). When boundary layers are thin, as in fast-flowing and turbulent water, oxygen diffuses over shorter distances between the water column and respiratory tissues, increasing supply rates (Pinder & Feder, 1990; Hall & Ulseth, 2019). Thus, aquatic ectotherms can tolerate deeper hypoxia and warmer temperatures when water velocities are sufficiently high (Frakes et al., 2021).

Taken together, climate change and other anthropogenic disturbances, like eutrophication and dams, are raising water temperatures, depressing oxygen concentrations, and lowering water velocities (at least seasonally) in many rivers and streams. These changes threaten aquatic communities in part by depressing the ratio of oxygen supply:demand (Jacobsen et al., 2008; Deutsch, et al., 2015; Verberk et al., 2016b). Predicting how aquatic communities will respond, however, will require a better understanding of the mechanisms that aquatic insects use to mitigate oxygen limitation. A broad literature now addresses the mechanisms by which ectotherm thermal performances, thermal limits, and acclimation responses depend on oxygen and flow (Verberk et al., 2011; Verberk et al., 2016; Rubalcaba et al., 2020; Collins et al., 2021; Frakes et al., 2021). Much less literature has focused on behavioral mitigation of the oxygen problem (but see Verberk & Bilton, 2015), even though behavior is well known to provide small terrestrial and intertidal ectotherms with the means to thermoregulate (Ebersole & Frissell, 2001; Helmuth et al., 2007; Scheffers et al., 2014; Woods et al., 2015; Birrell et al., 2020; Pincebourde & Woods, 2020). Whether aquatic insects can mitigate oxygen shortages by moving among microclimates will depend on (i) whether diverse conditions are available locally and (ii) whether insects actually exploit that diversity. We expect that these two conditions are related to one another – in the sense that aquatic insects should evolve behavioral mechanisms to exploit microclimatic mosaics only when lineages are exposed regularly to biologically meaningful gradients in nature. Assessing both the availability of microclimates and the capacity for individuals to exploit them will be critical to understanding how aquatic insects will respond to future climate change and has so far received little attention (e.g., Helmuth et al., 2007; Pincebourde & Woods, 2020).

Whether temperature, oxygen, and flow are likely to show strong local gradients depends on complex interactions among physical, hydrogeomorphic, and chemical factors. Because water has a high heat capacity, equilibrates slowly with local gas partial pressures, and is typically well-mixed (in lotic systems), we predict that microclimatic diversity in temperature and oxygen will be slight (Denny, 1993). Warm microclimates can form near warm springs or near shallow, slow-moving stream margins where solar radiation is high, and cool microclimates can form in association with groundwater inputs, side channels, springs, deep-water pools, and shading (Mosley, 1983; Matthews & Berg, 1996; Clark et al., 1999; Ebersole & Frissell, 2001; Ebersole et al., 2003). However, the spatial grain at which these differences are manifested appears too coarse for individual insects to exploit (e.g., Ebersole & Frissell, 2001). Moreover, cold patches are often associated with inputs of hypoxic groundwater, which would at least partially counteract the benefits of colder water (Matthews & Berg, 1996; Torgersen et al., 1999; Elliot, 2000). Small gradients in temperature and oxygen may also form in stream riffles in summer as a consequence of patterns of exchange between in-stream and hyporheic flows, with slightly cooler, more oxygenated water at riffle heads and slightly colder, less oxygenated water at the tail (from upwelling of hyporheic water) (Davy-Bowker et al., 2006).

By contrast, microclimatic diversity in flow velocities is often very high in lotic systems. In streams, flow velocities often vary from ~ 0 within the substrate to > 100 cm s^{-1} in the free stream environment (White, 1990; Comiti et al., 2007), and individuals may often be able to alter local flows past their bodies by moving just a few cm. Indeed, gradients in flow velocity strongly drive stream insect microdistributions (Mérigoux & Dolédec, 2004). Some aquatic insects choose more exposed surfaces and perform more respiratory movements when flows are low or temperatures high, presumably to increase rates of oxygen supply (Kovalak, 1976; Kovalak, 1979; Wiley & Kohler, 1980; Genkai-Kato et al., 2005). Higher flows can also increase the upper temperature tolerances and lower oxygen limits of stream insects (Frakes et al., 2021).

Here, we examine potential behaviors by which aquatic insects can mitigate oxygen shortages. We first measured the spatial diversity of temperature, oxygen saturation, and flow velocity available to aquatic insects within riffles at small spatial scales in two streams in western Montana (USA). We then established experimental gradients of each factor (temperature, oxygen, flow) in laboratory flow chambers and measured how strongly nymphs of the giant salmonfly, the stonefly *Pteronarcys californica*, chose among them. Broadly, we expected that when nymphs are subjected to oxygen deficiencies, they should move to ameliorating microclimates (lower temperatures, higher concentrations of oxygen, or higher flows). However, we also predicted that nymphs would make stronger choices among levels of the factors, e.g., flow, that show strong spatial gradients in nature.

Methods

Field microclimate measurements

In July, 2019, we sampled water temperatures, oxygen saturation, and flow velocities on the Blackfoot River and Rock Creek in western Montana, USA (see Fig. 1A for schematic of sampling methodology). The Blackfoot River and Rock Creek have similar hydrology and geomorphology. Both are large, cold-water, cobbled mountain streams fed by snowmelt, precipitation, and groundwater. The Blackfoot River originates in the Flathead Mountains and flows ~120 km southwest through open sage-prairie, rangeland, and canyons into the Clark Fork River near Missoula, Montana. From its west fork, Rock Creek flows ~100 km north through the Sapphire Mountains, cutting through rangeland and narrow canyons. It joins the Clark Fork River 40 km upstream of the Blackfoot.

We sampled at 9 sites on the Blackfoot River and 10 on Rock Creek. Sites were separated at relatively even intervals (~10 km) across the elevation gradients of both rivers (1000-1630 m, and 1090-2060 m, respectively). At each site, microclimates were measured surrounding 15 large cobbles (15-50 cm wide) within a 10 × 5 m quadrant in the middle of riffles. Cobbles were chosen haphazardly every meter along three transects within the quadrant. Transects were separated by 5 m (i.e., one at 0, 5, and 10 m within the quadrant). We measured water temperature and oxygen saturation under the bottom surface toward the rear and under the bottom surface toward the front of each cobble and in the free stream ~ 3/4 of the way up from the stream bed to the water surface. We measured flow velocity on the top surface, at the leading edge, and tail edge of each cobble as well as in the free stream ~ 3/4 of the way up from the stream bed to the water surface. Measurements therefore represent different microclimates around and among cobbles. To measure temperature and oxygen saturation, we used an optical temperature and oxygen meter (Pyroscience, OXROB3; Aachen, Germany) connected to a meter (Pyroscience, FSO2-C2). The sensor was fitted into a hollow metal rod, which allowed us to insert the tip into the substrate. At each location, the tip was held in place for 30 s. Flows were measured using a portable velocity meter (Global Water, FP111 Flow Probe; Davison, Michigan, USA).

Laboratory choice experiments

In the laboratory, we measured the preferences of *Pteronarcys californica* nymphs (salmonflies) for different temperature, oxygen, and flow conditions (see Fig. 1B,C for schematic of experimental setup and Fig. S1 for photos of a salmonfly nymph and the flow chambers). Salmonflies were collected from the Blackfoot River or Rock Creek with a kick screen (91×91 cm with 1 mm mesh openings) and returned to the University of Montana, where they were transferred to 20-liter buckets filled with de-chlorinated tap water and held in a temperature-controlled incubator (Percival Scientific, I-66LLC8; Perry Iowa, USA). Nymphs were held at ~ 10 °C and fed cottonwood leaves (*Populus spp.*) from the Blackfoot River or Rock Creek. Leaves were soaked in stream water (i.e., conditioned) for two weeks before being given to the nymphs, which softens them and helps bacterial and fungal biofilms establish on their surfaces, from which salmonflies derive much of their nutrition (Eggert & Wallace, 2007). Water in the buckets was oxygenated and stirred by air directed through air stones. Nymphs were held for a minimum of two days and up to three weeks before experimentation. During this pre-experimental period, there was no mortality and we observed vigorous feeding and frequent molting.

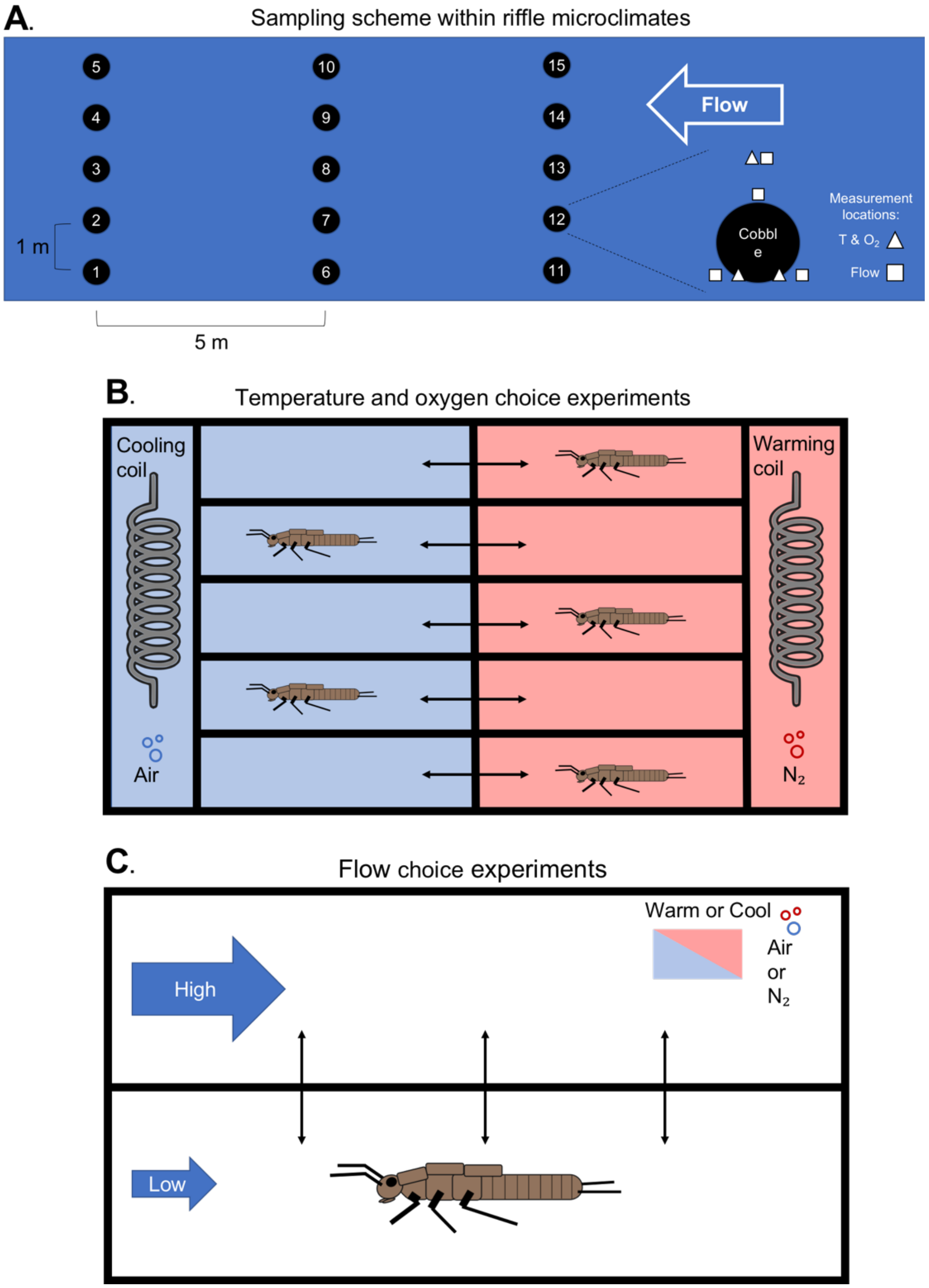

Fig. 1: Schematic of methods for sampling temperature, oxygen, and flow microclimates in the field (A) and for temperature and oxygen (B) and flow choice (C) experimental design. In the field, microclimates were measured surrounding 15 cobbles within a 10 × 5 quadrant in the middle of riffles. In oxygen experiments, nymphs chose between normoxic and hypoxic conditions when temperatures were either cool or warm. In the temperature experiments, nymphs chose between warm and cool conditions when oxygen levels were either normoxic or hypoxic. In the flow choice experiments, nymphs chose between decreasing flows and constantly high flows at variable temperature and oxygen levels.

Oxygen choice experiments

We performed oxygen choice experiments during the spring and summer of 2019 by measuring where nymphs distributed themselves between two static levels of oxygen saturation (hypoxic versus normoxic) within an experimental aquarium (53×39×16.5 cm). The oxygen gradient was sustained by bubbling air or nitrogen gas through air stones at each end of the aquarium. We placed stainless steel coils connected to cooling recirculating water baths to control the temperature (VWR Scientific, 1160A; Radnor, Pennsylvania, USA & Active Aqua, AACH10HP; Petaluma, CA, USA) and small water pumps to provide flow (PULACO, PL-118, 200 L h^{-1}; Guangzhou, China). Mesh walls divided the air stones, coils, and pumps from the compartment in which the nymphs were kept, while still allowing temperature- and oxygen-controlled water to circulate. Plexiglas walls divided the central chamber into five lanes and kept the nymphs separate. Median Plexiglas walls divided the aquarium (and its five lanes) in half, preventing the normoxic and hypoxic water from mixing. However, nymphs were able to move between the hypoxic and normoxic sides by crawling through a 1-cm diameter hole cut in the bottom-center of each median wall. Cobbles and conditioned cottonwood leaves were placed on both sides of all lanes to provide food and substrate for the nymphs. High-grit sandpaper (Gator, P150; Fairborn, Ohio, USA) glued to the floor of the chamber provided footing.

During oxygen experiments, we measured choices made by 25 nymphs – 13 at 5 °C and 12 at 16 °C. At the onset of each trial, one nymph was placed on a random side (left or right) of each lane and left to acclimate overnight, during which oxygen levels were held at ~ 100% of air saturation (~ 19.0 kPa oxygen at our elevation, 1000 m.a.s.l.) on both sides of the aquarium. On the following morning, the oxygen level on a randomly chosen side of the chamber was decreased to ~ 30%, 50% or 70% of saturation (~ 5.7, 9.5, 13.3 kPa, respectively) by bubbling in nitrogen gas, or kept at ~ 100% of saturation (~ 19 kPa) (control). Nymphs were left in the chamber for 2-3 hours, after which we recorded their locations (hypoxic vs. normoxic side) and reset the oxygen level on each side of the aquarium to 100% of saturation (19 kPa). Nymphs were then acclimated until the next morning, being free to eat and move between sides of the chamber. Each nymph experienced every oxygen-choice combination over the course of 5 days. Afterward, each nymph was lighted patted dry with paper towels (Kimtech, Kimwipes; Roswell, Georgia, USA) and weighed. New nymphs were then put in the chamber to experience each choice.

Temperature choice experiments

During the summer and fall of 2020, we measured how salmonfly nymphs distributed themselves between two static temperatures in an experimental aquarium (49×68×18.5 cm). The arena consisted of two insulated containers (Coleman, 24 Can Party Stackers; Chicago, Illinois, USA) connected via half-inch PVC pipes, providing a route for movement between differing temperatures on either side. Containers were divided into five lanes via mesh walls. Additional mesh walls separated the experimental lanes from the air stones, stainless steel coils, and water pumps that controlled the temperature, oxygen, and flow conditions of each side of the chamber. High-grit sandpaper (Gator, P150) glued to the floor of the chamber provided footing for the nymphs.

We measured temperature preferences of 51 nymphs – 32 in normoxia (100% of air saturation; 19 kPa) and 19 in hypoxia (60 - 95% of air saturation; 11.4 - 18.05 kPa). At the onset of each trial, one nymph was placed on a random side of each lane of the aquarium and left to acclimate for 6 - 12 hours. During this period, oxygen levels were ~ 100% of saturation (19 kPa) on both sides of the aquarium and temperatures were either 15 °C or 20 °C. For treatments with a 15 °C reference temperature, nymphs were given a choice the following morning between 15 °C versus 15 (control), 20, or 25 °C. These occurred at either normoxia (n = 22) or hypoxia (n = 19). For 20 °C reference trials, nymphs were given a choice of 20 °C versus 20 (control), 15, 25 and 30 °C at normoxia only (10 nymphs). Nymphs were left in the chamber for 6-12 hours, after which we recorded the location (warm or cool side) of each nymph and reset the temperature on each side of the aquarium to the reference temperature (15 or 20 °C). Nymphs were then acclimated until the next morning. Each nymph was assigned to either the 15 or the 20 °C reference experiment, but within that experiment they experienced every temperature-choice combination. Once nymphs experienced each choice-trial, we weighed them and placed new nymphs in the chamber for a new round of trials.

Flow choice experiments

In the flow experiments, nymphs were subjected to ramps of decreasing flow, and we measured the water velocities at which they relocated to higher flows in an adjacent chamber. We conducted these experiments in winter, 2021. The flow chamber (15×17×7.5 cm) was made of glued Plexiglas and divided lengthwise into two lanes by a median wall with a small gap (~ 0.5 cm high) allowing nymphs to move between sides. High-grit sandpaper (Gator, P150) glued to the floor of the chamber provided footing for the nymphs. Variable flows on the two sides were generated by water pumps (SeaFlo, SFBP1-G500; Xiamen, China) positioned inline in closed loops (PVC) connected to either side of each lane of the chamber via milled nylon end caps (designed in Autodesk based on designs from Mike Nishizaki and milled at The Friday Harbor Laboratories by Adam Summers). Mesh walls inserted between the flow chamber and the end caps prevented nymphs from escaping into the PVC loops. Calibrated flow velocities on each side were controlled by a two-way variable DC power supply (RSR, HY3005F-3; Manheim, PA, USA) connected to the pumps.

We established how flow velocity varied as a function of supply voltage by filming neutrally buoyant microscopic glass particles (TSI, 8-12 μm diameter, 1.5 g/cc; Shoreview, Minnesota, USA) moving through the chamber with a cellphone camera (Samsung Galaxy S10e; Suwon-si, South Korea). A ruler was placed within the chamber in the field of view of the camera during each recording. We used MATLAB (function: DLTdv7) to measure how many pixels spanned 1cm on the ruler, the average pixels the glass beads moved per frame, and the frame rate of the video (MATLAB ver. R2019a). We then calculated the velocity of the water via the following equation: $1/(px/cm) * px/frame * frames/s = cm\ s^{-1}$. Flow velocity values used in the analyses were estimated by interpolating these data via linear regression (Fig. S2, Table S1).

During each experiment, we controlled temperature by submerging the apparatus into a temperature-controlled water bath. Water temperatures were monitored throughout each experiment by a Type-T thermocouple and meter (Barnant, 600-1020; London, Ontario, Canada). Oxygen levels were controlled by bubbling air or mixtures of air and N_2 gas into both lanes via small drilled holes on top of the chamber. Oxygen levels were monitored with an oxygen optode (Pyroscience, OXROB3) connected to a meter (Pyroscience FireSting-O2) inserted through an additional hole.

To determine how temperature and oxygen interact with flow to impact microclimate preferences of nymphs, we performed flow choice experiments at two temperatures (10 and 18 °C) and two oxygen conditions (100 and 60% of air saturation; ~ 19.0 and 11.4 kPa). Ten nymphs were used in each treatment (40 nymphs total). At the onset of the experiment, a nymph was put into a random side of the flow chamber. Preliminary experiments suggested that 9 cm s^{-1} was the maximum velocity in which nymphs could move freely within the chamber without losing their footing and being swept into the mesh at the end. Preliminary experiments also showed that most nymphs explored both sides of the chamber for ~ 40 minutes before settling into a single lane for at least 2 hours. Based on these observations, nymphs were allowed to acclimate within the chamber for 50 minutes with flow velocities on both sides set to 9 cm s^{-1}. Subsequently, we recorded the location (left vs. right side) of the nymph and began to ramp down the flow velocity on that side of the chamber at 10 minute intervals (7, 5, 3, 2, 1.5, 1, 0.75, 0.5, 0.25, and 0 cm s^{-1}), while the flow velocity on the other side was kept constant at 9 cm s^{-1}. The location of the nymph was recorded at each interval.

Statistical Analyses

Before analysis, we removed extreme outliers from the microclimate dataset. This was done post hoc by pooling the measurements of each variable and discarding values that were < Q1 – 3 * IQR or > Q3 + 3 * IQR. We used linear mixed-effects models implemented in the R package 'nlme' (function: lme) (Pinheiro et al., 2021; R Core Team, 2021) to test whether significant variation in water temperature, oxygen saturation, and flow velocity existed among cobble microclimates (i.e., bottom-fronts, bottom-backs, and tops of cobbles and free stream) and along the length of riffles in the Blackfoot River and Rock Creek. Because we were not interested in conditions at particular sites and cobbles per se, site- and cobble-identity were modeled as random effects. We included the duration of time elapsed from the first sample to each sample per site as a covariate to account for changing conditions throughout the sampling period at each site.

To determine the effect of factor – oxygen, temperature, or flow – on the variability of measurements within riffles and around individual cobbles, we used ANOVA models (function: lm) (R Core Team 2021). We tested the effect of factor on the coefficients of variation (standard deviation / mean) of measurements for each variable at the whole site and individual cobble scales. For each model, river was included as a covariate.

After performing the oxygen choice trials, we discarded data for which oxygen levels on the normoxic side fell below 85% of saturation (16.15 kPa), which was due to equipment malfunction. To analyze effects of temperature and oxygen on salmonfly choices, we used zero-inflated negative binomial mixed models (function: glmm.zinb) in the R package 'NBZIMM' (Zhang & Yi 2020). More standard linear mixed effects models could not be used because they resulted in model singularity, likely because the data in many cases were non-normal. NBZIMM uses a non-parametric approach that resolved this issue. Because choices of individual nymphs were measured repeatedly, we used nymph identity as a random effect. In some trials, we were unable to maintain desired temperature and oxygen gradients precisely enough within chambers. To account for this variation, we included the difference between choice conditions (e.g., warm-side temperature – cold-side temperature) on both sides of the chambers as a covariate. In addition, because nymphs in the temperature choice experiment experienced different reference temperatures (i.e., 15 °C and 20 °C), reference temperature was also included as a covariate. For both experiments, preferences for the left versus right side of the chamber in control trials were analyzed by performing exact binomial tests (function: binom.test) (R Core Team).

To test the effects of flow, water temperature, and oxygen saturation on choices nymphs made in flow experiments, we used a mixed-effects logistic regression model, a type of generalized linear mixed-effects model, in the R package 'lme4' (function: glmer) (Bates et al., 2007). Because individual nymphs were measured repeatedly, we included nymph ID as a random effect. We also included mass as a covariate. In addition, we analyzed the effects of temperature and oxygen using linear regression models (function: lm) (R Core Team). We first analyzed whether temperature and oxygen levels influenced the water velocity at which nymphs switched between chambers for the final time (i.e., moved to a higher flow). Some nymphs, however, switched to the high flow chamber early during the ramp but later moved back and forth near the end of the trial. This resulted in several low 'last switch' flow velocity values, which may have skewed the results of the first model. To account for this potential bias, we also analyzed total time nymphs spent on the high flow side of the chamber.

For all of the above investigations, we did not pre-specify a target effect size. None of these experiments has been done previously, and we thus did not know what the variances would be within groups – either for the observational data collected on abiotic conditions within streams or the experimental data on nymph choices made in response to sets of conditions in the lab. Rather, we strived to maximize the number of replicates in each observation or experiment given existing limitations on time and resources.

Results

Field microclimate measurements

Oxygen saturation varied little at small scales within riffles of the Blackfoot River and Rock Creek (Fig. 2, Table 1). The mean range within riffles was < 6% of air saturation (1.14 kPa) in both streams. In the Blackfoot, oxygen varied with distance and time elapsed ($F_{1,111}$ = 5.34, P = 0.023 and $F_{1,111}$ = 21.90, P < .001), but not micro-position. In Rock Creek, oxygen varied significantly with micro-position and time elapsed ($F_{2,272}$ = 3.63, P = 0.028 and $F_{1,137}$ = 4.14, P = 0.044, respectively), but not distance. Differences between micro-positions were slight even when significantly different (i.e., < 0.4% of air saturation; 0.08 kPa) and are likely of little relevance to invertebrates, as shown below.

Water temperature varied modestly within stream riffles (Fig. 2, Table 1). The mean temperature range within riffles was ~ 1.7 °C in both streams. Temperature varied significantly with distance along riffles (Blackfoot: $F_{1,111}$ = 36.19, P < 0.001 and Rock Creek: $F_{1,137}$ = 14.00, P < 0.001), even after accounting for the effect sampling time on micro-thermal variation, which was significant in Rock Creek but not the Blackfoot ($F_{1,137}$ = 8.36, P = 0.005 and $F_{1,111}$ = 1.43, P = 0.234, respectively). On average, temperatures were ~ 0.7 °C warmer at riffle heads than tails, though they varied more strongly at some sites (e.g., Blackfoot River site 2 where riffle head ~ 3.2 °C warmer than riffle tail). Micro-position surrounding cobbles also had a significant effect on temperature ($F_{2,235}$ = 5.07, P = 0.007 and $F_{2,276}$ = 6.39, P = 0.002 for Blackfoot and Rock, respectively). However, mean thermal differences between micro-positions were small, particularly between the front and rear of cobbles (i.e., < 0.06 °C), and are likely of little biological importance, as demonstrated below.

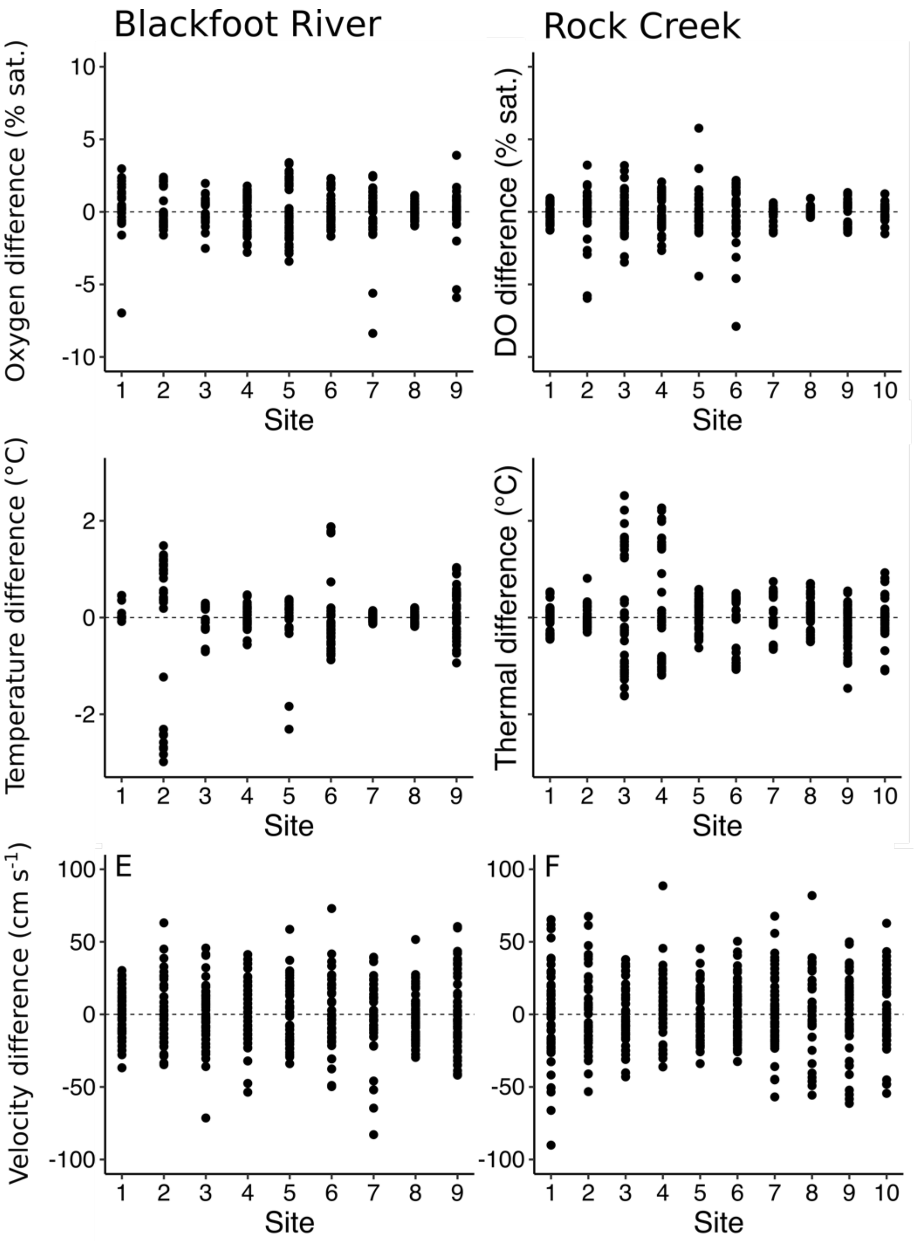

Fig 2: Scatter plots of oxygen saturation (A, B), temperature (C, D), and flow velocity (E, F) samples minus the mean for each site from the Blackfoot River and Rock Creek, respectively. Absolutely values are not reported because they varied strongly with time, and measurements were taken at different days and times.

Flow velocity varied strongly within stream riffles (Fig. 2, Table 1). The mean range of flow velocities at sites in both rivers were > 125 cm s^{-1}. Although flows did not vary along the lengths of riffles ($F_{1,113} = 0.49$, $P = 0.486$ and $F_{1,113} = 0.81$, $P = 0.370$ for Blackfoot and Rock Creek, respectively), flow velocity depended strongly on micro-position in both rivers (Blackfoot: $F_{3,361} = 232.83$, $P < 0.001$; Rock Creek: $F_{3,432} = 337.42$, $P < 0.001$). The lowest flows – mean ~ 20 cm s^{-1} but often near 0 cm s^{-1} – occurred at the front and rear of rocks. Flows were usually high on the tops of cobbles and higher still in the free stream, with a mean of ~ 64 cm s^{-1} and ~ 87 cm s^{-1} for both rivers, respectively.

Interactions among covariates were largely non-significant for temperature, oxygen, and flow data in each river and are therefore excluded from Table 1. See Table S2 for all the statistics, including interactions, of each microclimate model.

Factor identity had a significant effect on the coefficients of variation of measurements made within stream riffles ($F_{2,187} = 155.03$, $P < .001$) and around individual cobbles ($F_{2,852} = 90.26$, $P < .001$) (Table S3). The effect of river was non-significant for each model; interactions were non-significant. Oxygen, temperature, and flow measurements had mean coefficients of variation of 0.01, 0.03, and 0.68, respectively, at the site scale for each river. At the individual cobble scale, oxygen, temperature, and flow measurements had mean coefficients of variation of < 0.01, < 0.01, and 0.32, respectively, for each river.

Laboratory choice experiments

Oxygen saturation had a significant effect on the microdistributions of salmonfly nymphs in the oxygen choices experiments ($F_{1,43} = 16.78$, $P < 0.001$) (Fig. 3A) (Table 2). Choices were strongest when nymphs were exposed to steep oxygen gradients. 100% and ~ 87% of nymphs were found on the normoxic side when given a choice of 10-35% (1.9 – 6.65 kPa) and 35-60% (6.65 – 11.4 kPa) saturation versus normoxia, respectively. However, when presented with more modest oxygen gradients of 60-85% (11.4 – 16.15 kPa) of saturation versus normoxia, only 45% of nymphs were found on the normoxic side. Initial explorations of the data showed that differences between the oxygen levels on both sides, temperature (Fig. S3A), and nymph mass (range: 0.06 – 1.32g) had no significant effect on oxygen choices, and these covariates were thus excluded from the final model. In addition, there was no significant difference between nymph movements towards the left versus right side of the chamber in the control trials ($P = 0.832$).

Temperature had a near-significant effect on distributions of nymphs in the temperature choice experiments ($F_{1,77} = 3.55$, $P = 0.063$) (Fig. 3B, Table 2). There was a significant interaction between temperature and oxygen ($F_{1,77} = 7.19$, $P = 0.009$), such that nymphs made stronger choices based on temperature when oxygen levels were low (Fig. S3B). However, the effect of this interaction was small, with an interaction coefficient of -0.01. Nymph mass (range: 0.10 – 1.56 g), difference between the temperatures on both sides, and reference temperature had no significant effect and were excluded from the final model. There was also no significant difference between nymph movements towards the left versus right side of the chamber in the control trials ($P = 0.850$).

In the flow choice experiments, flow had a significant effect on the locations of nymphs ($z = -4.08$, $P < 0.001$); nearly all nymphs ($n = 37/40$) moved to the high-flow side before the low-flow side reached 0 cm s^{-1} (Fig. 4, Table 3). The effect of temperature was significant ($z = 2.07$, $P = 0.039$), such that nymphs had a stronger preferences for high flows when temperatures were warm. In addition, there was a significant interaction between oxygen and flow ($z = -1.99$, $P = 0.047$), such that nymphs relocated to higher flows more readily when oxygen levels were low. Mass (range: 0.18 – 1.40g) had no effect and was dropped from the final model. Supplemental analyses yielded similar results: Oxygen saturation ($F_{1,36} = 6.16$, $P = 0.018$) and temperature ($F_{1,36} = 5.45$, $P = 0.025$) had a significant effect on the time nymphs spent on the high flow side (Fig. 5A, Table 4); oxygen saturation had a significant effect on the water velocity of the low flow side at the final time nymphs moved from the low to the high flow side of the chamber ($F_{1,36} = 11.00$, $P = 0.002$), and temperature had a near significant effect ($F_{1,36} = 3.48$; $P = 0.070$) (Fig. 5B, Table 4). No significant interactions were found. Mass (range: 0.18 – 1.40g) had no effect and was dropped from both models.

Discussion

For insects, aquatic life is challenging because water contains little oxygen (Woods & Moran, 2020; Harrison et al., 2018; Harrison et al., 2012; Verberk et al., 2011). This basic problem of oxygen scarcity can be further exacerbated by warm temperatures and low flows, which increase oxygen demand and decrease oxygen supply, respectively (Verberk et al., 2016; Frakes et al., 2021). For insects, one potential solution is to exploit mosaics of local microclimates as a way of obtaining combinations of temperature, oxygen, and flow that promote high performance or that minimize low-oxygen stress (Birrell et al., 2020). Whether insects do so, however, depends on (1) whether biologically meaningful variation in conditions exists at spatial scales accessible by individuals and (2) whether individuals can in fact detect and choose to exploit local variation. Here, we demonstrate that flow velocities in mountain streams varied much more strongly at small scales than did either temperature or oxygen. Accordingly, and in support of our hypothesis, nymphs exploited laboratory gradients of flows far more readily than they did temperature or oxygen; nymphs made choices on temperature and oxygen only when presented with unrealistically strong gradients, which do not reflect field observations.

Table 1: Linear mixed effects models for the effects of micro-position, distance along riffles, and time elapsed during sampling at each site on oxygen saturation, water temperature, and flow velocity.

Oxygen Saturation Microclimates				
	Numerator DF	Denominator DF	F-value	P-value
Blackfoot River				
Micro-position	2	231	2.23	0.110
Distance	1	111	5.34	0.023
Time elapsed	1	111	21.90	< 0.001
Rock Creek				
Micro-position	2	272	3.63	0.028
Distance	1	137	1.08	0.302
Time elapsed	1	137	4.14	0.044

Water Temperature Microclimates				
	Numerator DF	Denominator DF	F-value	P-value
Blackfoot River				
Micro-position	2	235	5.07	0.007
Distance	1	111	36.19	< 0.001
Time elapsed	1	111	1.43	0.234
Rock Creek				
Micro-position	2	276	6.39	0.002
Distance	1	137	14.00	< 0.001
Time elapsed	1	137	8.36	0.005

Flow Velocity Microclimates				
	Numerator DF	Denominator DF	F-value	P-value
Blackfoot River				
Micro-position	3	361	232.83	< 0.001
Distance	1	113	0.49	0.486
Time elapsed	1	113	0.81	0.371
Rock Creek				
Micro-position	3	432	337.43	< 0.001
Distance	1	136	0.81	0.370
Time elapsed	1	136	8.49	0.004

See Table S1 for full tables showing interactions among covariates.

Table 2: Zero-inflated negative binomial mixed models for the effect of oxygen and temperature on the location of nymphs in oxygen and temperature choice experiments.

Oxygen choice experiments				
	Numerator DF	Denominator DF	F-value	P-value
Oxygen	1	43	16.78	< 0.001

Temperature choice experiments				
	Numerator DF	Denominator DF	F-value	P-value
Temperature	1	77	3.55	0.063
Oxygen	1	77	0.43	0.514
Temperature : Oxygen	1	77	7.19	0.009

We measured variation in temperature, oxygen, and flow at two spatial scales in Montana streams – around individual cobbles (scale of ~ 5 to 50 cm) and along the length of riffles (scale of ~ 5 to 10 m). Differences in temperature and oxygen were slight around individual cobbles: < 0.06 °C and < 0.4% of air saturation (0.8 kPa), respectively. Weak gradients like these offer little opportunity to shift levels of oxygen supply and demand (Verberk et al., 2011) and are probably not actively exploited as evidenced by the results from our choice experiments. Within riffles, oxygen and temperature varied more substantially: oxygen saturation varied irregularly by ~ 6%

of air saturation (1.14 kPa), and temperature varied by ~ 1.7 °C, with water near the tail of the riffle being consistently cooler (~ 0.7°C) than water near the head. These patterns likely arise because water downwells at riffle heads and is partially replaced by upwelling groundwater at the tails (Davy-Bowker et al., 2006). At several sites, temperatures changed more strongly with distance, being ~ 3-4 °C cooler at riffle trails. Strong, but inconsistent cold patches have been recorded in other streams, and are likely important refugia for fish (Ebersole & Frissell, 2001; Ebersole et al., 2003). For stream insects, moving more than a few meters likely raises risks of dislodgement or predation. Indeed, in a rare mark-recapture study of salmonfly nymphs, individuals moved an average of only 1.8 m in three months (Freilich, 1991). In our system, movements at this scale would allow individuals to modify local temperatures and oxygen saturations by ~ 0.31 °C and 0.11% of saturation (0.02 kPa), respectively. Drifting can of course lead to much greater movement and may be important for exploiting distant microclimates (Lancaster & Downes 2013).

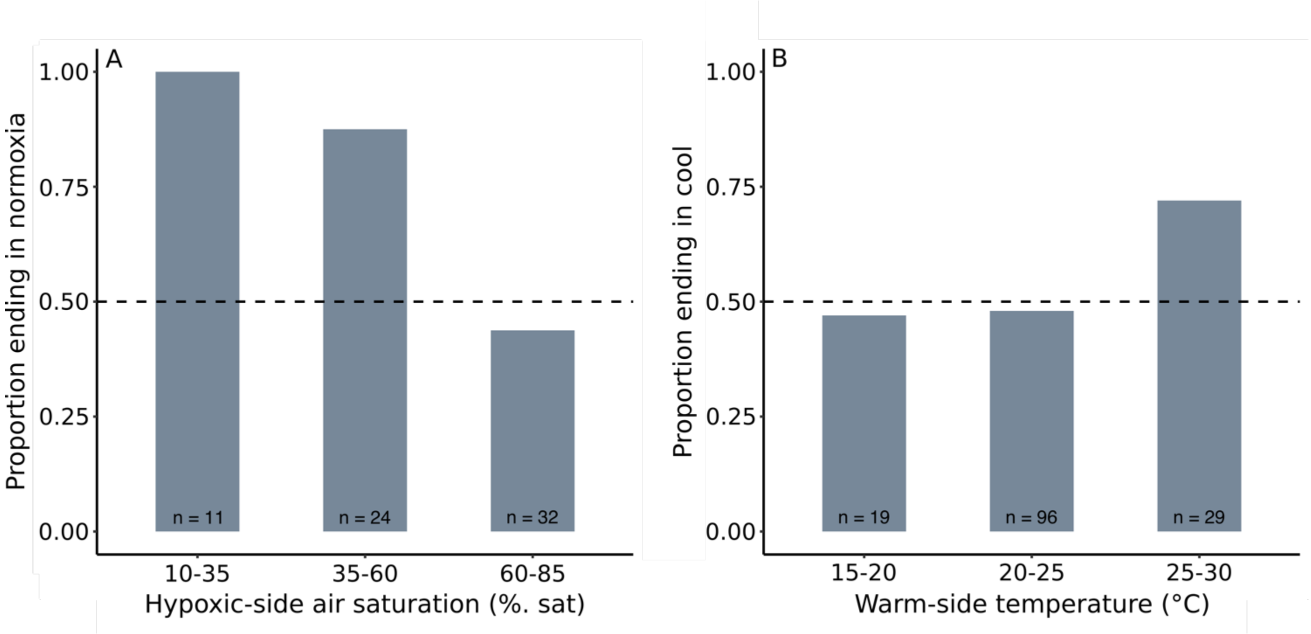

Fig. 3: Barplots summarizing choices made by nymphs in oxygen (A) and temperature (B) choice experiments. Nymphs responded strongly to oxygen but, only when presented with strong oxygen gradients. Nymphs made weak temperature-choices and only when presented with extreme thermal gradients, which are likely rare in nature. Because temperature had no effect on oxygen choices and oxygen level had little effect on temperature choices, we do not distinguish

the results from the various treatments of each experiment in this figure. See Fig. S3 for more detail. Sample sizes differ strongly across treatments because in some trials, a stronger or weaker temperature or oxygen gradient formed than was intended.

Flow velocities, by contrast, varied substantially at small spatial scales, often by more than 125 cm s^{-1} around a single cobble. Local flows available to insects are likely even more variable still, as the flow meter we used was too large to fit underneath cobbles where the lowest flows occur. By moving only a few cm, therefore, individual insects should be able to strongly alter their local flow environments – from < 1 cm s^{-1} under cobbles to > 60 cm s^{-1} on the tops of rocks, decreasing boundary layer thicknesses and increasing rates of oxygen supply (Denny, 1993). Moving greater distances to exploit high flows (e.g., by drifting) is likely ineffective for increasing oxygen supply, as we found that flow velocities did not vary significantly along the lengths of riffles.

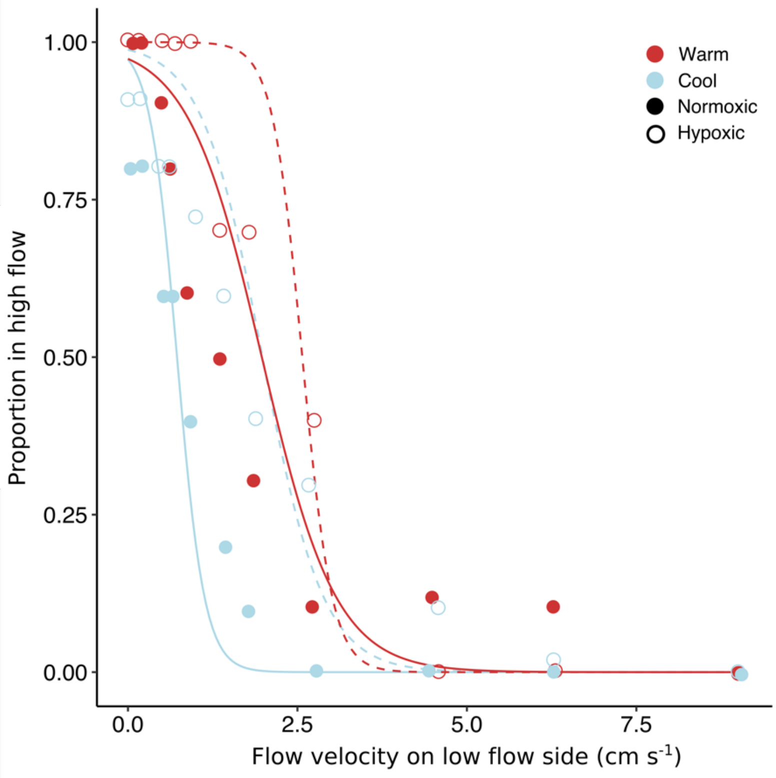

Fig. 4: Scatter plot of the proportion of individuals on the high-flow side of the chamber as flows were ramped down on the low flow side during flow choice experiments. The majority of nymphs in each treatment moved to the high-flow side during the ramps. Individuals moved to the high flow side at higher low-flow velocities when exposed to warm or hypoxic conditions. N = 110 for each treatment.

In support of our hypothesis that insects should evolve to exploit strong gradients in their local environments, salmonfly nymphs exploited laboratory variation in flow much more readily than they did temperature and oxygen. For example, the temperatures and oxygen levels that induced nymphs to make choices represented gradients that were ~ 1-2 orders of magnitude greater than those observed in the field. In contrast, nymphs made consistently strong choices in response to ecologically relevant gradients of flow. This suggests that while differences in design between experiments – i.e., two static choices for temperature and oxygen versus a ramp for flow – may have had some influence on the results, nymphs nonetheless exploit local flows more readily than temperature or oxygen. Similar responses to laboratory flows have been observed in mayflies (Wiley & Kohler 1980). In addition, we show some of the first experimental evidence that aquatic insects move to areas of high flow more readily, and spend more time in them, when temperatures are high and oxygen levels low. Our results corroborate field observations of caddisflies and stoneflies, showing that individuals occur more frequently in high-flow microhabitats when bulk flows were low or temperatures high (Kovalak, 1976; Kovalak, 1979; Genkai-Kato et al., 2005). Taken together, these results support the idea that interactions among temperature, oxygen, and flow strongly influence aquatic ectotherm metabolism, performance, and survival (Pörtner, 2007; Rubalcaba et al., 2020; Verberk et al., 2016a b; Harrison et al., 2018; Frakes et al., 2021). In addition, they suggest that aquatic insects exploit mosaics of flow to increase ratios of oxygen supply:demand in nature. Curiously, however, mass had no effect on the choices made by nymphs even though larger nymphs typically have greater demand for oxygen (e.g., Malison et al. 2022). We suggest that compensatory mechanisms may allow nymphs to maintain adequate rates of oxygen supply, e.g., by growing disproportionately larger gills or by increasing the density of tracheal tubes in their tissues in later instars.

Table 3: Mixed effects logistic models of the effect of flow velocity, temperature, and oxygen saturation on the locations of nymphs in flow choice experiments.

Flow Choice experiments				
	Estimate	Std. Error	Z-value	P-value
Flow	-2.22	0.54	-4.08	< 0.001
Temperature	7.23	3.50	2.07	0.039
Oxygen	0.83	2.27	-0.37	0.715
Flow : Temperature	-2.30	1.25	-1.84	0.066
Flow : Oxygen	-2.72	1.36	-1.99	0.047
Temperature : Oxygen	-7.19	4.24	-1.70	0.090
Flow : Temperature : Oxygen	5.41	2.07	2.62	0.009

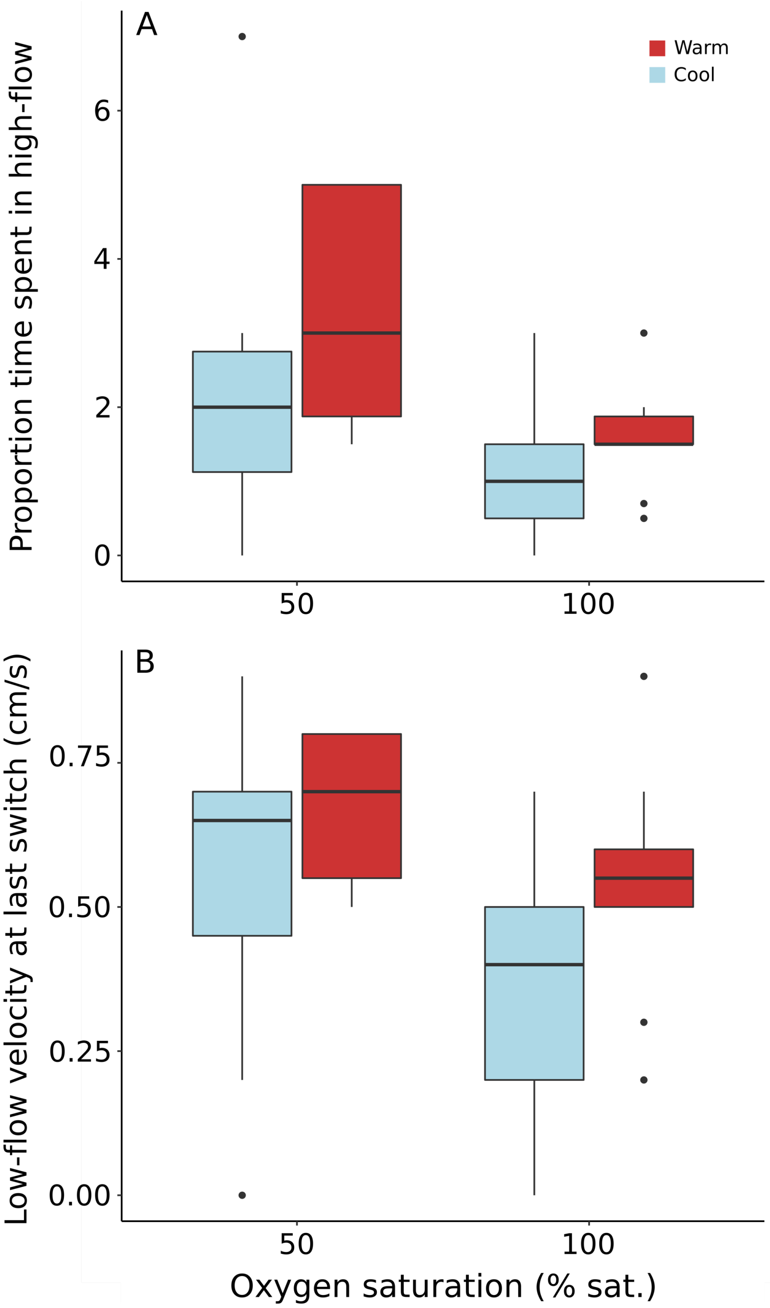

Fig. 5: Boxplots of nymph choices in flow choices experiments. Nymphs spent more time in the high-flow side under warm and hypoxic conditions (A). Nymphs also moved to the high-flow side at higher low-flow side velocities when conditions were warmer and more hypoxic (B).

As predicted from the weak gradients in temperature and oxygen measured at the scale of individual cobbles, nymphs made weak choices based on temperature and only when presented with ecologically unrealistic gradients (i.e., 20 vs. 28°C). Nymphs made stronger choices based on oxygen, but as in the temperature experiments, only when gradients were stronger than those found commonly in nature (i.e., 10-55% vs. 100% of air saturation; 1.9-10.5 kPa vs. 19.0 kPa). Counter to our predictions, oxygen choices were not affected by temperature, possibly because 15 °C was not warm enough to raise metabolic demand significantly (Malison et al. 2022). In addition, although hypoxia led nymphs to make stronger temperature choices, the effect of this interaction was weak (interaction coefficient -0.01). This is perhaps due to the apparently weak abilities of nymphs to sense and respond to temperature and oxygen. However, they clearly do have at least *some* ability exploit these conditions, which may allow them to avoid stressful conditions that occur irregularly in nature – likely avoiding deep, hypoxic pools or inflows from hot-springs. In more usual circumstances, salmonflies, and other aquatic insects, may rely, at least in part, on their strong acclimation abilities to buffer against warming and hypoxia (Gunderson & Stillman, 2015).

Table 4: Linear models for the effect of oxygen and temperature on the low-flow velocity at time of last switch and the proportion of time spent in the high-flow side of the chamber.

Proportion time spent in high-flow side					
	DF	Sum squared error	Mean square	F-value	P-value
Temperature	1	0.26	0.26	5.45	0.025
Oxygen	1	0.29	0.29	6.16	0.018
Temperature : Oxygen	1	0.01	0.01	0.19	0.664

Low-flow velocity at last switch					
	DF	Sum squared error	Mean square	F-value	P-value
Temperature	1	6.56	6.56	3.48	0.070
Oxygen	1	20.74	20.74	11.00	0.002
Temperature : Oxygen	1	1.16	1.16	0.61	0.439

In the face of climate change and other anthropogenic disturbances, organisms may: go extinct, shift their ranges, evolve new capacities or tolerances, or employ different forms of plasticity (e.g., Doneleson et al., 2019). Our study shows that behavioral plasticity – i.e., moving among locally available flows – may allow stream insects to mitigate oxygen limitation arising from climate change, river dewatering and damming, and nutrient-driven eutrophication (Birrell et al., 2020). Aquatic insects may also rely on other behaviors, like more frequent ventilatory movements, to increase ratios of oxygen supply:demand (Genkai-Kato et al., 2000; Frakes et al., 2021). Other forms of plasticity – i.e., morphological and physiology plasticity – will likely also be important (Angilletta, 2009). For example, aquatic insects like stoneflies and caddisflies tend to grow larger gills (Witchard, 1974; Badcock et al., 1987) and tolerate high temperatures better following acclimation to high temperatures or hypoxia (Gundersen & Stillman, 2015; Malison et al., in review). Indeed, aquatic taxa have about twice the acclimation abilities of terrestrial taxa, which may help buffer them from future functional hypoxia from droughts, warming, and other disturbances (Gunderson & Stillman, 2015).

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References

Angilletta, M. (2009) Thermal adaptation: a theoretical and empirical synthesis. Oxford: Oxford University Press.

Badcock, R. M., Bales, M. T. and Harrison, J. D. (1987). Observations on gill number and respiratory adaptation in caddis larvae. In *Proceedings of the Fifth International Symposium on Trichoptera* (ed. Bournaud, M.) and Tachet, H.), pp. 175–178. Dordrecht: Dr. W. Junk Publishers. https://doi.org/10.1007/978-94-009-4043-7_32

Bates, D., Mächler, M., Bolker, B., Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Jour. of Stat. Soft*. 67, 1–48. https://doi.org/10.48550/arXiv.1406.5823

Birrell, J. H., Shah, A. A., Hotaling, S., Giersch, J. J., Williamson, C. E., Jacobsen, D. and Woods, H. A. (2020). Insects in high-elevation streams: Life in extreme environments imperiled by climate change. *Glob. Chang. Biol.* 26, 6667–6684. https://doi.org/10.1111/gcb.15356

Clark, E., Webb, B. W. and Ladle, M. (1999). Microthermal gradients and ecological implications in Dorset rivers. *Hydrol. Process.* 13, 423–438. https://doi.org/10.1002/(SICI)1099-1085(19990228)13:3<423::AID-HYP747>3.0.CO;2-%23

Collins, M., Truebano, M., Verberk, W. C. E. P. and Spicer, J. I. (2021). Do aquatic ectotherms perform better under hypoxia after warm acclimation? *J. Exp. Biol.* 224, 232512. https://doi.org/10.1242/jeb.232512

Comiti, F., Mao, L., Wilcox, A., Wohl, E. E. and Lenzi, M. A. (2007). Field-derived relationships for flow velocity and resistance in high-gradient streams. *J. Hydrol.* 340, 48–62. https://doi.org/10.1016/j.jhydrol.2007.03.021

Davy-Bowker, J., Sweeting, W., Wright, N., Clarke, R. T. and Arnott, S. (2006). The distribution of benthic and hyporheic macroinvertebrates from the heads and tails of riffles. *Hydrobiologia* 563, 109–123. https://doi.org/10.1007/s10750-005-1482-9

Denny, M. W. (1993). *Air and water: the biology and physics of life's media*. Princeton: Princeton University Press.

Deutsch, C., Ferrel, A., Seibel, B., Portner, H.-O. and Huey, R. B. (2015). Climate change tightens a metabolic constraint on marine habitats. *Science.* 348, 1132–1135. https://doi.org/10.1126/science.aaa1605

Donelson, J. M., Sunday, J. M., Figueira, W. F., Gaitán-Espitia, J. D., Hobday, A. J., Johnson, C. R., Leis, J. M., Ling, S. D., Marshall, D., Pandolfi, J. M., et al. (2019). Understanding interactions between plasticity, adaptation and range shifts in response to marine environmental change. *Philos. Trans. R. Soc. B Biol. Sci.* 374, 20180186. https://doi.org/10.1098/rstb.2018.0186

Ebersole, J. L., Liss, W. J. and Frissell, C. A. (2001). Relationship between stream temperature, thermal refugia and rainbow trout Oncorhynchus mykiss abundance in arid-land streams in the northwestern United States. *Ecol. Freshw. Fish* 10, 1–10. https://doi.org/10.1034/j.1600-0633.2001.100101.x

Ebersole, J. L., Liss, W. J. and Frissell, C. A. (2003). Cold water patches in warm streams: Physicochemical characteristics and the influence of shading. *J. Am. Water Resour. Assoc.* 39, 355–368. https://doi.org/10.1111/j.1752-1688.2003.tb04390.x

Eggert, S. L. and Wallace, J. B. (2007). Wood biofilm as a food resource for stream detritivores. *Limnol. Oceanogr.* 52, 1239–1245. https://doi.org/10.4319/lo.2007.52.3.1239

Elliott, J. M. (2000). Pools as refugia for brown trout during two summer droughts: Trout responses to thermal and oxygen stress. *J. Fish Biol.* 56, 938–948. https://doi.org/10.1111/j.1095-8649.2000.tb00883.x

Frakes, J. I., Birrell, J. H., Shah, A. A. and Woods, H. A. (2021). Flow increases tolerance of heat and hypoxia of an aquatic insect. *Biol. Lett.* 17, 20210004. https://doi.org/10.1098/rsbl.2021.0004

Freilich, J. E., (1991). Movement patterns and ecology of Pteronarcys nymphs (Plecoptera) observations of marked individuals in a Rocky Mountain stream. *Freshw. Biol. 25*, 379-394. https://doi.org/10.1111/j.1365-2427.1991.tb00500.xC

Genkai-Kato, M., Mitsuhashi, H., Kohmatsu, Y., Miyasaka, H., Nozaki, K. and Nakanishi, M. (2005). A seasonal change in the distribution of a stream-dwelling stonefly nymph reflects oxygen supply and water flow. *Ecol. Res.* 20, 223–226. https://doi.org/10.1007/s11284-004-0029-2

Gunderson, A. R. and Stillman, J. H. (2015). Plasticity in thermal tolerance has limited potential to buffer ectotherms from global warming. *Proc. R. Soc. B Biol. Sci.* 282, 20150401. https://doi.org/10.1098/rspb.2015.0401

Hall, R. O. and Ulseth, A. J. (2019). Gas exchange in streams and rivers. *WIREs Water* 7, 1–18. https://doi.org/10.1002/wat2.1391

Harrison, J. F., Greenlee, K. J. and Verberk, W. C. E. P. (2018). Functional Hypoxia in Insects: Definition, Assessment, and Consequences for Physiology, Ecology, and Evolution. *Annu. Rev. Entomol.* 63, 303–325. https://doi.org/10.1146/annurev-ento-020117-043145

Harrison, J. F., Woods, H. A., & Roberts, S. P. (2012). *Ecological and environmental physiology of insects*. Oxford: Oxford University Press.

Helmuth, B., Kingsolver, J. G. and Carrington, E. (2005). Biophysics, physiological ecology, and climate change: does mechanism matter? *Annu. Rev. Physiol.* 67, 177–201. https://doi.org/10.1146/annurev.physiol.67.040403.105027

Hutchinson, G. E. (1981). Thoughts on aquatic insects. *Bioscience* 31, 495–500. https://doi.org/10.2307/1308491

Hynes, H. B. N. (1970). *The Ecology of Running Waters*. Liverpool: Liverpool University Press.

Jacobsen, D., Rostgaard, S. and Vásconez, J. J. (2003). Are macroinvertebrates in high altitude streams affected by oxygen deficiency? *Freshw. Biol.* 48, 2025–2032. https://doi.org/10.1046/j.1365-2427.2003.01140.xC

Jacobsen, D. (2008). Low oxygen pressure as a driving factor for the altitudinal decline in taxon richness of stream macroinvertebrates. *Oecologia* 154, 795–807.

Jones, J. D. (1972). *Comparative physiology of respiration*. London: Edward Arnold.

Kovalak, W. P. (1976). Seasonal and diel changes in the positioning of Glossosoma nigrior Banks (Trichoptera: Glossosomatidae) on artificial substrates. *Can. J. Zool.* 54, 1585–1594. https://doi.org/10.1139/z76-184

Kovalak, W. P. (1979). Day-night changes in stream benthos density in relation to current velocity. *Arch. Hydrobiol.* 87, 1–18.

Lancaster, J. and Downes, B. J. (2013). *Aquatic Entomology*. Oxford: Oxford University Press.

Malison, R.L., Frakes, J.I., Andreas, A.L., Keller, P.R., Hamant, E., Shah, A.A., and Woods, H.A. (2022). Plasticity of salmonfly (Pteronarcys californica) respiratory phenotypes in response to changes in temperature and oxygen. *J. Exp. Biol.*, *225*, 244253. https://doi.org/10.1242/jeb.244253

Matthews, K. R. and Berg, N. H. (1997). Rainbow trout responses to water temperature and dissolved oxygen stress in two southern California stream pools. *J. Fish Biol.* 50, 50–67. https://doi.org/10.1111/j.1095-8649.1997.tb01339.x

Mérigoux, S. and Dolédec, S. (2004). Hydraulic requirements of stream communities: a case study on invertebrates. *Freshw. Biol.* 49, 600–613. https://doi.org/10.1111/j.1365-2427.2004.01214.x

Mosley, M. P. (1983). Variability of water temperatures in the braided Ashley and Rakaia rivers. *New Zeal. J. Mar. Freshw. Res.* 17, 331–342. https://doi.org/10.1080/00288330.1983.9516007

Pincebourde, S. and Woods, H. A. (2020). There is plenty of room at the bottom: microclimates drive insect vulnerability to climate change. *Curr. Opin. Insect Sci.* 41, 63–70. https://doi.org/10.1016/j.cois.2020.07.001

Pinder, A. W. and Feder, M. E. (1990). Effect of boundary layers on cutaneous gas exchange. *J. Exp. Biol.* 154, 67–80. https://doi.org/10.1242/jeb.154.1.67

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., & R Core Team. (2016). Nlme: linear and nonlinear mixed effects models. *R package version*, 3, 1-89. https://doi.org/10.1080/10705511.2017.1396187

Pörtner, H. O. and Knust, R. (2007). Climate Change Affects Marine Fishes Through the Oxygen Limitation of Thermal Tolerance. *Science (80-.).* 315, 95–97. https://doi.org/10.1126/science.1135471

R Core Team. (2019) R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing.

Rubalcaba, J. G., Verberk, W. C. E. P., Jan Hendriks, A., Saris, B. and Woods, H. A. (2020). Oxygen limitation may affect the temperature and size dependence of metabolism in aquatic ectotherms. *Proc. Natl. Acad. Sci. U. S. A.* 117, 31963–31968. https://doi.org/10.1073/pnas.2003292117

Scheffers, B. R., Edwards, D. P., Diesmos, A., Williams, S. E. and Evans, T. A. (2014). Microhabitats reduce animal's exposure to climate extremes. *Glob. Chang. Biol.* 20, 495–503. https://doi.org/10.1111/gcb.12439

Torgersen, C. E., Price, D. M., Li, H. W. and McIntosh, B. A. (1999). Multiscale thermal refugia and stream habitat associations of chinook salmon in northeastern Oregon. *Ecol. Appl.* 9, 301–319. https://doi.org/10.1890/1051-0761(1999)009[0301:MTRASH]2.0.CO;2

Verberk, W. C. E. P. and Atkinson, D. (2013). Why polar gigantism and Palaeozoic gigantism are not equivalent: Effects of oxygen and temperature on the body size of ectotherms. *Funct. Ecol.* 27, 1275–1285. https://doi.org/10.1111/1365-2435.12152

Verberk, W. C. E. P. and Bilton, D. T. (2015). Oxygen-limited thermal tolerance is seen in a plastron-breathing insect and can be induced in a bimodal gas exchanger. *J. Exp. Biol.* 218, 2083-2088. https://doi.org/10.1242/jeb.119560

Verberk, W. C. E. P., Bilton, D. T., Calosi, P. and Spicer, J. I. (2011). Oxygen supply in aquatic ectotherms: Partial pressure and solubility together explain biodiversity and size patterns. *Ecology* 92, 1565–1572. https://doi.org/10.1890/10-2369.1

Verberk, W. C. E. P., Overgaard, J., Ern, R., Bayley, M., Wang, T., Boardman, L. and Terblanche, J. S. (2016). Does oxygen limit thermal tolerance in arthropods? A critical review of current evidence. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 192, 64–78. https://doi.org/10.1016/j.cbpa.2015.10.020

Verberk, W. C. E. P., Durance, I., Vaughan, I. P. and Ormerod, S. J. (2016). Field and laboratory studies reveal interacting effects of stream oxygenation and warming on aquatic ectotherms. *Glob. Chang. Biol.* 22, 1769–1778. https://doi.org/10.1111/gcb.13240

Vogel, S. (1994). *Life in moving fluids: The physical biology of flow*. 2nd ed. Princeton: Princeton University Press.

White, D. S. (1990). Biological relationships to convective flow patterns within stream beds. *Hydrobiologia* 196, 149–158. https://doi.org/10.1007/BF00006106

Wichard, W. (1974). Zur morphologischen Anpassung von Tracheenkiemen bei Larven der Limnephilini Kol. (Insecta, Trichoptera). *Oecologia* 15, 159–167.

Woods, H. A. (1999). Egg-mass size and cell size: Effects of temperature on oxygen distribution. *Am. Zool.* 39, 244–252. https://doi.org/10.1093/icb/39.2.244

Woods, H. A. and Moran, A. L. (2008). Temperature-oxygen interactions in Antarctic nudibranch egg masses. *J. Exp. Biol.* 211, 798–804. https://doi.org/10.1242/jeb.014621

Woods, H. A., Dillon, M. E. and Pincebourde, S. (2015). The roles of microclimatic diversity and of behavior in mediating the responses of ectotherms to climate change. *J. Therm. Biol.* 54, 86–97. https://doi.org/10.1016/j.jtherbio.2014.10.002

Woods, H. A and Moran, A. L. (2020). Reconsidering the oxygen-temperature hypothesis of polar gigantism: Successes, failures, and nuance. *Integr. Comp. Biol.* 60, 1438–1453. https://doi.org/10.1093/icb/icaa088

Zhang, X., & Yi, N. (2020). NBZIMM: negative binomial and zero-inflated mixed models, with application to microbiome/metagenomics data analysis. *BMC bioinformatics*, *21*, 1-19. https://doi.org/10.1186/s12859-020-03803-z

Supplemental information

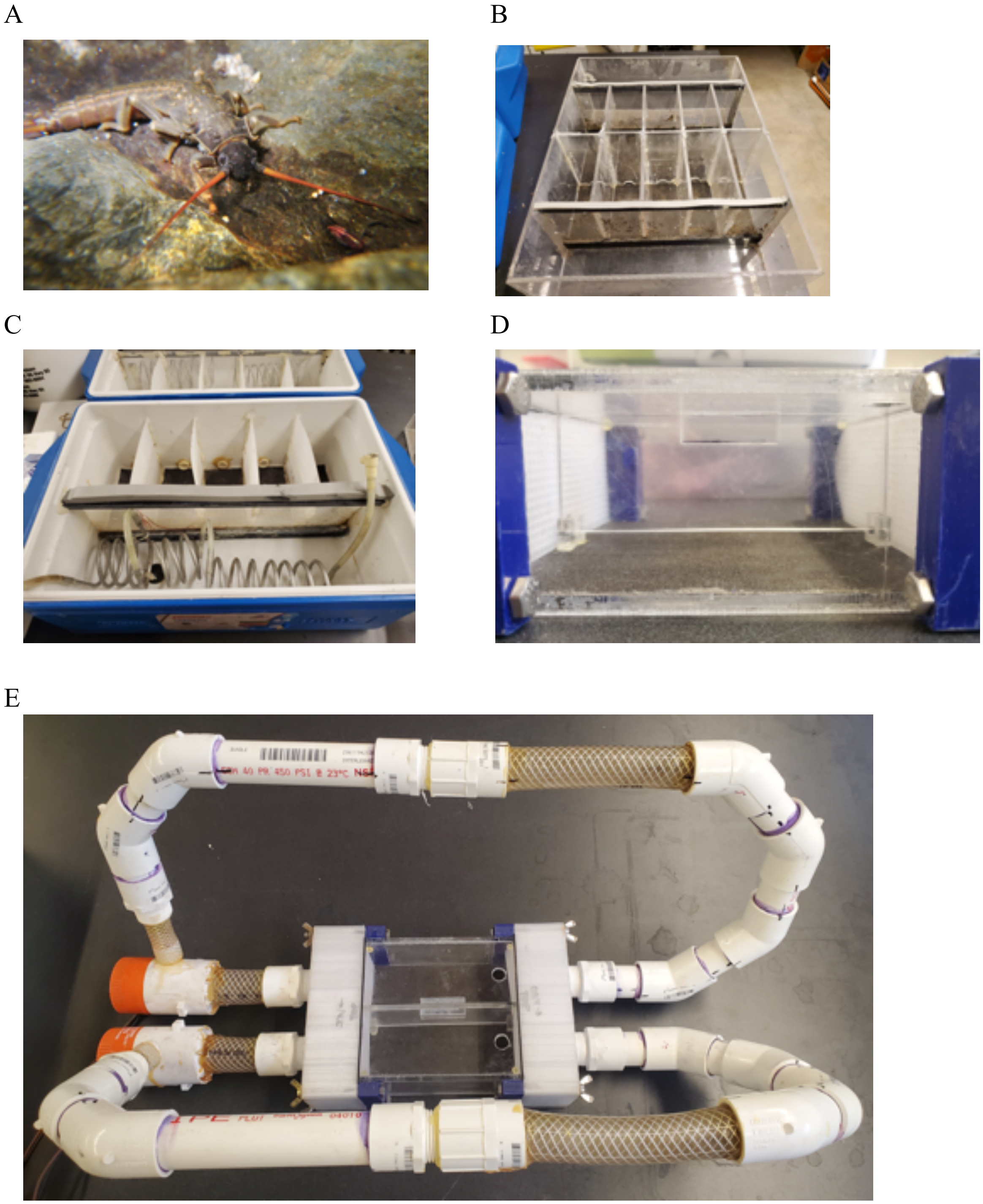

Fig. S1: Photo of *Pteronarcys californica* nymph (A), 5-lane aquariums used in oxygen (B) and temperature (C) choice experiments, and two-lane flow chamber with recirculating loops (D) and center wall with gap (E) used in flow choice experiments.

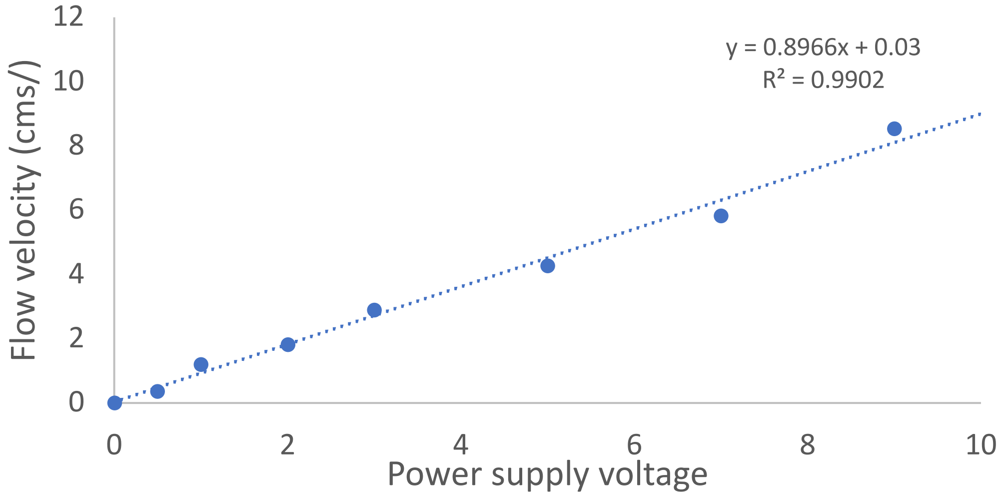

Fig. S2: Measured water velocities in flow chamber at different power supply voltages with linear regression line. Flow velocities used in the analysis were estimated by interpolating these data via linear regression. Y = 0.8966X + 0.03; R2 = 0.9902.

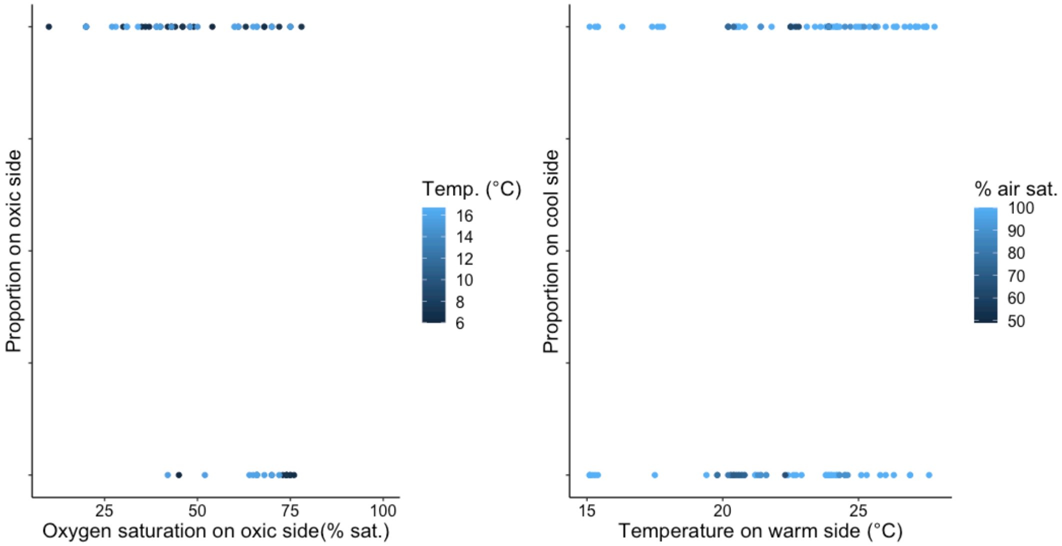

Fig. S3: Dot plots of choices made by nymphs in oxygen (A) and temperature (B) choice experiments at different temperatures and oxygen levels, respectively. Nymphs responded strongly to oxygen but, only when presented with strong oxygen gradients. Nymphs made weak temperature-choices and only when presented with extreme thermal gradients, which are likely rare in nature. Temperature had no effect on oxygen choices and oxygen level had little effect on temperature choices.

Table S1: Interpolated water velocity values in flow chamber at different power supply voltages. Regression equation from which flow estimates are based: Y = 0.8966X + 0.03; R2 = 0.9902.

Voltage	Interpolated water velocity (cm/s)
0.0	0.03
0.2	0.21
0.5	0.48
0.7	0.66
1.0	0.93
1.5	1.37
2.0	1.82
3.0	2.72
5.0	4.51
7.0	6.31
10.0	9.00

Table S2: Linear mixed effects models for the effects of micro-position, distance along riffles, and time elapsed during sampling at each site on oxygen saturation, water temperature, and flow velocity.

Oxygen Saturation Microclimates				
	Numerator DF	Denominator DF	F-value	P-value
Blackfoot River				

Micro-position	2	231	2.23	0.110
Distance	1	111	5.34	0.023
Time elapsed	1	111	21.90	< 0.001
Micro-position : Distance	2	231	0.23	0.797
Micro-position: Time elapsed	2	231	0.04	0.963
Distance : Time elapsed	1	111	0.76	0.387
Micro-position : Distance : Time elapsed	2	231	0.34	0.710
Rock Creek				
Micro-position	2	272	3.63	0.028
Distance	1	137	1.08	0.302
Time elapsed	1	137	4.14	0.044
Micro-position : Distance	2	272	0.54	0.583
Micro-position: Time elapsed	2	272	0.54	0.585
Distance : Time elapsed	1	137	2.94	0.089
Micro-position : Distance : Time elapsed	2	272	3.53	0.031

Water Temperature Microclimates

	Numerator DF	Denominator DF	F-value	P-value
Blackfoot River				
Micro-position	2	235	5.07	0.007
Distance	1	111	36.19	< 0.001
Time elapsed	1	111	1.43	0.234
Micro-position : Distance	2	235	1.73	0.179
Micro-position: Time elapsed	2	235	0.10	0.902
Distance : Time elapsed	1	111	5.98	0.016
Micro-position : Distance : Time elapsed	2	235	0.81	0.448
Rock Creek				
Micro-position	2	276	6.39	0.002
Distance	1	137	14.00	< 0.001
Time elapsed	1	137	8.36	0.005
Micro-position : Distance	2	276	1.40	0.248
Micro-position: Time elapsed	2	276	4.99	0.007
Distance : Time elapsed	1	137	4.00	0.048
Micro-position : Distance : Time elapsed	2	276	0.39	0.674

Flow Velocity Microclimates

	Numerator DF	Denominator DF	F-value	P-value
Blackfoot River				
Micro-position	3	361	232.83	< 0.001
Distance	1	113	0.49	0.486

Time elapsed	1	113	0.81	0.371
Micro-position : Distance	3	361	0.70	0.554
Micro-position: Time elapsed	3	361	1.01	0.388
Distance : Time elapsed	1	113	0.01	0.925
Micro-position : Distance : Time elapsed	3	361	2.91	0.034
Rock Creek				
Micro-position	3	432	337.43	< 0.001
Distance	1	136	0.81	0.370
Time elapsed	1	136	8.49	0.004
Micro-position : Distance	3	432	0.55	0.647
Micro-position: Time elapsed	3	432	8.44	< 0.001
Distance : Time elapsed	1	136	1.29	0.258
Micro-position : Distance : Time elapsed	3	432	2.09	0.102

Table S3: ANOVA model for the effect of variable identify and river on coefficients of variation of measurements within sites and individual cobbles

Coefficients of Variation - Site					
	DF	Sum Square	Mean Square	F-value	P-value
Variable	2	19.97	9.98	155.03	< 0.001
River	1	0.13	0.13	1.95	0.164
Variable : River	2	0.18	0.09	1.37	0.257
Coefficients of Variation - Cobble					
	DF	Sum Square	Mean Square	F-value	P-value
Variable	2	18.26	9.13	90.262	< 0.001
River	1	0.00	0.00	0.031	0.860
Variable : River	2	0.00	0.00	0.016	0.984

Chapter 4

Plasticity overshadows effects of duration during long-term exposure to high but variable temperatures in an aquatic insect nymph.

Plasticity overshadows effects of duration during long-term exposure to high but variable temperatures in an aquatic insect nymph

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Keywords:
Acclimation, climate change, oxygen limitation, thermal tolerance, thermal variation, streams

Abstract

Climate change is exposing ectotherms to more prolonged but variable periods of warming along with shifts in multiple other factors. How will species respond? Expectations are unclear because the mechanisms driving responses to long-term, multivariate challenges are rarely studied together. Indeed, while exposure duration is often hypothesized to *lower* the long-term tolerances of ectotherms via effects of prolonged heat stress, individuals can also *raise* their thermal limits by acclimation. Predictions are further complicated by interactions between temperature and oxygen, which can lower performance and survival during acute exposure to heat due to mismatches between oxygen supply and demand. In this study, we examine how the long-term performance and survival of an aquatic insect nymph (*Pteronarcys californica*) depend on interactions between warming and oxygen availability under ecologically relevant thermal regimes. We do this by exposing nymphs to three temperature profiles separated by 3 °C, which were ramped over three months at rates similar to those in nature, with realistic levels of diel thermal variation (± 2.5 °C). Ramps were replicated in normoxia and modest hypoxia. Overall, duration of exposure and temperature, but not hypoxia, had significant negative effects on survival, feeding, and growth of *P. californica* nymphs. Nymphs were, however, surprisingly resilient, tolerating high temperatures (in both normoxia and hypoxia) more than 10 °C above typical stream temperatures. Indeed, lethal temperatures of some nymphs exceeded upper thermal limits of nymphs from short-term tolerance experiments from the literature. High long-term thermal limits and a weak effect of hypoxia likely arose via plasticity, as the length of gill filaments and upper thermal maxima of nymphs increased after long-term exposure to warming and hypoxia. Additionally, nymphs in the long-term ramps had lower mortality rates and survived for longer periods for a given rise in temperature than those in a complementary, constant-temperature experiment. Overall, our study shows that, in ecologically realistic warming scenarios, plasticity may overshadow the negative effects of duration and temperature-induced oxygen-limitation. Further, our study suggests that in ectotherms with strong acclimation abilities, temperature may only rarely kill organisms outright, indicating that long-term decrements in performance may be the most important mechanism of mortality under natural thermal regimes.

Introduction

Climate change is rapidly altering patterns of thermal variation across nearly all ecosystems, with increases in mean temperatures and in the frequency and duration of extreme events (Diffenbaugh et al. 2013; van Vliet et al. 2013; Cheng et al. 2019; IPCC 2022). Biologists are thus seeking to understand anthropogenic impacts to better prioritize conservation strategies (Pacifi et al. 2015). Understanding organismal responses to climate change will be particularly important due to its ubiquity and strong impacts on fitness-related traits at multiple levels of organization (Woodward et al. 2010; Birrell et al. 2020). A common approach is to examine how the performance and survival of individuals respond to stressful conditions in the laboratory, and then to use the results in combination with models and climate projections to predict the vulnerability of future populations (Kingsolver et al. 2013; Sinclair et al. 2016; Jørgensen et al. 2018;).

Although such approaches can be powerful (e.g., Sunday et al. 2019; Shah et al. 2017a), they often rely on short-term experiments, in which individuals are exposed to single sets of abiotic factors (usually warming temperatures) over hours or days (Sinclair et al. 2016). Short-term experiments, however, best reveal responses to extreme, acute conditions (Frakes et al. 2021) and, thus, may poorly predict distributions or range shifts of threatened aquatic species (e.g., Hotaling et al. 2020). Indeed, climate change also imposes thermal challenges over much longer time frames – i.e., weeks, seasons, and years – and can interact strongly with other abiotic factors (e.g., oxygen availability) that influence organismal performance and survival (Rohr & Palmer 2012; Birrell et al. 2020). Understanding how organisms respond to such long-term, multivariate challenges will be critical for forecasting species' vulnerabilities (Todgham & Stillman 2013; Sinclair et al. 2016). Nevertheless, tolerance experiments that examine long-term effects have been rare (but see Shah et al. 2023), especially those that incorporate interactions among abiotic factors. In this paper, we lay out hypotheses for how warming water temperatures may interact with temporal patterns of variation in temperature and low oxygen availability to impact the long-term thermal limits of aquatic ectotherms, and we test these hypotheses using an ecologically important but declining North American stonefly.

Exposure duration versus acclimation

For ectotherms, impacts of warming are predicted to depend on both the intensity and duration of exposure, with longer durations causing thermal limits to decrease (Santos et al. 2011) (Fig. 1A, Duration Hypothesis). Rezende et al. (2014) demonstrated this relationship across a wide set of taxa (insects, bivalves, and fishes) exposed to constant stressful temperatures for different durations. They found that a 100-fold increase in exposure duration causes thermal limits to decline, in some cases, by > 18 °C. Indeed, this relationship between duration and warming intensity in constant-temperature experiments has been shown repeatedly (e.g., Bigelow 1921; Smith 1957; Armstrong et al; 2009). However, predictions from the duration-intensity paradigm may underestimate long-term tolerance limits because prolonged exposure to high temperatures, especially if those temperatures are approached slowly, can also allow individuals to acclimate, leading to *higher* thermal limits and lower vulnerabilities (Angilletta 2009, Harrison et al. 2012).

Indeed, under the right circumstances, acclimation to recent warming can help individuals *increase* their thermal tolerance limits and is predicted to mitigate potential impacts of climate change (Stillman 2003; Birrell et al. 2020) (Fig. 1B, Acclimation Hypothesis). In a large meta-analysis, for example, Gunderson & Stillman (2015) show that diverse lineages of ectotherms, including fish, crustaceans, and amphibians, can shift their upper thermal limits by 1-3 °C with a 10 °C increase in acclimation temperature. Many other ectotherms, including insects, also have strong acclimation abilities (e.g., Shah et al, 2017b; Birrell et al. 2023). Acclimation (or acclimatization in nature) typically occurs over periods of days to weeks and can stem from changes in physiology and morphology (Angilletta 2009). For example, prolonged exposure to warm temperatures can trigger the production of heat shock proteins, which can prevent or reverse damage from denatured proteins (Tomanek & Somero 2000; Dong et al. 2008). In addition, warming can lead to gill hypertrophy in fish and aquatic insects, allowing individuals to take up more oxygen as metabolic demand for oxygen increases (Lefevre 2017; Malison 2022) Ectotherms can also survive higher temperatures via plasticity in behavior– e.g., by seeking out cooler microhabitats (Woods et al. 2021; Birrell et al. 2023) or engaging more frequently in motions that ventilate respiratory surfaces (Verberk & Bilton 2013; Frakes et al. 2021). Incorporating the effects of plasticity into vulnerability predictions is thus of great importance, particularly for aquatic ectotherms, which have stronger acclimation abilities than do terrestrial or marine taxa (Gunderson & Stillman 2015).

What determines the relative importance of duration and acclimation in setting long-term upper thermal limits? The answer will depend in part on whether individuals are presented with conditions that facilitate acclimation. In the laboratory, acclimation is most often induced by either short-term exposure to extreme-high temperatures (i.e., heat shock) or prolonged exposure to warm but non-extreme temperatures (Angilletta 2009). However, if temperatures are held constant and high for prolonged periods (but below acute tolerance limits), as commonly occurs in tolerance experiments (Sinclair et al. 2016), individuals may be unable to devote sufficient resources to acclimation and, instead, experience negative sublethal effects, such as reduced feeding and fertility, or eventually, death (e.g., Nebeker 1971; Dallas & Ross-Gillespie 2015; Walsh et al. 2021). This could explain why plasticity can appear to play a limited role in shaping thermal limits during constant-temperature experiments (e.g., Rezende et al. 2014). In contrast, the combination of relatively slow ramping rates and diel thermal variation, which are common in natural environments, may facilitate plasticity by providing individuals opportunities to experience successively higher temperatures but with chances to recover during relatively cool nights.

Temperature-oxygen interactions

Accurately forecasting the effects of climate change will depend also on understanding how high temperatures interact with other abiotic factors, like oxygen availability (Birrell et al. 2020). Temperature-oxygen interactions can play especially strong roles in the lives of aquatic ectotherms because, in water, rates of oxygen flux are low and costs of ventilating respiratory surfaces are high (compared to air) (Verberk & Bilton 2013; Verberk et al. 2016a). This is because water imposes high diffusion coefficients, holds relatively little oxygen (20-30 times less than air), and generates thick boundary layers around respiratory surfaces due to its high dynamic viscosity (Denny 1993; Verberk & Atkinson 2013; Woods and Moran 2020). For ectotherms, high water temperatures can exacerbate this oxygen problem by causing metabolic demand for oxygen to increase exponentially, eventually causing levels of oxygen demand to surpass supply (Woods 1999; Verberk et al. 2011; Jacobsen et al. 2003). Temperature-induced oxygen limitation can thus lead to decrements in aerobic scope and performance and may underlie the upper thermal limits and range contractions of aquatic ectotherms across wide taxa (Pörtner & Knust 2007; Verberk et al. 2016a,b; Deutsch et al. 2015; Harrison et al. 2018; Rubalcaba et al. 2020) (Fig. 1A).

However, the role of oxygen in setting upper thermal limits remains controversial (Verberk et al. 2016a, Jutfelt et al. 2019). Substantial evidence shows that oxygen-limitation may be important primarily during exposure to acute, extreme temperatures (Brijs et al. 2015; Ern et al. 2016; Kim et al. 2017) (Fig. 1B). Indeed, individuals can overcome short-term oxygen limitation by altering behaviors (e.g., increasing ventilation rates), morphologies (e.g., increasing the size of gills), and underlying physiological systems (e.g., reducing tissue oxygen levels to create higher inward fluxes of oxygen) (Verberk & Bilton 2013; Malison et al. 2022; Birrell et al. unpublished data), but plastic adjustments to long-term temperature-oxygen problems have been rarely shown. Additional experiments are thus needed to understand how strongly interactions among temperature and oxygen drive patterns of performance and survival over prolonged periods, especially under variable thermal regimes, which may support plasticity.

Here, we test the relative importance of duration versus plasticity in two levels of oxygen availability for the long-term performance and survival of aquatic ectotherms. We do this by performing a long-term thermal tolerance experiment on nymphs of a common but declining species of North American stonefly, the giant salmonfly, *Pteronarcys californica,* under ecologically relevant ramping rates and levels of diel thermal variation. We exposed nymphs to three different temperature profiles separated by 3 °C, which were slowly ramped over an 87-day period at rates similar to those that occur in nature. In addition, each ramp included ecologically relevant levels of diel thermal variation (± 2.5 °C) and was replicated in normoxia (100% of air sat.) and hypoxia (70% of air sat.). Throughout the experimental duration, we measured nymph survival and multiple aspects of performance (feeding, growth, and molting success) and plasticity (CT_{MAX} and gill size). Finally, to further examine the roles of slow ramping and diel variation, we carried out a more standard temperature-duration tolerance experiment by measuring survival rates and times of *P. californica* nymphs held at different, constant temperatures.

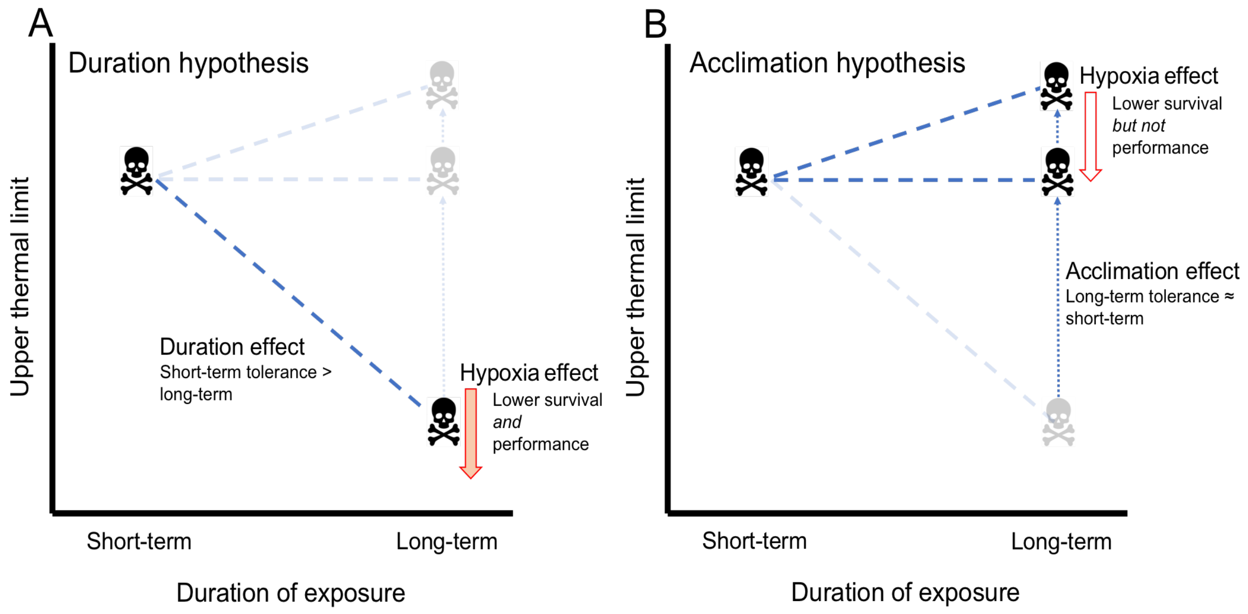

Fig. 1: Schematics of predictions of the Duration Hypothesis (A) and Acclimation Hypothesis (B) and how hypoxia is expected to impact survival and performance under both scenarios. The Duration Hypothesis predicts lower upper thermal limits with increased duration of exposure because of prolonged, cumulative heat stress. The Acclimation Hypothesis predicts that long-term upper thermal limits will be similar to, or even exceed, short-term thermal limits due to beneficial plasticity. Hypoxia is expected to lower both survival and performance under the Duration Hypothesis due to reduced aerobic scope for feeding and growth and increased long-term stress from lower ratios of oxygen supply to demand. Hypoxia is expected to lower survival but not performance under the Acclimation Hypothesis due to plastic adjustments in oxygen

delivery mechanisms, which minimize oxygen supply to demand mismatches until extreme, high temperatures.

Methods

Study species

P. californica is one of the largest stoneflies in the world and is native to Western North America. They inhabit medium to large mountain streams with cold, fast-flowing water and large, unconsolidated cobbles. As nymphs, *P. californica*, help cycle in-stream nutrients by shredding leaves (Merritt et al. 2008). They also make up a substantial component of the diets of economically important fish, like trout, especially during their large, synchronous adult emergences (Nehring et al. 2011). For this reason, *P. californica* emergences are followed closely by anglers. Adult emergences also transfer large pulses of carbon to terrestrial ecosystems, supporting riparian insectivores (Walters et al. 2018).

Despite their importance, *P. californica* are undergoing population declines and local extirpations across western North America, yet little is known about why (Birrell et al. 2019; Stagliano 2010; Kowalski & Richer 2020; Anderson et al. 2019). In some cases, however, population densities and declines are correlated with water temperature, with fewer salmonflies and larger declines occurring in warmer stream reaches, especially where mean monthly summer temperatures > 19 °C (Huff et al. 2006; Anderson et al. 2020). Such temperatures are well below published upper thermal limits derived from short-term thermal tolerance experiments (i.e., 29 – 37°C, depending on acclimation temperature) (Frakes et al. 2021; Malison et al. 2022). By providing some of the first long-term thermal tolerance data of *P. californica* nymphs, our study also seeks to provide additional insights into the mechanisms driving their distributions and declines and to help to improve vulnerability assessments.

Long-term ramping experiment

From July-September, 2021 we measured the survival and performance of salmonfly nymphs exposed to warm temperatures with ecologically-relevant ramping rates and levels of diel thermal variation. Medium-sized *P. californica* nymphs (mean: 0.43 g, range: 0.21-0.83 g) were collected in late June, 2021 using a kick screen (91 x 91 cm with 1 x 1 mm mesh) from lower Rock Creek in Montana, USA (46.6980, −113.6695). Nymphs were transferred to the

University of Montana, where they were weighed and sorted in the laboratory into three temperature treatments (cool, warm, hot) crossed by two oxygen (normoxic and hypoxic) treatments, with 12 nymphs of similar size distributions per temperature-oxygen treatment.

Before the start of the long-term thermal ramps, nymphs were acclimated to their respective starting temperatures and oxygen regimes. For the first three days of acclimation, temperatures were held at the mean water temperature of the week nymphs were collected from Rock Creek (14.75 °C ± 1.25 °C diel thermal variation) (USGS 2023). During days four to seven, this thermal regime was maintained for nymphs in the cool treatment while those in the warm and hot treatments experienced consecutively higher mean temperatures every two days – 0.75 and 1.50 °C (± 1.25 °C diel variation), respectively. Thus, at the start of the ramping period, nymphs were acclimated to starting mean temperatures of their respective treatments (14.75, 16.25, 17.75 °C ± 1.25 °C) for the cool, warm, and hot treatments, respectively. Throughout the entire acclimation period, oxygen levels were held ~ 100% air sat. (~ 19.5 kPa O_2) in the normoxic treatment and ~ 70% of air sat. in the hypoxic treatment.

Experimental thermal regimes exposed nymphs to different magnitudes of warming separated by 3 °C for each treatment (but only 1.5 °C for the first two weeks), but with ramping rates and levels of diel thermal variation similar to those occurring in local streams. Throughout the 87-day ramping period, mean temperatures were increased every two weeks. Temperatures were not ramped at finer intervals because reprograming the incubators more regularly was impractical. Biweekly rates of warming and diel levels of thermal variation were based on USGS temperature data from lower Rock Creek from 2018-2020, which showed that water temperatures typically warmed by ~ 1.5 °C every two weeks from mid-June to mid-August, with 2-4 °C of thermal variation per day, before steadily cooling each week starting in late August (USGS 2023). In our experiment, however, we continued to increase temperatures every two weeks – a kind of 'endless summer' designed to explore the limits of long-term acclimation. In addition, diel thermal variation was set to 2.5°C per day as varying the temperatures 3-4 °C was not possible under our experimental setup.

Throughout both the acclimation and long-term ramping periods, nymphs from each treatment were held in separate covered mesocosms (17 L plastic storage containers) filled with 10 L of dechlorinated tap water placed within laboratory incubators (Percival Scientific I-66LLC8), with both normoxic and hypoxic mesocosms of the same temperature treatment in the same incubator. Individual nymphs were held within custom-sewn mesh bags (1 x 1 mm mesh) sealed with labelled zip ties. Mesh bags were relatively rigid, allowing nymphs to move about the internal spaces. Thermal regimes were controlled by altering the air temperature regimes of the incubators. In the normoxic treatments, oxygen levels were held near 100% of air saturation by bubbling room air (barometric pressure 93 kPa) through air pumps (Danner, AP-8) and aquarium air stones. In the hypoxic treatments, oxygen levels were held near 70% of air saturation by bubbling compressed nitrogen through air stones distributed by a series of manifolds from a gas tank. Two submersible pumps (Rio, 600-200 GPH), which were adhered to the inside of each mesocosm, provided turbulent flow to support nymph gas exchange. To better simulate natural conditions, light:dark cycles were changed biweekly to match current light regimes in our region. Throughout the experiment, nymphs were fed cottonwood leaves *ad libitum*, which were first conditioned in stream water for two weeks. We also added leaves to one unoccupied bag in each container as a feeding control. Levels of ammonia within each mesocosm were checked weekly (API, Ammonia test kit) and were always low (< 0.25 mg/l). In addition, water in each mesocosm was replaced every two weeks with clean, dechlorinated tap water. In early weeks of the experiment, corners of some mesh bags were cut open after being sucked into the pumps within the mesocosms. This occasionally allowed some nymphs to escape their mesh bags. When found, and if alive (some were swept up in the pumps and killed), nymphs were immediately returned to a new bag. Each time an escapee was found, its identity was recorded so we could account for this during analysis (see Statistical analysis). After realizing the problem, we tied mesh around each pump, preventing the problem from recurring.

Temperature and oxygen levels were measured in the mesocosms by two methods. First, temperature and oxygen point measurements were made once a day with a thermocouple probe (Barnant, 600-1020) and an oxygen optode (Pyroscience, OXROB3) connected to a meter (Pyroscience, FireSting-O2). These measurements were usually taken during daytime hours (8 am – 6 pm). To supplement these measurements and to better capture the full variation of temperature and oxygen, continuous measurements were also made every 10 minutes with two temperature-oxygen data loggers (PME, miniDot). Data loggers were kept only within the hypoxic mesocosms – one in the hot treatment and the other in either the cool or warm treatment. See Figure S1 for temperature and oxygen plots for each treatment.

We measured nymph survival and three aspects of performance – feeding, growth, and molting – at two-week intervals throughout the ramp (i.e., at the beginning and end of each new, ramped thermal regime). Survival was measured by inspecting whether each nymph moved when gently prodded. Nymphs that did not respond to repeated stimulation were considered dead. Mesh bags (without their dead occupant) were returned to their respective mesocosms to prevent changing flow dynamics within containers. Feeding was measured by taking the difference between the mass of new conditioned leaves (4-5 leaves; 3-5 g), which were placed inside the bags of nymphs at the onset of each 14-day thermal regime, and the mass of old (eaten) leaves at the end of each thermal regime. The same was done for leaves in unoccupied, control bags. Similarly, growth was measured by taking the difference between nymph masses at the beginning and end of each 14-day period. Leaves and nymphs were gently blotted dry with paper towel then weighed on a microbalance (Mettler Toledo, ME54TE) and . Dead nymphs were not weighed, as they rapidly took on water by osmosis after death. Molting was measured by inspecting bags for exuviae. Because exuviae can decompose rapidly in water, we also considered nymphs to have molted if they underwent a drastic lightening in color within the 14-day period, a common response to molting for aquatic insects. During the final two weeks of the experiment, we checked nymphs more frequently – every 3 or 4 days – because we expected mortality to be high in the warmest treatment. The experiment was terminated when all nymphs in the hot treatment died, at 83 days. We were unable to extend the experiment further (until all nymphs had died in all treatments) due to other logistical constraints.

After the ramping experiment ended, nymphs in the cool and warm treatments remained in the mesocosms for an additional seven days at the same thermal regime they experienced for the final two weeks of the ramp. During this period, nymphs had continual access to conditioned leaves, yet three additional nymphs died from the warm treatment. These mortalities were not counted in our dataset as they occurred after the termination of the ramping experiment. We then evaluated the acute upper thermal limits of nymphs by measuring their critical thermal maxima (CT_{MAX}). CT_{MAX} was measured as the highest temperature experienced during thermal ramps before nymphs lost the ability to right themselves after being flipped on their backs (Lutterschmidt and Hutchison, 1997; Shah et al., 2017a). CT_{MAX} assays were performed at normoxia (100% of air sat.) within an insulated container (20 L). Each nymph was held in a separate reusable coffee filter submerged in ~ 5 cm of dechlorinated tap water. Oxygen levels were maintained at normoxia, as above. A submersible water pump was also added to stir the water and provide turbulent flow. The starting water temperature of each ramp mimicked the approximate mean minimum daily temperatures of the final two weeks of the treatments (and additional seven-day acclimation period) – 25 and 28 °C for the cool and warm treatments, respectively. Although we acknowledge that different starting temperatures can bias measurements of CT_{MAX} (Terblanche et al., 2007; Rezende et al., 2014), this likely had minimal influence on our data: CT_{MAX} of warm treatment nymphs were *lower* than expected – nearly the same as that of the cool treatment, possibly due to poor nymph condition from prolonged exposure to extreme, warm temperatures (see Results). In addition, although CT_{MAX} is usually measured after starving individuals for several days, we chose to feed them continually to avoid excess mortality under the extreme acclimation conditions. During CT_{MAX} ramps, water was heated with a programmable temperature controller (PolyScienctific, 7312) at 0.3 °C/min, and water temperatures and ramping rates were monitored using a timer and thermocouple probe (Barant, 600-1020). After nymphs lost righting response, they were transferred to well-oxygenated, cool water for recovery. All individuals recovered.

Following CT_{MAX} assays, three individuals from each temperature-oxygen treatment were randomly selected for gill imaging and were each placed in vials with 10 ml of of fixative (2% paraformaldehyde in cacodylate, pH 7.2); the remaining nymphs were stored in 70% ethanol. The following June, five new, non-acclimated nymphs were collected from lower Rock Creek from the same site, using the same methods, as above, and placed in 10 ml of fixative. Salmonfly nymphs have ~ 20 ventral gills tufts along thoracic and abdominal segments, each with dozens to hundreds of gill filaments. From each individual we counted the gill filaments of the four largest tufts on the left side of the body (thoracic segments 1, 2, and 3 and abdominal segment 1) and measured the lengths and widths of all visible filaments of the largest tuft (2nd thoracic, left side). All measurements were made blind (measurements taken without knowledge of acclimation treatment). Filament number was counted under a dissection microscope (Nikon, SMZ1500) by combing through target tufts with a small pick to separate clumps of filaments. Filament lengths and widths were measured by mounting a tuft on a slide and imaging its filaments under at 20X (Plan Apo 0.8NA objective) with a confocal aperture of 1AU on a Zeiss 880 laser scanning confocal microscope (Carl Zeiss Microscopy, LLC., White Plaines, NY). Multiple images were taken of each tuft but not of all filaments, as some were tangled together and others were facing the bottom surface of the slide. Images were then analyzed with ImageJ (tool: segment line) to obtain the lengths (base to gill terminus) and widths (at filament mid-point) (Abramoff et al. 2009). Total filament volumes were then estimated as π × (mean width/2)^2 × mean height × number of gill filaments.

Constant-temperature experiment

In April and May, 2022, we measured the mortality and survival time of salmonfly nymphs exposed to constant-temperatures thermal regimes after being acclimated to cool (8 °C) and warm (18 °C) conditions. Medium-sized *P. californica* nymphs were collected and sorted at the same locations and using the methods described above but with 60 nymphs per 8 °C and 18 °C acclimation treatment. Nymphs from each treatment were placed in separate 20 L buckets filled with 12 L of dechlorinated tap water. Water temperature was controlled by circulating water from each bucket into separate cooling water baths (Active Aqua, AACH10HP) and back into each bucket via submersible pumps (Rio, 2500-785 GPH). Nymphs were fed conditioned cottonwood leaves, which were scattered freely in each bucket, and mesh bags were tied around the pumps to prevent damage to nymphs. Oxygen levels were kept at normoxia with air pumps and air stones. Nymphs were left to acclimate for seven days at 14L:12D hour cycles (our approximate day:night cycle in April). Afterwards, nymphs were sorted into five constant-temperature exposure treatments (27, 30, 32, 33, 34.5 °C), with 12 nymphs of similar size distributions per treatment. Nymphs were observed for a maximum of two weeks, after which the experiment was terminated.

For 27 - 33 °C temperature treatments, nymphs from each treatment were housed and abiotic conditions were monitored in the same way as in the long-term ramping experiment (see Figure S2 for temperature plots). However, although we intended to expose nymphs to 14L:12D hour cycles, the lights failed to shut off for the first two nights. After noticing this, we exposed the nymphs to constant ambient light to keep conditions consistent across all 14 days. Throughout the experiment, mortality was measured at different intervals between 8 am and 8 pm, as checking throughout the night was not possible. Over the first several days, mortality was checked every 20-30 minutes. Once mortality rates slowed, mortality was checked hourly and then every several hours until the end of the experiment. For the 34.5 °C temperature treatment, nymphs for each treatment were housed as in the CT_{MAX} assays, above. Oxygen levels were checked at the start and end of the experiment (and were always near air saturation). Water temperatures were checked using a thermocouple thermometer. Mortality and survival duration were measured continuously. At all exposure temperatures, when dead nymphs were found, they were removed from their bags, following the same methods as above, and weighed after being dried in a drying oven (Thermo Scientific, Hermatherm OMH750) for several weeks.

Statistical analysis

Before analysis, we discarded extreme outliers from the long-term ramp dataset. This was done *post hoc* by combining the measurements for each variable and removing values that were < Q1–3×IQR or > Q3+3×IQR, where Q1 is the lower quartile, Q3 is the upper quartile, and IQR is the interquartile range. In addition, we removed data on nymphs that had escaped for each two-week period. To explore collinearity among predictors, we calculated Spearman correlation coefficients among covariates (mean temperature, duration of exposure, oxygen saturation, and nymph body mass) using the R function, cor (R Core Team 2023). Although correlations were generally weak (< 0.15), they were high between mean temperature and duration (0.84), which was expected because temperatures were raised systematically through time. To avoid biasing the results in subsequent analyses, therefore, the effects of mean temperature and duration were modelled additively. In addition, to ensure models fit the data well, residuals were calculated and visually analyzed for each test using the R function, residuals (R Core Team 2023).

To test the effect of mean temperature, duration of exposure, oxygen saturation, and body mass on survival and performance response variables, we primarily used generalized linear mixed effects models (i.e., biweekly survival and molting) and linear mixed effects models (i.e., biweekly feeding and growth) implemented in the R packages lme4 (function: glmm) and nlme (function: lme), respectively, with nymph identity as a random effect to account for repeated measures made on each response (Bates 2023; Pinheiro 2023). In initial analyses, we explored whether each predictor had a significant effect on responses, both as a main effect and in an interaction with other predictors. Because body mass had no significant effect on survival, growth and molting, it was dropped from these analyses. In addition, no significant effects were found on molting and all predictors were dropped. Oxygen also no effect on response variables and was dropped from analyses of the full dataset. However, because nymphs in the hot treatment appeared to die more rapidly under hypoxia than normoxia in the final two weeks of the ramp, we ran an additional test for the effect of oxygen for a subset of the data that only included nymphs in the hot treatment for the final fourteen days. At this scale, we found a significant effect, and we included this model below (see Results).

To analyze the effect of recent, mean, acclimation temperature and oxygen saturation on CT_{MAX} after the long-term ramp concluded, we used linear regression models (package: Base R; function: lm) (R Core Team 2023). However, when analyzing the entire dataset – CT_{MAX} of nymphs from the cool and warm treatments – no significant effects were found. This is likely due to the relatively low CT_{MAX} values of warm treatment nymphs, which suffered from significant additional mortality in the additional 7-day acclimation period. We, thus, ran a supplementary analysis for the effect of oxygen on CT_{MAX} of nymphs from the cool treatment, only, which had experienced no mortality during recent acclimation. In addition, we used linear mixed effects models with individual as a random effect, as above, to test the effect of acclimation temperature and oxygen level – control, cool and warm, normoxia and hypoxia – on the number, lengths, widths, and volume of salmonfly gill filaments. No significant effects were found, so we merged individuals from cool and warm ramping treatments to improve statistical power. We then found a significant effect of treatment – control, ramped-normoxic, ramped-hypoxic – on gill filament length and included this model below (see Results). The effects of all other predictors were non-significant and dropped from the analysis.

Before analyzing data from the constant-temperature experiment, we removed extreme outliers, following the same methods, as above. To test the effect of exposure temperature on survival, we used generalized linear models (R package: Base R; function: glm) because they can be applied binomial data (R Core Team, 2023). The effect of acclimation temperature could not be formally analyzed, however, because only one nymph survived the cool acclimation treatment, which caused the model to fit poorly. We tested the effect of exposure and acclimation temperature on the survival duration of nymphs using censored regression (Tobit) models with the R package, censReg (function: censReg) (Henningsen 2010). Censored regression was used because the data was strongly right-skewed, which led to skewed model residuals with more traditional methods, like linear regression.

Estimating tolerance parameters

To quantify differences in thermal tolerance and the effect of duration on tolerance among individuals from tolerance experiments using different methodologies (i.e., ramping vs. constant-temperature), we calculated two parameters: the estimated upper critical limit (eCT$_{\mathrm{MAX}}$) and the constant of thermal sensitivity (z). eCT$_{\mathrm{MAX}}$ and z can are derived from the following linear model from Rezende et al. (2014), which describes how upper lethal temperatures decrease linearly with exposure duration:

$$Eq1\colon T_{KO} = \mathrm{eCT}_{\mathrm{MAX}} - z\log_{10}t.$$

with T_{KO} as the upper lethal limit and t as duration of exposure. The ability of organisms to cope with heat for any duration of time is thus shown to depend on eCT$_{\mathrm{MAX}}$, the estimated upper critical limit at $\log_{10} t = 0$ (i.e., 1 minute), and z, the constant of thermal sensitivity, which describes how rapidly thermal limits decline with exposure duration (i.e., the decline of T_{KO} per 10-fold increase in t).

We estimated eCT$_{\mathrm{MAX}}$ and z using data from our long-term ramp, CTmax, and constant-temperature experiments, following the methods of Rezende et al. (2014). For ramping experiments, eCT$_{\mathrm{MAX}}$ and z is derived as the y-intercept and slope, respectively, for the regression of $\log_{10} t$ on T_{KO}. These parameters can be also derived from regressions for the effect of T (constant experimental temperature) on log t (knockdown time) from constant temperature experiments from back-transformation, where eCT$_{\mathrm{MAX}}$ = -intercept/slope and z = -1/slope, according to the following, rearranged equation of Eq1:

$$Eq2\colon \log_{10}t = \frac{(\mathrm{eCT}_{\mathrm{MAX}} - T)}{z}$$

In particular, for the ramping data, we compiled data from the CT$_{\mathrm{MAX}}$ assays and long-term ramp and ran a linear model (package: base R, function: lm) for the effect of the $\log_{10}$ of exposure time on survival duration (in seconds). We calculated the eCT$_{\mathrm{MAX}}$ and as intercept and slope, respectively. For the constant temperature experiment, we ran a censored regression for the effect of exposure temperature on the $\log_{10}$ of survival duration (in seconds) and calculated eCT_{MAX} and z as -intercept/slope and 1/slope, respectively.

Results

Survival

In the variable-temperature, long-term ramping experiments, *Pteronarcys californica* nymphs tolerated repeated daily exposure to mean and maximum temperatures up to 31.7 and 33.3 °C, respectively, despite ~ 80 days of previous exposure to rising temperatures (Fig. 2A). Across all treatments, mean temperature had a significant effect on survival (Table 1; $P < 0.0001$). By the end of the experiment and 87 days of exposure, no nymphs remained alive in either (normoxic and hypoxic) hot treatments (final mean temperature = 31.7 °C), while 71.4% and 75.0% remained in the normoxic and hypoxic warm treatments (final mean temperature = 28.7 °C), and 100% and 91.7% remained in the normoxic and hypoxic cool treatments (final mean temperature = 25.7 °C). Duration of exposure also had a significant effect on survival ($P < 0.05$), reflecting an increase in mortality with exposure time at similar temperatures both among (Fig 2A) and within (Fig 2B) treatments. Oxygen saturation had no significant effect on survival in any ramp, except in the warmest treatment during the final two weeks of exposure. In that final period, nymphs in hypoxia died significantly more rapidly than did those in normoxia ($P < 0.05$) (Fig. 2B, Table 1).

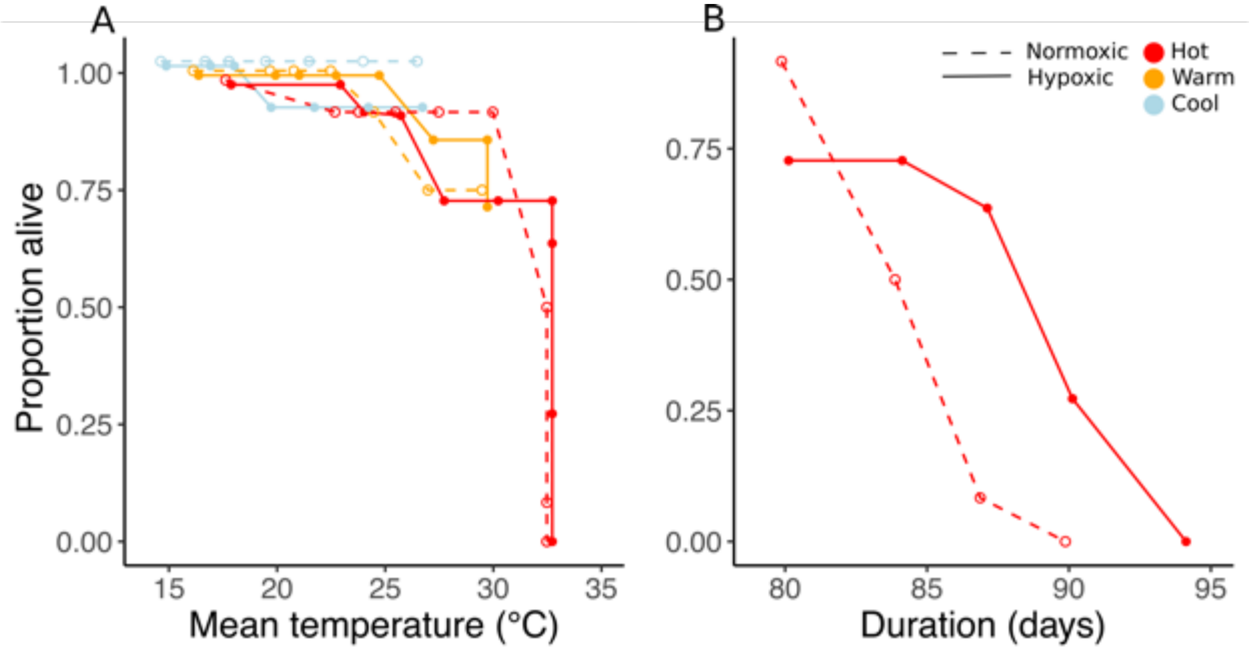

Fig. 2: Line plots of the effect of mean temperature (A) and duration of exposure (B) on the proportion of nymphs alive in long-term ramping experiments.

Table 1: Linear mixed effects (lme) models for the effect of mean temperature, duration and oxygen level on nymph survival.

Long-term ramp: Survival - entire dataset (lme)				
Fixed effect	Estimate	Std. Error	z-value	p-value
Mean temperature	0.58462	0.10716	5.456	**< 0.0001**
Duration	-0.04839	0.02048	-2.363	**0.0181**
Long-term ramp: Survival - final two weeks for hot treatment, only, at 31.7 °C (lme)				
Fixed effect	Estimate	Std. Error	z-value	p-value
Oxygen	-0.05052	0.02574	-1.962	**0.0497**

In the constant-temperature experiment, survival of *P. californica* nymphs depended strongly on both acclimation and exposure temperatures (Fig. 3). After two weeks of exposure, no nymphs survived 32, 33 or 34.5 °C (from either acclimation group). At 27 and 30 °C, however, higher proportions nymphs survived – 10% and 0% and 70% and 25% of nymphs in the cool and warm acclimation treatments, respectively. These results reflect a significant effect of exposure temperature of nymphs, with more nymphs surviving at cooler exposure temperatures (Table 2, $P < 0.0001$). In addition, acclimation and exposure temperatures had a significant effect on the duration of survival of nymphs (Fig 3B; Table 2), with individuals surviving longer in cool than hot temperatures and in warm versus cool acclimation treatments.

For the long-term ramp and constant temperature experiments, estimated $\mathrm{CT}_{\mathrm{MAX}} = 37.77$ and 35.91 °C and $z = 1.18$ and 1.71 °C/$\log_{10}$s, respectively. Values reflect that nymphs in the long-term ramp had both higher upper lethal limits and were less sensitive to negative impacts of exposure duration than those from constant temperature experiments.

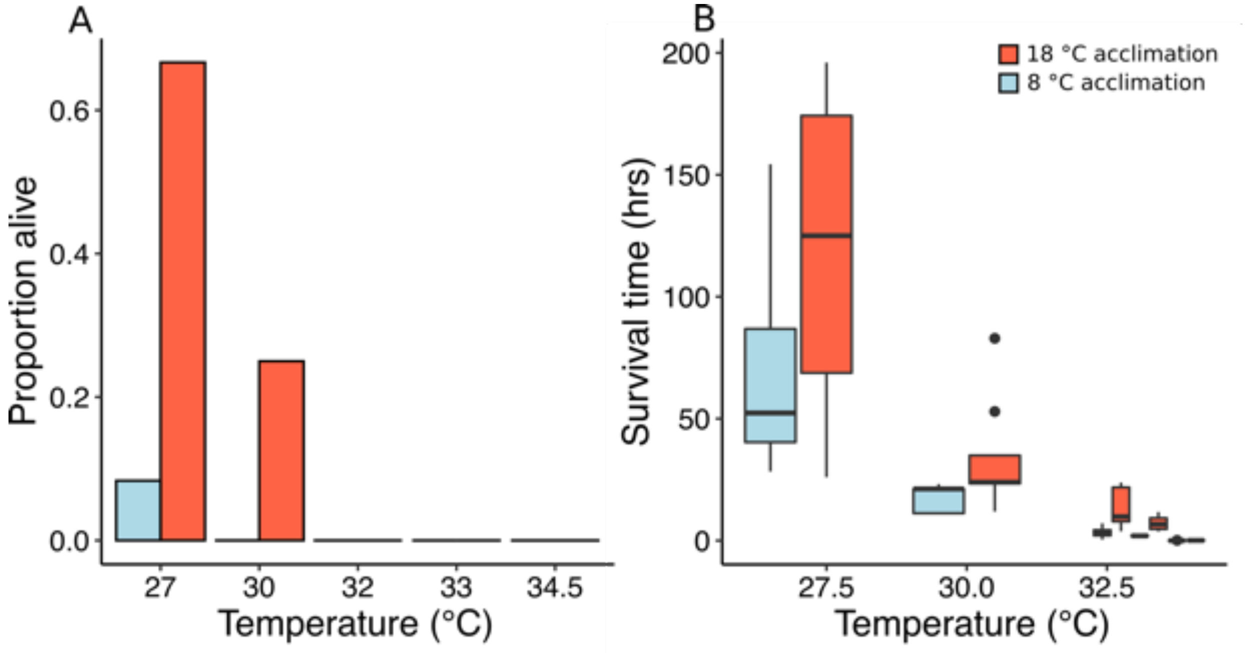

Fig. 3: Boxplots and bar plots of the effect of constant temperature proportion of nymphs alive (A) and nymph survival time (B), respectively, after one week acclimation to 8 or 18 °C.

Table 2: Generalized linear model (glm) and censored regression (cr) model for the effect of exposure and acclimation temperatures on the survival and survival time of nymphs in constant-temperature experiments, respectively.

Two-week constant-temperature: Survival (glm)				
Fixed effect	Estimate	Std. Error	z-value	p-value
Exposure temperature	0.7091	0.1952	3.634	**0.0002**
Two-week constant-temperature: Survival duration (cr)				
Fixed effect	Estimate	Std. Error	t-value	p-value
Exposure temperature	- 9.43304	1.75451	- 5.376	**< 0.0001**
Acclimation temperature	- 141.39954	62.35670	2.268	**0.0234**
Exposure : Acclimation temperature	-4.02724	2.21425	-1.819	0.0689

Performance and plasticity

In the long-term ramping experiment, levels of weekly leaf mass consumed were nearly always positive at all exposure temperatures, whereas weekly growth rates were nearly always negative, particularly when mean temperatures exceeded 18 °C (Fig. 4). However, weekly feeding and growth declined significantly with temperature across all treatments ($P < 0.0001$). In addition, duration had a significant effect on feeding ($P < 0.001$) and a near-significant effect on growth ($P = 0.068$), reflecting lower feeding and growth values of nymphs from cooler treatments at warm mean temperatures (Table 3). Oxygen had no significant effect on feeding or growth and nymph mass had a significant effect on feeding ($P < 0.0001$) but not growth (Table 3).

Nymphs molted across a wide range of conditions (14 – 27.5 °C in both normoxia and hypoxia), and were not significantly related to either temperature or oxygen levels (Fig. 5). Only one nymph died while molting, at 18 °C in normoxia.

CT_{MAX} of surviving nymphs from the cool and warm treatments were statistically indistinguishable when taking into account the entire dataset. However, CT_{MAX} of nymphs from the cool treatment, only, were significantly affected by oxygen level ($P < 0.05$) (Table 4; Fig. 6), with higher CT_{MAX} achieved by nymphs in hypoxic treatments. CT_{MAX} of cool treatment nymphs under hypoxia and normoxia were 36.5 and 35.2 °C respectively, while CT_{MAX} of warm treatment nymphs under hypoxia and normoxia were 35.5 and 35.4 °C, respectively.

Lengths of gill filaments were significantly affected by acclimation treatment ($P < 0.05$), with lengths being lowest in individuals from the control treatment (mean = 75.00 mm) but nominally higher in those from the ramped-normoxic treatment (mean = 83.00) and significantly higher in those from the ramped-hypoxic treatment (mean = 90.25 mm) (Table 5; Fig. 7-8).

Discussion

Climate change is forcing ectotherms to cope with more severe, prolonged periods of warming and associated shifts in other abiotic factors. Predictions of species responses to long-term, interactive challenges, however, remain unclear. Indeed, while prolonged exposure to high temperatures can greatly reduce the thermal limits of ectotherms (Rezende et al. 2014) (the Duration Hypothesis), under the right circumstances, exposure to heat can also *raise* thermal limits via acclimation (Angilletta 2009) (the Acclimation Hypothesis). Extrapolations from thermal tolerance experiments to vulnerability may therefore depend on whether individuals are exposed to conditions in the laboratory that facilitate acclimation, including very slow rates of ramping and diel thermal variation. Such patterns are common in nature yet are rarely incorporated into thermal tolerance experiments (Sinclair et al. 2016). Predicting impacts of climate change is complicated further by interactions between rising temperatures and changes in other factors, like oxygen. For aquatic ectotherms, rising temperatures can cause metabolic demand for oxygen to exceed rates of supply, which can depress performance and survival, especially for aquatic species exposed acutely to high-temperature hypoxia (Verberk et al. 2016a). Whether such interactions occur over prolonged periods, however, has rarely been shown. A key question is whether they are mitigated by acclimation to ecologically relevant thermal regimes.

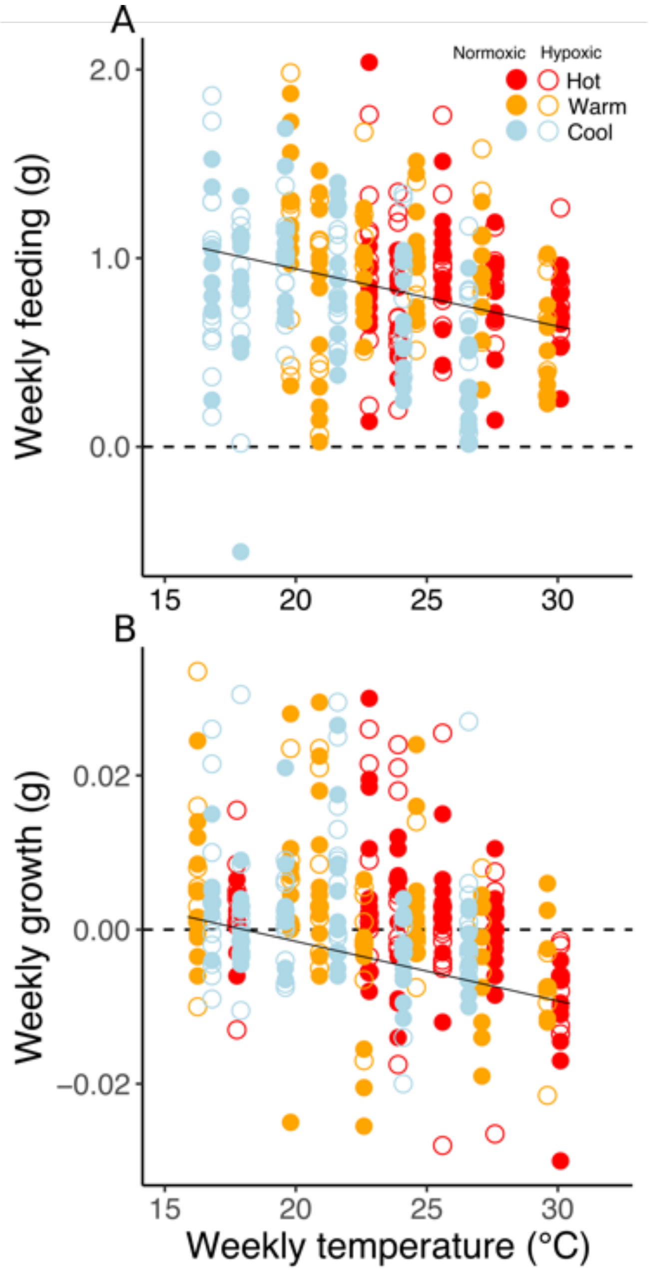

Fig. 4: Scatterplots of the effect of mean temperature on mass of leaves consumed per week (A) and growth of nymphs per week (B) in long-term ramping experiments.

Table 3: Linear mixed effects (lme) models for the effect of mean temperature and duration on feeding and growth

Long-term ramp: Feeding (lme)					
Fixed effect	Estimate	Std. Error	DF	t-value	p-value
Mean temperature	0.126094	0.0366889	256	3.436839	**0.0007**
Nymph mass	7.941881	1.6843459	256	4.715113	**< 0.0001**
Duration	-0.011803	0.003004	256	-3.929153	**0.0001**
Mean temperature : nymph mass	-0.253647	0.0704807	256	-3.598811	**0.0004**
Long-term ramp: Growth (lme)					
Fixed effect	Estimate	Std. Error	DF	t-value	p-value
Mean temperature	-0.0008945	0.00041937	301	-2.132853	**0.0337**
Duration	-0.0001216	6.6402E-05	301	-1.831284	0.0680

In this study, we examine how the performance and survival of aquatic nymphs of a large-bodied stonefly (*Pteronarcys californica*) depend on interactions between rising temperatures and altered oxygen availability under ecologically relevant thermal regimes. We exposed nymphs to normoxic or hypoxic temperature ramps over 83 days, with warmings rates that mimic natural rates in local streams, with ecologically relevant levels of diel thermal variation (± 2.5 °C). Overall, both temperature and exposure duration, but not oxygen, had significant effects on the long-term performance and survival of nymphs. Individuals acclimated strongly throughout the long-term ramp, however, allowing them to mitigate the effects of exposure duration and to cope better with chronically high, but variable, temperatures and low oxygen availability. Our results demonstrate that under ecologically relevant conditions, acclimation can largely offset the effects of exposure duration.

Survival

The data provide some support for the Duration Hypothesis, as both temperature and exposure duration had significant negative effects on nymph survival. Exposure duration led to higher mortality the longer nymphs were exposed and appeared to drive mortality in the hot treatment during the final two weeks of the experiment. In addition, average upper thermal limits of nymphs in the long-term ramp were 2.7 °C lower than those in the short-term CT_{MAX} assay.

Altogether, however, there is more support for the Acclimation Hypothesis. Long-term lethal temperatures were remarkably high: significant mortality under normoxia and hypoxia did not begin until mean and maximum temperatures exceeded 31 and 33 °C, respectively, for several days in a row. In addition, CT_{MAX} of cool and warm treatment nymphs measured after exposure to the ramps, were the highest ever recorded for *P. californica* and were ~ 4 °C higher than those of nymphs from past experiments acclimated to 12-16 °C, the approximate starting temperatures of the pre-ramp acclimation period (Frakes et al. 2021; Malison 2022; Frakes 2022). The physiological mechanisms underlying the observed acclimation remain unknown, although one possibility is changes in gill morphology. Gill lengths of nymphs from the ramped, hypoxic treatments were ~ 20% longer than those of control nymphs, which did not experience long-term warming and hypoxia. Gill hypertrophy may also explain the negligible effect of oxygen in the long-term ramps, until the final stages of the ramps when temperatures were acutely high (see Oxygen). Plastic increases in gill size in response to warming or hypoxia have been documented in other aquatic taxa (Malison et al. 2022, Lefevre et al. 2017) and is consistent with literature showing that aquatic ectotherms generally acclimate better than do terrestrial taxa (Gunderson & Stillman et al. 2015).

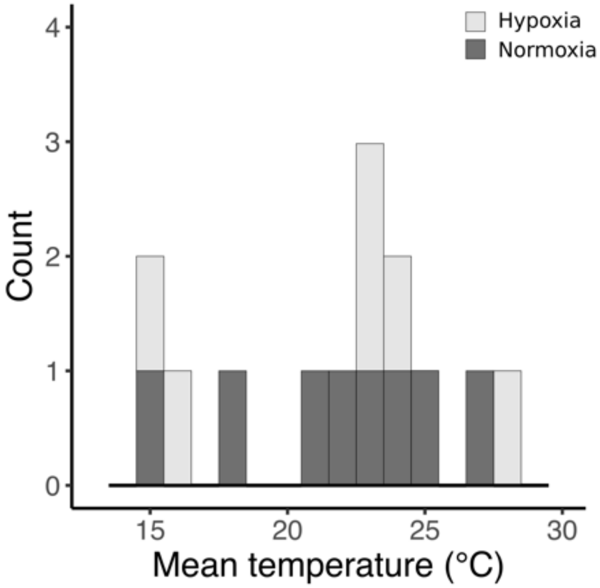

Fig. 5: Bar chart of the number of molts of nymphs across mean temperatures and between hypoxic and normoxic treatments.

Regardless of the underlying mechanisms, our results suggest that acclimation ability was facilitated by slow ramping rates and diel thermal variation. For example, eCT_{MAX} was ~ 1.9 °C higher in nymphs from the long-term ramp than those in the constant-temperature experiment, reflecting a rise in acute, thermal tolerance in the presence of natural patterns of thermal variation. In addition, the thermal sensitivity parameter, z, was ~ 0.5 °C/$\log_{10}$s lower in nymphs from the long-term ramp, demonstrating that upper lethal limits dropped ~ 1.1 °C less per 100-fold increase in exposure duration in the long-term ramp. Thus, while *P. californica* nymphs may be predisposed to handling long-term heat problems (i.e., a low z), both higher eCT_{MAX} and lower z of nymphs in the long-term ramp suggest that plastic shifts facilitated by thermal variation improved this tendency. Indeed, selection for low eCT_{MAX} and z is perhaps not surprising for stream insects, like *P. californica*, as water temperatures are generally cooler and more stable than air temperatures due to the heat capacity of water and low temperatures of inputs from higher elevations. However, Rezende et al. (2014) showed that across fish, insects, and bivalves, eCT_{MAX} and z are tightly coupled, with species having either a high eCT_{MAX} and z or a low eCT_{MAX} and z (Rezende et al. 2014). Although the eCT_{MAX} and z values measured here follow this pattern, falling within lower the range of from the literature (18 – 60 °C and 0.82-9.8 °C, respectively; Rezende et al. 2014), the simultaneously higher eCT_{MAX} and lower z of nymphs from the long-term ramp suggest that plasticity can circumvent tradeoffs between thermal tolerance and sensitivity to duration (Rezende et al. 2014).

Overall, our results support the Acclimation Hypothesis and show that acclimation can help offset the effect of exposure duration in aquatic ectotherms, under ecologically relevant thermal regimes. Studies assessing the vulnerability of ectotherms to climate change based on extrapolations from short-term and constant-temperature experiments should account for potential effects of beneficial acclimation. This is especially important when organisms have low z (Rezende et al. 2014). Our results add to the growing literature showing that thermal tolerance estimates depend strongly on methodology (Chown et al. 2009; Terblanche et all. 2007; Rezende et al. 2014), and we suggest that additional attention be paid to the role of acclimation *during* exposure to thermal challenges.

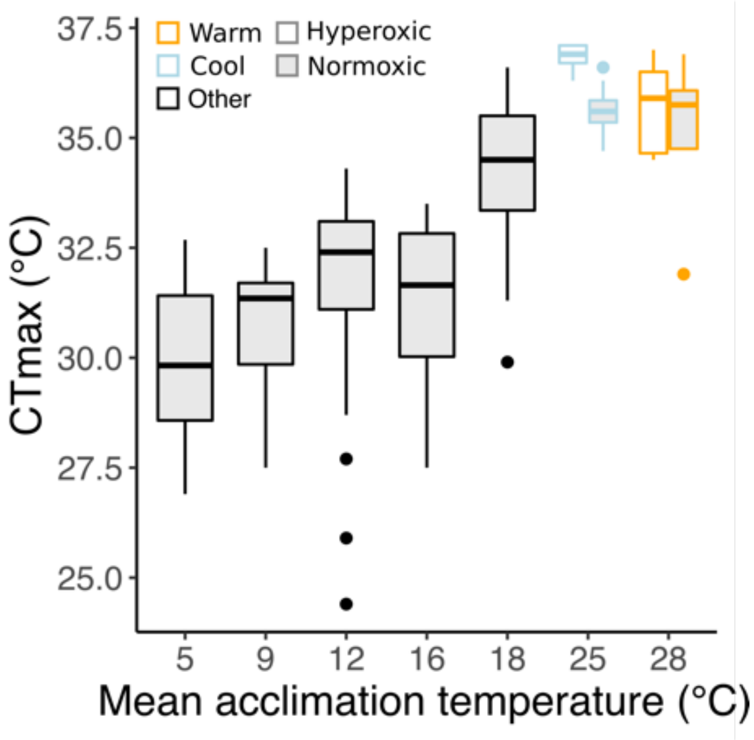

Fig. 6: Boxplots of the effect of mean acclimation temperature on CT_{MAX}. Other data are from Frakes et al. 2021, Malison et al. 2022, and Frakes et al. 2022.

Table 4: Linear model (lm) for the effect of acclimation mean temperature and oxygen level on CT_{MAX} of cool treatment nymphs.

Long-term ramp: CT_{MAX} (lm)				
Fixed effect	Estimate	Std Error	t-value	P-value
Oxygen	-0.040	0.006	-6.839	**< 0.0001**

Performance

In contrast to survival, performance was more sensitive to warming and exposure duration, with decrements in feeding and growth occurring earlier at cooler temperatures (e.g., 22 °C). Reductions in feeding at high, but ecologically relevant temperatures have been documented in other aquatic insect detritovores and may occur if nymphs allocate energy away from feeding and digestion and toward processes that support short-term survival (Nebeker et al. 1971). Reduced growth is likely a consequence of lower feeding rates and higher metabolic rates but may also occur from decreased quality of microbial communities on leaves or from difficulty digesting or assimilating nutrients at high temperatures (e.g., Cargill et al. 1985; Mas-Marti et al. 2015; Fenoy et al. 2020). Reduced feeding and growth may have severe long-term consequences. For example, aquatic insect nymphs in temperate regions typically grow rapidly in summer, when warmer temperatures support higher biological rates (e.g., Branham & Hathaway 1975). If temperatures are too warm, however, metabolic demand for resources may exceed income from feeding, which can stall or prevent development (Nebeker 1971). Severe warming may also depress subsequent production or viability of eggs and sperm (Dallas & Ross-Gillespie 2015; Walsh et al. 2021). Although long-term exposure to high temperatures may also reduce molting success (Nebeker et al. 1996; Shah et al. 2022), nymphs in our experiments had almost no trouble molting (only one died during a molt).

Climate change is currently warming streams at a rate of ~ 0.2 °C per decade and North American rivers are projected to see mean and high temperatures increase by 2-3 °C by the end of the century (Isaak et al. 2012; van Vliet et al. 2013). Given that nymphs readily acclimate, climate change is not projected to approach short- or long-term thermal limits of *P. californica* before 2100. Instead, sublethal effects of warming may be more important (Dallas et al. 2015; Carlo et al. 2018; Paratt et al. 2021), stressing the need to understand the consequences on later developmental stages (Kingsolver et al. 2011). Indeed, while *P. californica* only rarely occur where mean summer temperatures > 19 °C (Huff et al. 2006; Anderson et al. 2020), our experiments show that nymphs can tolerate temperature well over 19 °C for months, suggesting that direct mortality from heat exposure is not the primary mechanism driving the decline of this, and possibly other, species.

Oxygen

Our results suggest that acclimation may mitigate temperature-induced oxygen limitation during sublethal exposure. Indeed, although nymphs were subjected experimentally to conditions that both raise metabolic demand and lower rates of oxygen supply, hypoxia had no effect on survival and performance – except in the hot treatment, which experienced extreme, high temperatures during the final two weeks. The negligible effect of oxygen during sublethal heat exposure is remarkable given that *P. californica* prefer clean, fast-flowing, well-oxygenated water (Elder & Gaufin 1973). Indeed, for aquatic ectotherms, sublethal exposure to even moderate hypoxia can reduce aerobic scope (Rubalcaba et al. 2020), cause individuals to seek out microclimates with higher oxygen availability (Chapter 3; Birrell & Woods. 2023), and alter species distributions (Verberk et al. 2016b; Deutsch et al. 2015). High tolerance to chronic hypoxia likely reflects acclimation, as nymphs exposed to long-term hypoxia in the cool treatment of the long-term ramp had longer gill filaments than those exposed to normoxia. Longer gills are expected to increase rates of oxygen uptake via higher surface area for gas exchange. Similar changes to gill morphology have been previously documented (Malison et al. 2022) and likely mitigate mismatches between oxygen supply and demand at high temperatures. Strong behavioral, physiological, and morphological plasticity of aquatic ectotherms may thus explain why temperature-induced oxygen limitation does not always underlie thermal tolerance limits (e.g., Brijs et al. 2015; Ern et al. 2016).

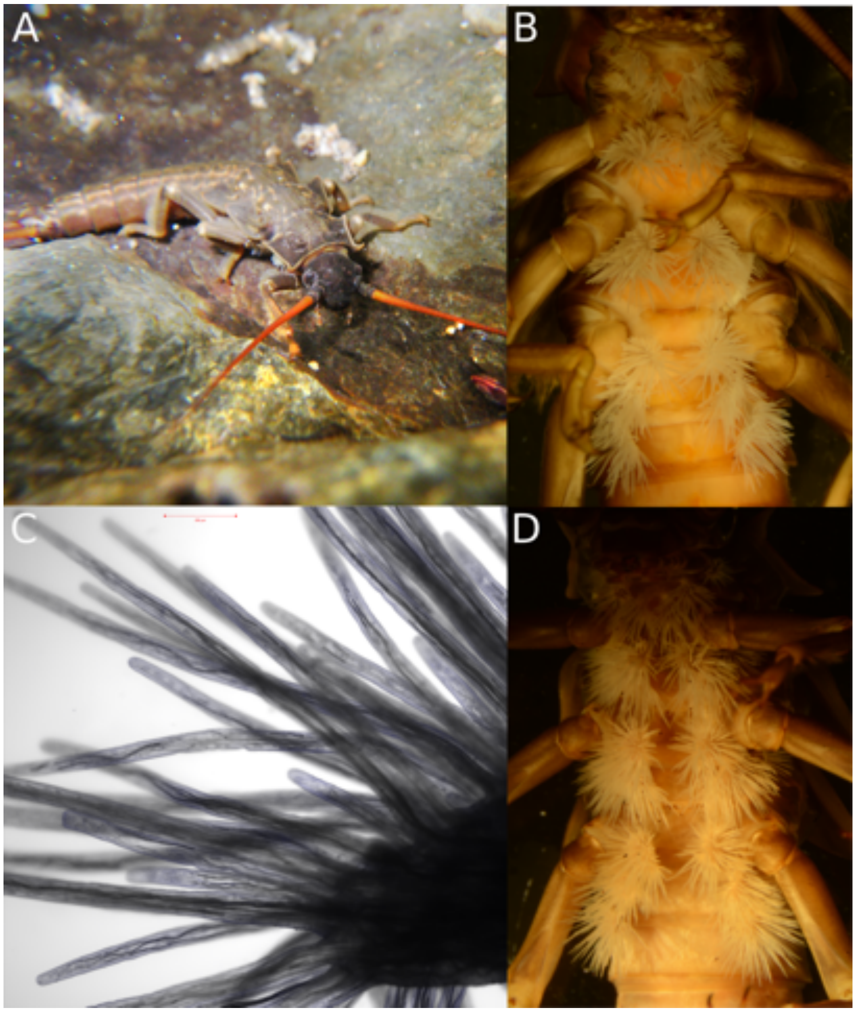

Fig. 7: Photo of *Pteronarcys californica* nymph (A), gill tufts of control nymph taken directly from river (B) fill filaments of nymph from warm-hypoxia treatment after long-term ramp, and (D) gill tufts of nymph from warm-hypoxia treatment after long-term ramp.

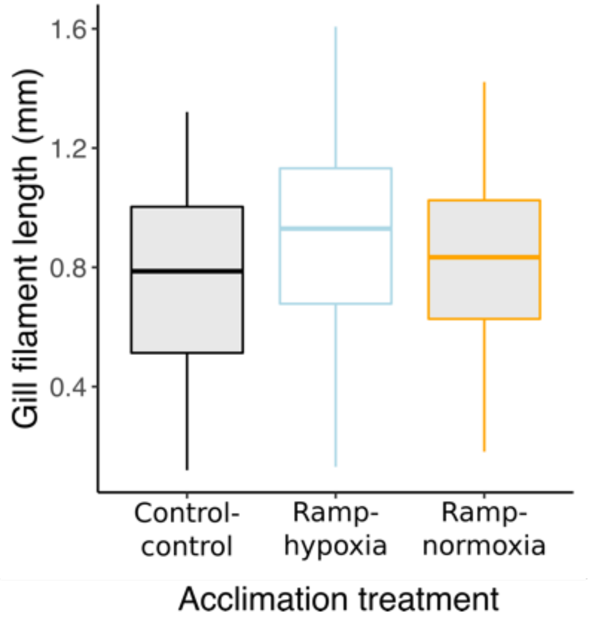

Fig. 8: Boxplots of the effect of acclimation treatment on gill filament length of control individuals and those from warm-hypoxic and warm-normoxic conditions.

Table 5: Linear mixed effects (lme) model for the effect of normoxia and hypoxia acclimation treatments compared to control treatments on gills lengths from nymphs from the cool and warm treatments.

Long-term ramp: Gill length (lme)					
Fixed effect	Value	Std. Error	DF	t-value	p-value
Normoxia	85.56	62.23	14.00	1.37	0.1908
Hypoxia	139.71	61.84	14.00	2.26	**0.0403**

Nevertheless, while moderate hypoxia had no effect on performance and survival during sublethal heat exposure, we observed a strong role of oxygen in setting acute, upper thermal limits. We propose that the negative effect of hypoxia on nymph survival during the final weeks of the hot treatment (when temperatures were acutely high) likely represents the tipping point where supply:demand mismatches are no longer preventable via plastic adjustments and an effect of oxygen thereby becomes detectable. Results from CT_{MAX} assays also point to an effect of oxygen on acute, upper thermal limits, as nymphs held in long-term hypoxia had higher CT_{MAX} than those held in normoxia. Similar results have been found by Malison et al. (2022), in which nymphs acclimated to hypoxia also had higher CT_{MAX}. Together, these results support the role of oxygen as an important driver of short-term, upper thermal limits in aquatic insect nymphs (Frakes et al. 2021).

Conclusion

We show that aquatic nymphs of *P. californica* exposed to ecologically realistic ramping rates and diel thermal variation can tolerate surprisingly high temperatures and moderate hypoxia for prolonged periods. Individuals underwent strong plastic adjustments to gill morphology, CT_{MAX}, and their sensitivity to exposure duration. Ultimately, our study shows that acclimation can mitigate negative effects of duration and temperature-induced oxygen limitation if individuals are given opportunities for plasticity. In addition, our study suggests that, in nature, high temperature may only rarely kill organisms outright. Sublethal effects of warming may be a more important mechanism of mortality under natural thermal regimes. Future research should incorporate the effects of plasticity into vulnerability assessments and should more thoroughly investigate the consequences of sublethal warming.

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References

Abramoff, M.D., Magalhaes, P.J., and Ram, S. J. (2004). Image processing with ImageJ. Biophotonics Int.. 11, 36–42,

Anderson, H.E., Albertson, L.K., and Walters, D.M. (2019). Water temperature drives variability in salmonfly abundance, emergence timing, and body size. River Res. Appl. *35*, 1013–1022. 10.1002/rra.3464.

Angilletta Jr., M.J. (2009). Thermal Adaptation: a theoretical and empirical synthesis (Oxford University Press).

Armstrong, J.W., Tang, J., and Wang, S. (2009). Thermal Death Kinetics of Mediterranean, Malaysian, Melon, and Oriental Fruit Fly (Diptera: Tephritidae) Eggs and Third Instars. J. Econ. Entomol. *102*, 522–532. 10.1603/029.102.0209.

Arthur Woods, H., and Moran, A.L. (2020). Reconsidering the oxygen-temperature hypothesis of polar gigantism: Successes, failures, and nuance. Integr. Comp. Biol. *60*, 1438–1453. 10.1093/icb/icaa088.

Bates, D. M. (2023). lme4: Mixed-effects modeling with R. 1.1-32. [https://cran.r-project.org/web/packages/lme4/lme4.pdf]

Bigelow, W.D. (1921). The Logarithmic Nature of Thermal Death Time Curves. J. Infect. Dis. *29*, 528–536.

Birrell, J.H., and Woods, H.A. (2023). Going with the flow – how a stream insect, Pteronarcys californica, exploits local flows to increase oxygen availability . J. Exp. Biol. 10.1242/jeb.244609.

Birrell, J.H., Frakes, J.I., Shah, A.A., and Woods, H.A. (2023). Mechanisms underlying thermal breadth differ by species in insects from adjacent but thermally distinct streams – A test of the climate variability hypothesis. J. Therm. Biol. *112*, 103435. 10.1016/j.jtherbio.2022.103435.

Birrell, J.H., Meek, J.B., and Nelson, C.R. (2019). Decline of the Giant Salmonfly Pteronarcys Californica Newport , 1848 (Plecoptera : Pteronarcyidae) in the Provo River, Utah, USA. Illiesia *15*, 83–97.

Birrell, J.H., Shah, A.A., Hotaling, S., Giersch, J.J., Williamson, C.E., Jacobsen, D., and Woods, H.A. (2020). Insects in high-elevation streams: Life in extreme environments imperiled by climate change. Glob. Chang. Biol. *26*, 6667–6684. 10.1111/gcb.15356.

Birrell, J.H., Verberk, W.C.E.P, Woods, H.A. (unpublished data). Remarkably low tissue oxygen levels in aquatic insects reflect a balance between toxicity and asphyxiation.

Branham, J.M., and Hathway, R.R. (1975). Sexual differences in the growth of Pteronarcys californica Newport andpteronarcella badia Hagen (Plecoptera). Can. J. Zool. *53*, 501–506.

Brijs, J., Jutfelt, F., Clark, T.D., Gräns, A., Ekström, A., and Sandblom, E. (2015). Experimental manipulations of tissue oxygen supply do not affect warming tolerance of European perch. J. Exp. Biol. *218*, 2448–2454. 10.1242/jeb.121889.

Cargill, A.S., Cummins, K.W., Hanson, B.J., and Lowry, R.R. (1985). The Role of Lipids, Fungi, and Temperature in the Nutrition of a Shredder Caddisfly, Clistoronia magnifica. Freshw. Invertebr. Biol. *4*, 64–78. 10.2307/1467178.

Carlo, M.A., Riddell, E.A., Levy, O., and Sears, M.W. (2018). Recurrent sublethal warming reduces embryonic survival, inhibits juvenile growth, and alters species distribution projections under climate change. Ecol. Lett. *21*, 104–116. 10.1111/ele.12877.

Cheng, L., Abraham, J., Hausfather, Z., and Trenberth, K.E. (2019). How fast are the oceans warming? Science (80-.). *363*, 128–129. 10.1126/science.aav7619.

Chown, S.L., Jumbam, K.R., Sørensen, J.G., and Terblanche, J.S. (2009). Phenotypic variance, plasticity and heritability estimates of critical thermal limits depend on methodological context. Funct. Ecol. *23*, 133–140. 10.1111/j.1365-2435.2008.01481.x.

Dallas, H., and Ross-Gillespie, V. (2015). Review: Sublethal effects of temperature on freshwater organisms, with special reference to aquatic insects. Water SA *41*, 712. 10.4314/wsa.v41i5.15.

Dallas, H., and Ross-Gillespie, V. (2015). Review: Sublethal effects of temperature on freshwater organisms, with special reference to aquatic insects. Water SA *41*, 712. 10.4314/wsa.v41i5.15.

Denny, M.W. (1993). Air and water: the biology and physics of life’s media (Princeton University Press).

Deutsch, C., Ferrel, A., Seibel, B., Portner, H.-O., and Huey, R.B. (2015). Climate change tightens a metabolic constraint on marine habitats. Science (80-.). *348*, 1132–1135. 10.1126/science.aaa1605.

Diffenbaugh, N.S., and Field, C.B. (2013). Changes in Ecologically Critical Terrestrial Climate Conditions. Science (80-.). 341, 486–492. 10.1126/science.1237123.

Dong, Y., Miller, L.P., Sanders, J.G., and Somero, G.N. (2008). Heat-Shock Protein 70 (Hsp70) Expression in Four Limpets of the Genus Lottia : Interspecific Variation in Constitutive and Inducible Synthesis Correlates With in situ Exposure to Heat Stress. Biol. Bull. *215*, 173–181. 10.2307/25470698.

Elder, J.A., and Gaufin, A.R. (1973). Notes on the occurrence and distribution of Pteronarcys Californica Newport (Plecoptera) within streams. Gt. Basin Nat. *33*, 218–220.

Ern, R., Norin, T., Gamperl, A.K., and Esbaugh, A.J. (2016). Oxygen dependence of upper thermal limits in fishes. J. Exp. Biol. *219*, 3376–3383. 10.1242/jeb.143495.

Fenoy, E., Moyano, F.J., and Jesús Casas, J. (2020). Warming and nutrient-depleted food: Two difficult challenges faced simultaneously by an aquatic shredder. Freshw. Sci. *39*, 393–404. 10.1086/709023.

Frakes, J.I., Birrell, J.H., Shah, A.A., and Woods, H.A. (2021). Flow increases tolerance of heat and hypoxia of an aquatic insect. Biol. Lett. *17*. 10.1098/rsbl.2021.0004.

Frakes, J.I., Malison, R.L., Sydor, M.J., and Arthur Woods, H. (2022). Exposure to copper increases hypoxia sensitivity and decreases upper thermal tolerance of giant salmonfly nymphs (Pteronarcys californica). J. Insect Physiol. *143*, 104455. 10.1016/j.jinsphys.2022.104455.

Gunderson, A.R., and Stillman, J.H. (2015). Plasticity in thermal tolerance has limited potential to buffer ectotherms from global warming. Proc. R. Soc. B Biol. Sci. *282*. 10.1098/rspb.2015.0401.

Harrison, J.F., Greenlee, K.J., and Verberk, W.C.E.P. (2018). Functional Hypoxia in Insects: Definition, Assessment, and Consequences for Physiology, Ecology, and Evolution. Annu. Rev. Entomol. *63*, 303–325. 10.1146/annurev-ento-020117-043145.

Harrison, J.F., Woods, H.A., and Roberts, S.P. (2012). Ecological and environmental physiology of insects. (Oxford University Press).

Henningsen, A. (2010). Estimating censored regression models in R using the censReg Package. R Pack. Vign., 5, 1-12.

Hotaling, S., Shah, A.A., McGowan, K.L., Tronstad, L.M., Giersch, J.J., Finn, D.S., Woods, H.A., Dillon, M.E., and Kelley, J.L. (2020). Mountain stoneflies may tolerate warming streams: Evidence from organismal physiology and gene expression. Glob. Chang. Biol. *26*, 5524–5538. 10.1111/gcb.15294.

Huff, D.D., Hubler, S.L., Pan, Y.D., and Drake, D.L. (2006). Detecting shifts in macroinvertebrate assemblage requirements: Implicating causes of impairment in streams. Oregon Department of Environmental Quality Watershed Assessment Report, Portland, OR, 38.

IPCC, 2022: Climate Change 2022: Impacts, Adaptation and Vulnerability. Contribution of Working Group II to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change [H.-O. Pörtner, D.C. Roberts, M. Tignor, E.S. Poloczanska, K. Mintenbeck, A. Alegría, et al., (eds.)]. (Cambridge University Press).

Isaak, D.J., Wollrab, S., Horan, D.L., and Chandler, G. (2012). Climate change effects on stream and river temperatures across the northwest U.S. from 1980–2009 and implications for salmonid fishes. Clim. Change *113*, 499–524. 10.1007/s10584-011-0326-z.

Jacobsen, D., Rostgaard, S., and Vásconez, J.J. (2003). Are macroinvertebrates in high altitude streams affected by oxygen deficiency? Freshw. Biol. *48*, 2025–2032. 10.1046/j.1365-2427.2003.01140.x.

Jørgensen, L.B., Malte, H., and Overgaard, J. (2019). How to assess Drosophila heat tolerance: Unifying static and dynamic tolerance assays to predict heat distribution limits. Funct. Ecol. *33*, 629–642. 10.1111/1365-2435.13279.

Jutfelt, F., Norin, T., Ern, R., Overgaard, J., Wang, T., McKenzie, D.J., Lefevre, S., Nilsson, G.E., Metcalfe, N.B., Hickey, A.J.R., et al. (2018). Oxygen- and capacity-limited thermal tolerance: Blurring ecology and physiology. J. Exp. Biol. *221*, 2016–2019. 10.1242/jeb.169615.

Kim, K.S., Chou, H., Funk, D.H., Jackson, J.K., Sweeney, B.W., and Buchwalter, D.B. (2017). Physiological responses to short-term thermal stress in mayfly (Neocloeon triangulifer) larvae in relation to upper thermal limits. J. Exp. Biol. *220*, 2598–2605. 10.1242/jeb.156919.

Kingsolver, J.G., Arthur Woods, H., Buckley, L.B., Potter, K.A., MacLean, H.J., and Higgins, J.K. (2011). Complex life cycles and the responses of insects to climate change. Integr. Comp. Biol. *51*, 719–732. 10.1093/icb/icr015.

Kingsolver, J.G., Diamond, S.E., and Buckley, L.B. (2013). Heat stress and the fitness consequences of climate change for terrestrial ectotherms. Funct. Ecol. *27*, 1415–1423. 10.1111/1365-2435.12145.

Kowalski, D.A., and Richer, E.E. (2020). Quantifying the habitat preferences of the stonefly Pteronarcys californica in Colorado. River Res. Appl. *36*, 2043–2050. 10.1002/rra.3733.

Lefevre, S., McKenzie, D.J., and Nilsson, G.E. (2017). Models projecting the fate of fish populations under climate change need to be based on valid physiological mechanisms. Glob. Chang. Biol. *23*, 3449–3459. 10.1111/gcb.13652.

Lindquist, S. (1992). Heat-shock proteins and stress tolerance in microorganisms. Curr. Opin. Genet. Dev. *2*, 748–755. 10.1016/S0959-437X(05)80135-2.

Lutterschmidt, W.I., and Hutchison, V.H. (1997). The critical thermal maximum: history and critique. Can. J. Zool. *75*, 1561–1574. 10.1139/z97-783.

Malison, R.L., Frakes, J.I., Andreas, A.L., Keller, P.R., Hamant, E., Shah, A.A., and Woods, H.A. (2022). Plasticity of salmonfly (Pteronarcys californica) respiratory phenotypes in response to changes in temperature and oxygen. J. Exp. Biol. *225*, 1–11. 10.1242/jeb.244253.

Mas-Martí, E., Romaní, A.M., and Muñoz, I. (2015). Consequences of warming and resource quality on the stoichiometry and nutrient cycling of a stream shredder. PLoS One *10*, 1–21. 10.1371/journal.pone.0118520.

Merritt, R.W., Cummins, K.W., and Berg, M.B. (2008). An introduction to the aquatic insects of North America. Kendall Hunt, 1996. (Kendall).

Nebeker, A. V (1971). Effect of Water Temperature on Nymphal Feeding Rate , Emergence , and Adult Longevity of the Stonefly Pteronarcys dorsata. J. Kansa Entomol. Soc. *44*, 21–26.

Nebeker, A. V (1971). Effect of Water Temperature on Nymphal Feeding Rate , Emergence , and Adult Longevity of the Stonefly Pteronarcys dorsata. J. Kansa Entomol. Soc. *44*, 21–26.

Nebeker, A. V., Onjukka, S.T., Stevens, D.G., and Chapman, G.A. (1996). Effect of low dissolved oxygen on aquatic life stages of the caddisfly Clistoronia magnifica (Limnephilidae). Arch. Environ. Contam. Toxicol. *31*, 453–458. 10.1007/BF00212427.

Nehring, R.B., Heinold, B., and Pomeranz, J. (2011). Colorado River Aquatic Resources Investigations Federal Aid Project F-237R-18. Color. Div. Wildl.

Pacifici, M., Foden, W.B., Visconti, P., Watson, J.E.M., Butchart, S.H.M., Kovacs, K.M. et al. (2015). Assessing species vulnerability to climate change. Nat. Clim. Change, 5, 215–224.

Parratt, S.R., Walsh, B.S., Metelmann, S., White, N., Manser, A., Bretman, A.J., Hoffmann, A.A., Snook, R.R., and Price, T.A.R. (2021). Temperatures that sterilize males better match global species distributions than lethal temperatures. Nat. Clim. Chang. *11*, 481–484. 10.1038/s41558-021-01047-0.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Heisterkamp, S., Van Willigen, B., & Maintainer, R. (2023). Package 'nlme'. *Linear and nonlinear mixed effects models, version*. 3.1-162. [https://cran.r-project.org/web/packages/nlme/nlme.pdf]

Pörtner, H.O., and Knust, R. (2007). Climate Change Affects Marine Fishes Through the Oxygen Limitation of Thermal Tolerance. Science (80-.). *315*, 95–97. 10.1126/science.1135471.

R Core Team. (2023). R: A language and environment for statistical com- puting. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://www.R-project.org

Rezende, E.L., Castañeda, L.E., and Santos, M. (2014). Tolerance landscapes in thermal ecology. Funct. Ecol. *28*, 799–809. 10.1111/1365-2435.12268.

Rohr, J.R., and Palmer, B.D. (2013). Climate change, multiple stressors, and the decline of ectotherms. Conserv. Biol. *27*, 741–751. 10.1111/cobi.12086.

Rubalcaba, J.G., Verberk, W.C.E.P., Jan Hendriks, A., Saris, B., and Arthur Woods, H. (2020). Oxygen limitation may affect the temperature and size dependence of metabolism in aquatic ectotherms. Proc. Natl. Acad. Sci. U. S. A. *117*, 31963–31968. 10.1073/pnas.2003292117.

Shah, A.A., Funk, W.. C., and Ghalambor, C.K. (2017b). Thermal Acclimation Ability Varies in Temperate and Tropical Aquatic Insects from Different Elevations. Integr. Comp. Biol. *57*, 977–987. 10.1093/icb/icx101.

Shah, A.A., Gill, B.A., Encalada, A.C., Flecker, A.S., Funk, W.C., Guayasamin, J.M., Kondratieff, B.C., Poff, N.L., Thomas, S.A., Zamudio, K.R., et al. (2017a). Climate variability predicts thermal limits of aquatic insects across elevation and latitude. Funct. Ecol. *31*, 2118–2127. 10.1111/1365-2435.12906.

Shah, A.A., Hotaling, S., Lapsansky, A.B., Malison, R.L., Birrell, J.H., Keeley, T., Giersch, J.J., Tronstad, L.M., and Woods, H.A. (2023). Warming undermines emergence success in a threatened alpine stonefly: a multi-trait perspective on vulnerability to climate change. 1–26. 10.1111/1365-2435.14284.

Sinclair, B.J., Marshall, K.E., Sewell, M.A., Levesque, D.L., Willett, C.S., Slotsbo, S., Dong, Y., Harley, C.D.G., Marshall, D.J., Helmuth, B.S., et al. (2016). Can we predict ectotherm responses to climate change using thermal performance curves and body temperatures? Ecol. Lett. *19*, 1372–1385. 10.1111/ele.12686.

Smith, J.M. (1957). Temperature Tolerance and Acclimatization in Drosophila Subobscura. J. Exp. Biol. *34*, 85–96. 10.1242/jeb.34.1.85.

Stagliano, D. (2013). Evaluation of salmonflies in Montana's rivers : are statewide populations really declining? Mont. Nat. Herit. Progr., 1–29. 10.5962/bhl.title.65536.

Steffensen, J.F. (1985). The transition between branchial pumping and ram ventilation in fishes: energetic consequences and dependence on water oxygen tension. *J. exp. Biol*, *114*, 141-150.

Stillman, J.H. (2003). Acclimation capacity underlies susceptibility to climate change. Science (80-.). *301*, 65. 10.1126/science.1083073.

Sunday, J., Bennett, J.M., Calosi, P., Clusella-Trullas, S., Gravel, S., Hargreaves, A.L., Leiva, F.P., Verberk, W.C.E.P., Olalla-Tárraga, M.Á., and Morales-Castilla, I. (2019). Thermal tolerance patterns across latitude and elevation. Philos. Trans. R. Soc. B Biol. Sci. *374*. 10.1098/rstb.2019.0036.

Terblanche, J.S., Deere, J.A., Clusella-Trullas, S., Janion, C., and Chown, S.L. (2007). Critical thermal limits depend on methodological context. Proc. R. Soc. B Biol. Sci. *274*, 2935–2943. 10.1098/rspb.2007.0985.

Todgham, A.E., and Stillman, J.H. (2013). Physiological Responses to Shifts in Multiple Environmental Stressors: Relevance in a Changing World. Integr. Comp. Biol. *53*, 539–544. 10.1093/icb/ict086.

Tomanek, L., and Somero, G.N. (2000). Time Course and Magnitude of Synthesis of Heat-Shock Proteins in Congeneric Marine Snails (Genus Tegula) from Different Tidal Heights. Physiol. Biochem. Zool. *73*, 249–256. 10.1086/316740.

U.S. Geological Survey, 2021, National Water Information System data available on the World Wide Web (USGS Water Data for the Nation), accessed [June 1, 2021], at URL [https://waterdata.usgs.gov/monitoring-location/12334510/]

Van Vliet, M.T.H., Franssen, W.H.P., Yearsley, J.R., Ludwig, F., Haddeland, I., Lettenmaier, D.P., and Kabat, P. (2013). Global river discharge and water temperature under climate change. Glob. Environ. Chang. *23*, 450–464. 10.1016/j.gloenvcha.2012.11.002.

Van Vliet, M.T.H., Franssen, W.H.P., Yearsley, J.R., Ludwig, F., Haddeland, I., Lettenmaier, D.P., and Kabat, P. (2013). Global river discharge and water temperature under climate change. Glob. Environ. Chang. *23*, 450–464. 10.1016/j.gloenvcha.2012.11.002.

Verberk, W.C.E.P., and Atkinson, D. (2013). Why polar gigantism and Palaeozoic gigantism are not equivalent: Effects of oxygen and temperature on the body size of ectotherms. Funct. Ecol. *27*, 1275–1285. 10.1111/1365-2435.12152.

Verberk, W.C.E.P., and Bilton, D.T. (2013). Respiratory control in aquatic insects dictates their vulnerability to global warming. Biol. Lett. *9*, 0–3. 10.1098/rsbl.2013.0473.

Verberk, W.C.E.P., Bilton, D.T., Calosi, P., and Spicer, J.I. (2011). Oxygen supply in aquatic ectotherms: Partial pressure and solubility together explain biodiversity and size patterns. Ecology *92*, 1565–1572. 10.1890/10-2369.1.

Verberk, W.C.E.P., Durance, I., Vaughan, I.P., and Ormerod, S.J. (2016). Field and laboratory studies reveal interacting effects of stream oxygenation and warming on aquatic ectotherms. Glob. Chang. Biol. *22*, 1769–1778. 10.1111/gcb.13240.

Verberk, W.C.E.P., Overgaard, J., Ern, R., Bayley, M., Wang, T., Boardman, L., and Terblanche, J.S. (2016). Does oxygen limit thermal tolerance in arthropods? A critical review of current evidence. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. *192*, 64–78. 10.1016/j.cbpa.2015.10.020.

Walsh, B.S., Parratt, S.R., Hoffmann, A.A., Atkinson, D., Snook, R.R., Bretman, A., and Price, T.A.R. (2019). The Impact of Climate Change on Fertility. Trends Ecol. Evol. *34*, 249–259. 10.1016/j.tree.2018.12.002.

Walters, D.M., Wesner, J.S., Zuellig, R.E., Kowalski, D.A., and Kondratieff, M.C. (2018). Holy flux: spatial and temporal variation in massive pulses of emerging insect biomass from western U.S. rivers. Ecology *99*, 238–240. 10.1002/ecy.2023.

Woods, H.A. (1999). Egg-mass size and cell size: Effects of temperature oxygen distribution. Am. Zool. *39*, 244–252. 10.1093/icb/39.2.244.

Woods, H.A., Pincebourde, S., Dillon, M.E., and Terblanche, J.S. (2021). Extended phenotypes: buffers or amplifiers of climate change? Trends Ecol. Evol. *36*, 889–898. 10.1016/j.tree.2021.05.010.

Woodward, G., Perkins, D.M., and Brown, L.E. (2010). Climate change and freshwater ecosystems: impacts across multiple levels of organization. Philos. Trans. R. Soc. B Biol. Sci. *365*, 2093–2106. 10.1098/rstb.2010.0055.

Supplemental Information

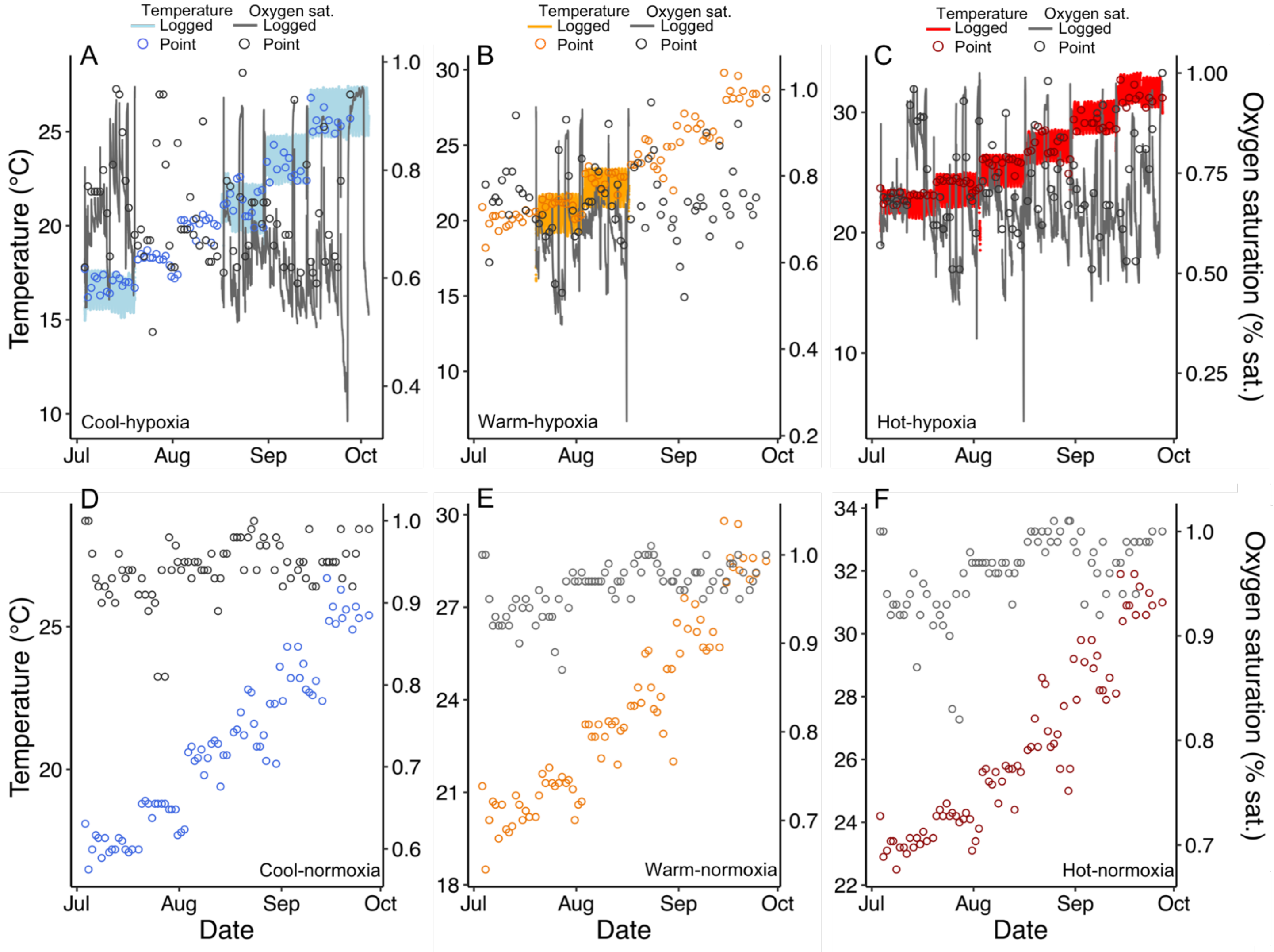

Fig. S1: Scatterplots of logged and point-measurement temperature over time in long-term ramp for cool-hypoxia (A), warm-hypoxia (B), hot-hypoxia (C), cool-normoxic (D), warm-normoxic (E), hot-normoxic (F) treatments.

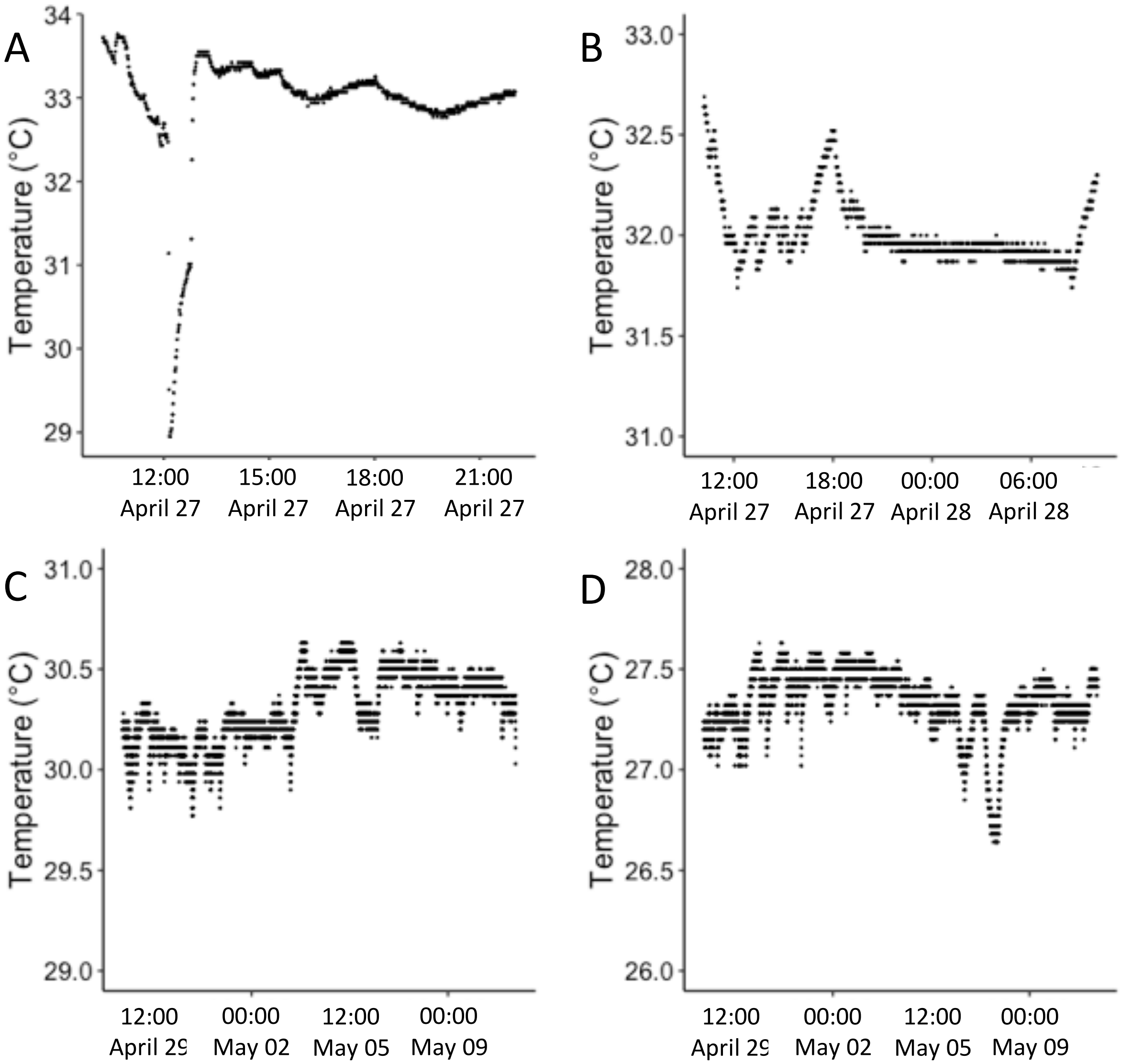

Fig. S2: Scatterplots of temperature over time in constant-temperature tolerance experiments for each exposure temperature treatment: 33 (A), 32 (B), 30 (C), and 27 °C(D). 33 °C treatment: mean = 32.95 °C, sd = 0.72 °C; 32 °C treatment: mean = 32.02 °C, sd = 0.16 °C.; 30 °C treatment: mean = 30.31 °C, sd = 0.17 °C; 27 °C treatment: mean = 27.33 °C, sd = 0.16 °C.