

RESEARCH ARTICLE

Tolerance of aquifer stoneflies to repeated hypoxia exposure and oxygen dynamics in an alluvial aquifer

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ABSTRACT

Aquatic insects cope with hypoxia and anoxia using a variety of behavioral and physiological responses. Most stoneflies (Plecoptera) occur in highly oxygenated surface waters, but some species live underground in alluvial aquifers containing heterogeneous oxygen concentrations. Aquifer stoneflies appear to be supported by methane-derived food resources, which they may exploit using anoxia-resistant behaviors. We documented dissolved oxygen dynamics and collected stoneflies over 5 years in floodplain wells of the Flathead River, Montana. Hypoxia regularly occurred in two wells, and nymphs of *Paraperla frontalis* were collected during hypoxic periods. We measured mass-specific metabolic rates (MSMRs) at different oxygen concentrations (12, 8, 6, 4, 2, 0.5 mg l⁻¹, and during recovery) for 111 stonefly nymphs to determine whether aquifer and benthic taxa differed in hypoxia tolerance. Metabolic rates of aquifer taxa were similar across oxygen concentrations spanning 2 to 12 mg l⁻¹ ($P > 0.437$), but the MSMRs of benthic taxa dropped significantly with declining oxygen ($P < 0.0001$; 2.9-times lower at 2 vs. 12 mg l⁻¹). Aquifer taxa tolerated short-term repeated exposure to extreme hypoxia surprisingly well (100% survival), but repeated longer-term (>12 h) exposures resulted in lower survival (38–91%) and lower MSMRs during recovery. Our work suggests that aquifer stoneflies have evolved a remarkable set of behavioral and physiological adaptations that allow them to exploit the unique food resources available in hypoxic zones. These adaptations help to explain how large-bodied consumers might thrive in the underground aquifers of diverse and productive river floodplains.

KEY WORDS: Aquifer and benthic stoneflies, Plecoptera, River floodplain, Hypoxia, Physiology, Adaptation, Metabolic rate

INTRODUCTION

Adaptations to hypoxia and anoxia are important because oxygen is essential for sustaining intracellular bioenergetics for most species on Earth. Low oxygen stress occurs for many species owing to fluctuating environmental conditions or conditions induced by different pathological states (Lee et al., 2019, 2020). Extreme hypoxia and anoxia are common in aquatic habitats, as well as in many terrestrial microhabitats, and present challenges for insects. Aquatic habitats like eutrophic lakes, ponds, wetlands and groundwater often contain persistent patches of extreme hypoxia or anoxia. Other terrestrial

habitats, such as soils, are flood-prone (Cavallaro and Hoback, 2014; Hoback and Stanley, 2001), becoming saturated at some times of the year from snowmelt, rainfall (Hoback et al., 1998) or flooding. Insects face more challenges in aquatic habitats because oxygen concentrations are much lower and oxygen also diffuses more slowly in water than it does in air (Aachib et al., 2004; Denny, 1993; Verberk and Bilton, 2011; Woods, 1999).

To cope with limited oxygen, aquatic insects employ a suite of behavioral and physiological adaptations, commonly using air stores, gas gills and cutaneous respiration to extract oxygen from water (see Jones et al., 2019). In moderate hypoxia, they often enhance oxygen uptake through behavioral changes such as moving gills or initiating push-ups (Baumer et al., 2000; Benedetto, 1970; Knight and Gaufin, 1963; Nagell and Landahl, 1978; Van Der Geest, 2007). Under more extreme conditions, groundwater taxa may maintain low metabolic rates over a range of oxygen levels (Malard and Hervant, 1999) or just in hypoxia (Hoback and Stanley, 2001). Other taxa have enlarged tracheal systems or switch from aerobic to anaerobic metabolism (Hoback and Stanley, 2001; Woods and Lane, 2016). Some groundwater beetles have cutaneous respiration that allows them to permanently remain in underground aquifers with few air–water interfaces (Jones et al., 2019). Many hypoxia-tolerant aquatic insects also enhance the oxygen-carrying capacity of hemolymph by expressing respiratory proteins such as hemoglobin or hemocyanin (Burmester and Hankeln, 2007; Weber, 1980).

Oxygen concentrations vary significantly in different aquatic habitats, and species assemblages have been defined in terms of the concentration of dissolved oxygen in the habitat (Dodds, 2002; Hoback and Stanley, 2001; Hynes, 1960). In flowing streams and rivers, water is generally well mixed and oxygenated, though some variation occurs temporally and spatially as a result of changes in partial pressure with altitude, temperature and light, flow velocity, groundwater inputs, decomposition of organic matter, instream photosynthesis, respiration by organisms and exchanges with the atmosphere (Allan and Castillo, 2007; Dodds, 2002; Hynes, 1960; Paerl et al., 1998). These highly oxygenated habitats are home to taxa commonly used as hypoxia-sensitive bioindicators of good water quality, including mayflies (Order: Ephemeroptera), stoneflies (Plecoptera) and caddisflies (Trichoptera) (Gaufin, 1973; Gaufin et al., 1974).

The alluvial aquifers that underlay gravel-bed river floodplains are generally well oxygenated by strong connections with the main channel (Stanford and Ward, 1988) and diffusion from the vadose zone (unsaturated zone between the land surface and groundwater; Smith et al., 2011). Water residence times in alluvial aquifers can be highly variable, ranging from hours to 3 years in the Nyack floodplain in Montana (Helton et al., 2014) where fast flow is facilitated in places by the presence of paleo channels (preserved cobble-boulder bed of now buried historic river channels; Poole et al., 1997). Compared with surface waters, it is likely that alluvial aquifers regularly have more heterogeneity in oxygen

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concentrations. Indeed, measured dissolved oxygen concentrations in the surface water of the Middle Fork of Flathead River at the Nyack floodplain ranged from 8.44 to 13.1 mg l⁻¹ whereas within the aquifer they ranged from 0.14 to 12.8 mg l⁻¹, varying with season, discharge and floodplain position (Helton et al., 2012).

Alluvial aquifers support a unique assemblage of groundwater stoneflies from two families: the Chloroperlidae and the Capniidae. Unlike the vast majority of the ~3500 stonefly species worldwide (Fochetti and de Figueroa, 2008), which inhabit highly oxygenated, flowing surface waters, obligate groundwater stoneflies spend the entirety of their juvenile growth phase (as nymphs) in the aquifer before returning to the river to emerge as adults (Stanford et al., 1994; Stewart and Stark, 2002). Alluvial aquifers with groundwater stoneflies are voluminous compared with surface waters and can extend several kilometers from the main river (Stanford and Ward, 1988; Stanford et al., 1994). In addition to potentially having more variable oxygen dynamics, these environments are dark, carbon-poor and sediment laden. Despite these challenging conditions, abundant populations of groundwater stoneflies are supported by the subsurface environment.

The presence of abundant populations of stoneflies in alluvial aquifers was viewed as a paradox until recently. In aquifers there is no possibility for photosynthesis, bioavailable dissolved organic carbon is scarce and microbial productivity is very low (Craft et al., 2002; Ellis et al., 1998; Gibert et al., 1994). In comparison, the food webs of surface waters are fueled by large inputs of allochthonous organic matter (material imported into streams, e.g. leaves) and algal primary production. It was not clear how a diverse and productive food web with large-bodied stonefly consumers could be supported in this ultra-oligotrophic environment until the recent finding that a large portion of the carbon making up aquifer stonefly biomass was derived from methane in the Nyack floodplain in Montana (DelVecchia et al., 2016). Furthermore, these organisms had ¹³C signatures indicating a preference for methane-derived carbon sources, and DelVecchia et al. (2019) recently found obligate anaerobic methanogens and aerobic methanotrophs in stonefly gut contents.

This type of food web contribution was unexpected because stoneflies are thought to require highly oxygenated water (Gaufin, 1973; Gaufin et al., 1974). In contrast, methanogenesis often occurs in anoxia and the resulting methane is consumed rapidly where oxygen is present, probably at oxic–anoxic interfaces (Bussmann et al., 2006). To access the resources produced at these locations, aquifer stoneflies would need to be adapted to tolerate anoxic and/or hypoxic conditions. Indeed, aquifer stoneflies do survive and have been observed moving after longer periods of exposure than surface dwelling benthic taxa in anoxia and hypoxia (Malison et al., 2020). This ability to tolerate and continue moving in zones of low oxygen could allow aquifer stoneflies to forage directly within extremely hypoxic zones, but it is not clear if aquifer stoneflies can tolerate the repeated exposure that would be required to regularly forage in these environments.

Exposure to anoxia can be highly stressful and even individuals tolerant to anoxia can experience large changes in metabolism and development (Harrison et al., 2006; Hoback and Stanley, 2001; Hochachka, 1997; Woods and Lane, 2016; Yocum and Denlinger, 1994). Additional physiological challenges occur during recovery from anoxia, including the need to re-establish energy and ion homeostasis and minimize oxidation damage to the tracheal system (Lighton and Schilman, 2007; Wegener, 1993). In many animals and insects, these challenges can disrupt development, reduce performance or lead to death (Woods and Lane, 2016). Like some other aquatic insects, most Plecoptera nymphs have a closed tracheal

system. As such, dissolved oxygen from the water enters by diffusion through tracheal gills and/or the body wall (Nagell, 1973; Nation, 2008). If aquifer stoneflies can tolerate and recover from exposure to anoxia or hypoxia repeatedly this suggests they may have specialized adaptations to anoxia that minimize oxidative damage.

The ability to exploit different environmental conditions varies between different organisms. Organisms with the ability to more strongly adjust physiological functions are able to move through and tolerate more heterogeneous environments (Mislán et al., 2016). For example, some marine taxa are able to repeatedly exploit anoxic and hypoxic habitats (Childress and Seibel, 1998; Stramma et al., 2012), because their oxygen carrier proteins have a range of oxygen affinities (Mislán et al., 2016). Some fishes are able to detect and avoid hypoxia while swimming (Herbert et al., 2011). Other taxa, including groundwater crustaceans, can also quickly and repeatedly recover from exposure to hypoxic conditions (Malard and Hervant, 1999), and blue crabs (*Callinectes sapidus*) can even continue feeding on vulnerable prey in hypoxic conditions in estuaries (Bell et al., 2003a,b). In contrast, mayflies have reduced growth rates from chronic exposures to sub-lethal levels of hypoxia (Winter et al., 1996). Both the short-term and long-term implications of hypoxia and anoxia exposure is unclear in many taxa, including aquifer stoneflies. A threshold of exposure, in degree and length of exposure, likely exists under which aquifer stoneflies may be able to repeatedly forage and minimize negative fitness effects. Additionally, the actual distribution and variability of patches of anoxia and extreme hypoxia in alluvial aquifers could strongly influence food availability and the likelihood that stonefly populations utilize these zones.

Here, we used sensor data collected from six wells across a floodplain aquifer to test for the occurrence of hypoxic conditions in locations where hyporheic stoneflies are often collected. We used intermittent respirometry to measure the metabolic rates of aquifer and benthic stoneflies at different levels of oxygen concentrations. We predicted: (1) that metabolic rates of aquifer taxa would remain more constant with changing oxygen levels compared to benthic taxa because they are adapted to live in environments that can have low oxygen; (2) that metabolic rates of aquifer taxa would recover to initial levels following short-term repeated exposure to extreme hypoxia (expected time scale of repeated foraging); but (3) that longer-term repeated exposure of many hours (much longer than would be required for foraging) of low oxygen would have negative effects on metabolic rates and survival.

MATERIALS AND METHODS

Study area

Aquifer-dissolved oxygen data and aquifer and benthic stoneflies were collected from the Nyack floodplain of the Middle Fork of the Flathead River in northwestern Montana (Fig. 1; 48°27'30" N, 113° 50' W). The Middle Fork forms part of the southern boundary of Glacier National Park and drains a 2300 km² catchment, originating in a pristine wilderness area. The Middle Fork is a 5th order river with a spring snowmelt hydrograph, an average peak annual discharge of 541 m³ s⁻¹ and average baseflow of 17 m³ s⁻¹ (Whited et al., 2007). The Nyack floodplain is approximately 10 km in length and 2 km wide constrained by bedrock canyons at the upstream and downstream ends. Within the floodplain, the river forms a braided channel with high hydrological connectivity between the river and the aquifer (Poole et al., 2006; Stanford and Ward, 1993), resulting in an expansive and voluminous alluvial aquifer. The floodplain was equipped with a network of aquifer wells drilled to 8–10 m in depth using a hollow auger drilling rig

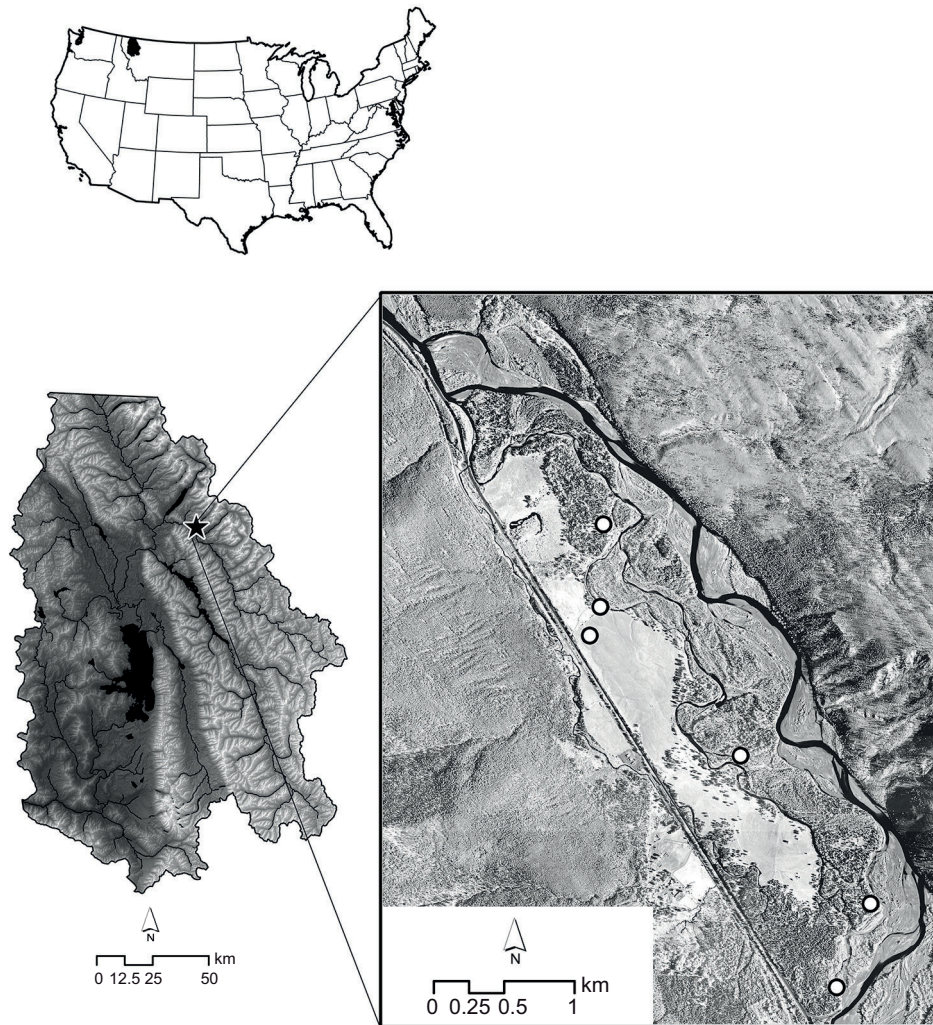


Fig. 1. Map of the Nyack floodplain in the Flathead Basin of western Montana.

Circles show the location of sampling wells on the floodplain that are instrumented with the RiverNet data loggers.

(see Helton et al., 2014 for more details). Wells were slotted with 2 mm openings throughout the length of the pipe and 6 of the wells were instrumented in 2013 with the RiverNet continuous monitoring system (see below).

Study design

Alluvial aquifer dissolved oxygen data have been collected hourly in the six wells since 2013. We determined when wells were hypoxic and summarized when aquifer stoneflies were collected from wells with hypoxic conditions in 2013 and 2014, the time period with the most extensive monthly sampling (see DelVecchia et al., 2016). To measure the physiological responses of aquifer [*Paraperla frontalis* (Banks 1902), *Isocapnia* spp. Banks 1938 and *Kathroperla perdita* Banks 1920] and benthic [*Hesperoperla pacifica* (Banks 1900), *Claassenia sabulosa* (Banks 1900) and *Pteronarcys californica* Newport 1848] stonefly species to hypoxia and anoxia, we sampled aquifer and river habitats during June–October 2018 and April–May 2019 and brought live samples back to the lab for individual respirometry trials. All individuals were acclimated to the lab for at least 24 h prior to experimentation. We measured the influence of anoxia and hypoxia on individual stoneflies using 3 different intermittent respirometry experiments, including: (1) incremental depletion trials, (2) short-term alternating normoxia/hypoxia trials

during June–October 2018 and (3) longer-term repeated normoxia/extreme-hypoxia trials during April–May 2019.

Field sampling

The RiverNet continuous monitoring system has recorded hourly measurements of depth, dissolved oxygen concentration and saturation, conductivity and temperature at approximately 3 m below the base flow water table in 6 wells since 2013. RDO sensors (RDO classic, RDO Pro and RDO Pro-X, *In-Situ*, in-situ.com, Fort Collins, Colorado, USA) measure dissolved oxygen, while C5450-L pressure transducers (Campbell Scientific, campbellsci.com, Logan, UT, USA) measure water table depth and 5547 Conductivity sensors (Campbell Scientific) measure water conductivity. All sensors are maintained on a monthly basis for biofilm removal and RDO probe calibration. The RDO sensors are calibrated with *Win-Situ* software using a one-point calibration procedure under 100% saturation conditions once each month.

Aquifer stonefly samples were collected from wells (Fig. 1) using a gas-powered diaphragm pump. We pumped wells for 10 min intervals from all wetted depths (~2–7 m) using a 2.5 or 5 cm diameter hose (depending on well size) and collected samples in a 500 μ m net. Samples collected during 2013 and 2014 were preserved in the field in ethanol. Samples collected in 2018 and 2019 for

physiology experiments were placed live into buckets of aerated well water, and transported back to FLBS. All live stoneflies remained in aerated containers in a walk-in cooler (5°C) prior to experimentation.

Benthic stonefly nymphs were collected from riffle habitat in the main Middle Fork River on the Nyack floodplain (Fig. 1) using Stanford-Hauer kick nets. Rocks were first scrubbed by hand and then we kicked the substrate to wash stoneflies into the net. After decanting the sample, we picked individual stoneflies stream side, placed live individuals in aerated buckets of river water, and transported them to FLBS for experimentation. Species level identification of all stoneflies was confirmed following collection or experimentation using a dissecting microscope.

Respirometry

We conducted individual intermittent respirometry trials on 158 individual aquifer and benthic stoneflies (Table 1) using a complete respirometry system with two Plexiglas boxes that housed 8 horizontal glass mini-chambers and a water bath reservoir to control temperature (Fig. 2; Loligo Systems, Denmark; www.loligosystems.com). Individual stoneflies were placed into 3 of the mini-chambers in each box, leaving a blank mini-chamber control in one box and an open chamber for oxygen sensing and regulation in the second box. Small (2.2 ml) and large (17 ml) mini-chambers were used (depending on the species and size of nymph being tested). The respirometry system contained 0.2 µm filtered water held at ambient aquifer temperatures (5.5–6.5°C). Cooled water (held constant by a refrigerated unit) circulated continuously between the water bath and the Plexiglas boxes. Temperature and dissolved oxygen were monitored continuously using two Witrox 4 oxygen meters for mini sensors. Dissolved oxygen concentration in each mini chamber was measured using polymer optical bare tip fibers and 2 mm sensor spots mounted onto the inside of each mini-chamber (thin planar oxygen mini sensors, PreSens Precision Sensing, Germany, www.presens.de). Sensor spots were calibrated for a zero baseline using a sodium sulphite solution (10 g per 500 ml water) and 100% saturation baseline in bubbled recirculated water. During trials, oxygen concentration was measured every second using AutoResp software (Loligo Systems). Initial oxygen saturation at normoxia was 11–12 mg l⁻¹ for all experiments. Ambient barometric pressure ranged from 1002 to 1027 hPa at an elevation of 892 m.

For intermittent respirometry the mini-chambers were connected to flush pumps and controlled by the program AutoResp. Each intermittent cycle consisted of an initial flush period (90 s) during which water was pulled from the box into the mini-chambers, then a waiting period (30 s) that allowed oxygen levels in the chambers to stabilize prior to measurement, and then finally, a closed period (600 s) during which pumps were off and oxygen was depleted by

stoneflies (and metabolic rates were later calculated). Measurement windows of 600 s for each cycle provided data with high *R*² values, allowed us to make replicate measurements (3–5 for each level) and still complete each experiment in a feasible amount of time.

Oxygen levels were lowered by bubbling nitrogen gas into the water bath reservoir and raised by bubbling air. The intermittent respirometry system can effectively be used to measure metabolic rates from normoxia down to ~0.5–1 mg l⁻¹ of oxygen, depending on the chamber size. While it would also be ideal to take intermittent measurements closer to anoxia (0.0 mg l⁻¹), it was not possible with the system because there was too much gas diffusion from the air in the top of the Plexiglas boxes (see Fig. 2). To measure metabolic rates as close to anoxia as possible, we closed the systems (turned off pumps) and allowed the stoneflies to deplete the chambers overnight before reaerating for recovery measurements.

Because only six individuals could be measured at once, individuals from different trials experienced different lengths of holding time. At a minimum, stoneflies were held in individual containers for at least 24 h prior to experimentation (at 5°C) for gut evacuation and all individuals remained unfed for the duration they were held. For experiment 1, individuals were held in the cooler for up to 13 days, and there was no mortality. For experiment 2 individuals were held for up to 4 days and for experiment 3 individuals were held for up to 12 days, except for five individuals that were held for 31–37 days. We recorded the collection date and holding time for each individual and tested for differences in survival and metabolic rates with holding time. Individuals held for 1–37 days in the aerated water displayed similar behaviors when observed and only individuals that were moving normally (moving all body parts fluidly, able to swim and right themselves) were used in trials for each experiment.

Experiment 1: Incremental depletion trials for aquifer and benthic taxa

To measure metabolic rates over decreasing oxygen levels and to test for differences in performance, recovery and survival between aquifer and benthic taxa, we conducted incremental depletion trials on 123 stoneflies (aquifer: 23 *P. frontalis*, 24 *Isocapnia* spp. and 1 *K. perdita*; benthic: 19 *C. sabulosa*, 23 *H. pacifica* and 21 *P. californica*; Table 1). We measured oxygen levels at normoxia, over incrementally decreasing levels of dissolved oxygen, in extreme hypoxia or anoxia, and during recovery in normoxia. Each trial lasted ~24 h, with oxygen levels lowered incrementally during the day, the system closed at night (to obtain extreme hypoxia or anoxia), and recovery measured the next morning. We repeated 5 complete intermittent cycles (flush, wait, measure) at each oxygen level, lowering oxygen concentrations in a step-wise fashion: at 12, 8, 6, 4, 2 and ~0.5–1.0 mg l⁻¹. After completing the last intermittent level

Table 1. Summary of stonefly species used in respirometry experiments

Species	Experiment 1 (Incremental depletion)	Experiment 2 (Short-term alternating)	Experiment 3 (Longer-term alternating)	Mass (g)	
				Mean±s.d.	Range
Aquifer					
<i>Isocapnia</i> spp.	24	10	8	0.03±0.01	0.016–0.049
<i>Paraperla frontalis</i>	23	10	8	0.03±0.01	0.012–0.051
<i>Kathroperla perdita</i>	1	–	11	0.05±0.01	0.028–0.068
Benthic					
<i>Claassenia sabulosa</i>	19	–	–	0.26±0.15	0.044–0.521
<i>Hesperoperla pacifica</i>	23	–	–	0.34±0.12	0.041–0.494
<i>Pteronarcys californica</i>	21	–	–	0.45±0.33	0.093–1.299
Total	111	20	27		
Grand total			158		

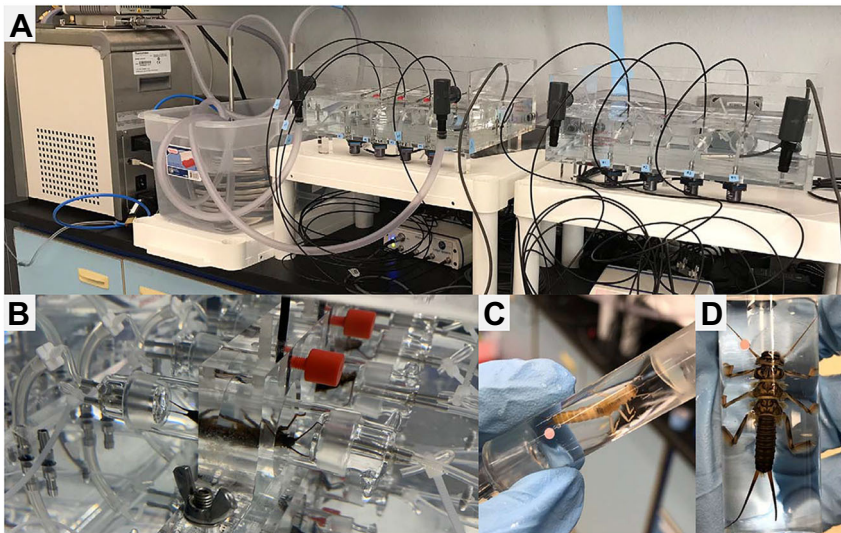


Fig. 2. Intermittent respirometry equipment.

(A) Intermittent respirometry set up (from left to right) includes water refrigeration unit, water bath with temperature control coil, and Plexiglas water chambers holding individual mini-chambers. (B) Close up of *Pteronarcys californica* nymphs in individual mini-chambers connected to flush pumps. (C) *Kathroperla perdita* and (D) *Claassenia sabulosa* nymphs in mini-chambers following experimentation.

we turned the pumps off and left the system closed for the night to let the stoneflies deplete the chambers as close to anoxia as possible (closed period mean oxygen level of $0.35 \pm 0.35 \text{ mg l}^{-1}$). Following closed overnight measurements, we recorded the disposition of each stonefly, changed the system back to the intermittent setting, and aerated the water bath and boxes to return the system and mini-chambers back to normoxia. After the system reached 11 mg l^{-1} or higher we measured metabolic rates over a minimum of 5 complete intermittent cycles. During the closed period all taxa experienced hypoxia, but due to limitations with the equipment (large chambers were ‘leakier’ and did not deplete as low) and the fact that most benthic taxa were larger, the aquifer species experienced lower oxygen levels during the closed period than benthic taxa (0.13 ± 0.24 vs. $0.49 \pm 0.33 \text{ mg l}^{-1}$).

Experiment 2: Short-term (24 h) alternating normoxia and hypoxia trials

To test the effect of short-term repeated exposure of hypoxia on aquifer taxa metabolic rates, recovery, and survival, we conducted short-term alternating normoxia/hypoxia trials on two focal aquifer species (10 *Isocapnia* spp. and 10 *P. frontalis*; Table 1). For this experiment we completed 5 intermittent cycles at 3 oxygen levels: (1) normoxia ($\sim 12 \text{ mg l}^{-1}$), (2) intermediate hypoxia (2 mg l^{-1}) and (3) deep hypoxia ($0.4\text{--}1.2 \text{ mg l}^{-1}$), and repeated the entire set of measurements 3 times over the course of ~ 9 h. After each set of intermittent cycles were completed at each oxygen level, oxygen was adjusted to the next level using nitrogen gas or by bubbling air. It took 15–25 min to go from 12 to 2 mg l^{-1} , 14–27 min from 2 to 0.5 mg l^{-1} and 31–45 min from 0.5 mg l^{-1} back to 12 mg l^{-1} . Following the completion of each trial we conducted intermittent cycles at normoxia continuously for 12–14 h to measure recovery. We recorded survival status at the end of each trial.

Experiment 3: Longer-term alternating normoxia and extreme hypoxia

To determine how longer repeated exposures to extreme hypoxia would affect aquifer stoneflies metabolic rates, recovery and survival we conducted 96 h intermittent respirometry trials on the 3 focal aquifer species (8 *Isocapnia* spp., 8 *P. frontalis* and 11 *K. perdita*; Table 1). We completed 5 intermittent cycles for each oxygen level lowered in a step-wise fashion: at 12, 4, 2 and $\sim 0.5 \text{ mg l}^{-1}$ (concentrations ranged from $0.5\text{--}0.7 \text{ mg l}^{-1}$). After finishing

measurements at the 0.5 mg l^{-1} level we turned off the pumps and left the system closed for the night to let the stoneflies deplete the chambers as close to anoxia as possible ($0\text{--}0.7 \text{ mg l}^{-1}$). Following closed overnight measurements we changed the system back to the intermittent setting, and aerated the water bath and boxes to return the system and mini-chambers back to normoxia. After the system reached 11 mg l^{-1} or higher we conducted intermittent cycles for 2 h to measure recovery. We then repeated the 24 h cycle 4 more times, for a total of 5 periods of normoxia, drawdown, extreme hypoxia and recovery measurements. Because trials ended at the end of the week, we collected extended recovery metabolic rate data at normoxia over the weekend for ~ 48 h at the end of each trial.

Data analysis

Dissolved oxygen (DO) data collected from the RiverNet wells was filtered to remove observations that were likely a result of sensor disturbance, which usually occurred during sampling or monthly sensor maintenance. Because aquifers are isolated from the atmosphere, their temperatures are generally stable. If any observation deviated more than 6°C from observations 4 h prior to and after the given observation, we removed it from the dataset. This was effective in removing observations which clearly were affected by sensor removal in all wells. However, well HA12 often had rapid biofilm buildup on the optical DO sensor and this caused DO readings to be somewhat erratic. Much of this data was cleaned by the filtering process, but some was still reflected in the dataset. To compensate further for this issue without making assumptions specific to well HA12, we used both the raw filtered data and a loess curve fit (locally estimated scatterplot smoothing) to visually represent DO data. We used DO data collected from 2013–2018 to demonstrate the annual patterns in aquifer conditions over as long a period as possible.

Metabolic rates were estimated from raw data on changes in oxygen concentrations using scripts written in R (version 3.5; <https://www.r-project.org/>). For each time period, we calculated oxygen consumption as the slope of the linear regression of oxygen concentration with time. To control for any microbial respiration or gas leakage, we corrected the data by subtracting the slope of the control chamber from the slope of each experimental chamber. We measured oxygen values every second during each 10 min closed measurement period and calculated mean metabolic rates for each individual for each oxygen level from the replicate 10 min windows within each level. For each measurement period, oxygen slopes were

converted to mass-specific metabolic rates (MSMRs) by multiplying the respiration volume by the adjusted slope (slope–control chamber slope) and dividing by insect mass. We then calculated the mean MSMR based on all replicate nymphs for each species for each oxygen level. Not all bins included data on all replicated individuals for each species when data points were missing. During analysis, we visually inspected the traces for each oxygen level and omitted traces for chambers or replicates where any traces behaved erratically (e.g. spikes in oxygen levels of short duration, control traces excessively steep, etc.) and in some cases data were missing due to power outages or computer restarts. All mean MSMR values for each species for each time period were based on 8–24 individuals, except for *K. perditia* in experiment 1 (only one individual) and some closed periods (2–25 individuals). Negative metabolic rates are a result of the control chamber having a greater slope than the measurement chamber, which we consider to be experimental error. Negative metabolic rates were most commonly calculated for very low oxygen levels when stonefly respiration was very low and for smaller individuals that had higher ratios of water volume to body mass in the chamber and did not deplete oxygen as strongly.

For experiment 1, we analyzed differences in survival rates between aquifer and benthic taxa using the Kruskal–Wallis rank sum test. We analyzed differences in MSMRs over different oxygen concentrations using a two-way ANOVA. Main effects were oxygen level (8 levels) and taxon type (aquifer versus benthic). We analyzed the effect of the interaction of oxygen and taxon type on metabolic rates. We conducted *post hoc* Tukey's tests to test for significant differences between different groups for both main effects and interaction of oxygen and taxon type. To verify that holding time did not affect metabolic rates we conducted *t*-tests for the 12 and 2 mg l⁻¹ oxygen levels for each species that had groups of individuals with holding times that differed by more than 3 days. Lastly, because maintaining lower metabolic rates can be a mechanism which improves hypoxia tolerance, we analyzed potential differences in MSMRs between aquifer and benthic taxa in normoxia using a two-sample *t*-test.

For experiment 2, we analyzed differences in MSMRs by oxygen level and repeated exposure period for *P. frontalis* and *Isocapnia* spp.

using a linear mixed effects model (Package nlme; <https://CRAN.R-project.org/package=nlme>). Individual was included as a random effect. We also included a first-order autoregressive function to test for autocorrelation between measurements on the same individuals at the same oxygen levels but different replicates. Independent variables were dissolved oxygen concentration (3 levels), number of repeated exposure period (total of 4, 3 different exposure periods plus the recovery period), and the interaction of oxygen and exposure period. Our full model tested for differences in metabolic rates over the first 3 repeated exposure periods. We explored the origins of the significant interaction between level of repeated exposure and oxygen level by partitioning the data set in several ways. For example, we ran a reduced model with the same structure but only the 12 mg l⁻¹ level for all 4 repeated exposure periods to determine if differences in metabolic rates measured during the first exposure period (e.g. pre-exposure rates at 12 mg l⁻¹) were disproportionately affecting model fit. These additional analyses suggest that the interaction between repeated exposure level and oxygen level arises because initial metabolic rates in fully saturated water in the first replicate were elevated, possibly because insects were stressed from having been recently introduced into the respiration chambers. All individuals were held for 4 days or less so we did not test for any effect of holding time.

For experiment 3, we analyzed differences in MSMR by oxygen level and repeated exposure period for *P. frontalis* and *Isocapnia* spp. using the same linear mixed effects models described above. For this experiment there were 5 oxygen levels and 6 repeated exposure periods (5 different exposure periods, plus the recovery period). Our full model tested for differences in metabolic rates over the first 5 repeated exposure periods and we tested for differences in recovery rates versus pre-exposure with a second model for all 6 repeated exposure periods at only the 12 mg l⁻¹ oxygen level. We also used the same methods to investigate the origins of significant interactions by partitioning the data set in several ways to run reduced models. Lastly, to verify that the long holding time (31–37 days) experienced by two *P. frontalis* and three *Isocapnia* did not affect metabolic rates we conducted *t*-tests for each of the species at the 12 and 2 mg l⁻¹ oxygen levels for replicates 1 and 5

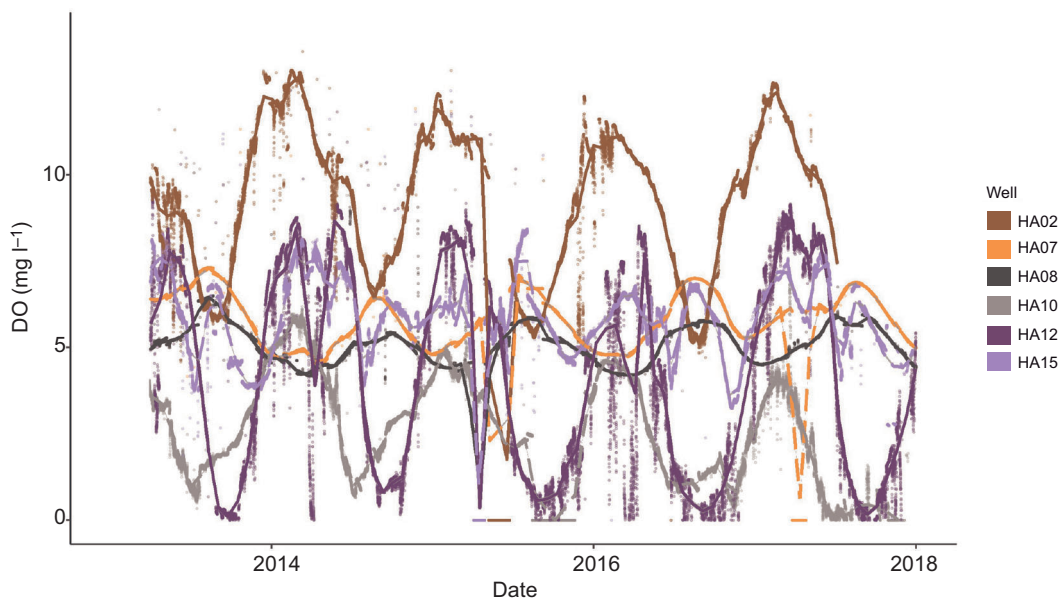


Fig. 3. Dissolved oxygen concentrations for each of the RiverNet wells between 2013 and 2018 are overlaid with loess fits. Data show that wells nearer the main channel (e.g. HA02), with shorter flowpath lengths, have more variable and higher dissolved oxygen (DO) levels. Wells with the longest flowpath lengths (e.g. HA07, HA10) tend to have lower variability in DO concentrations.

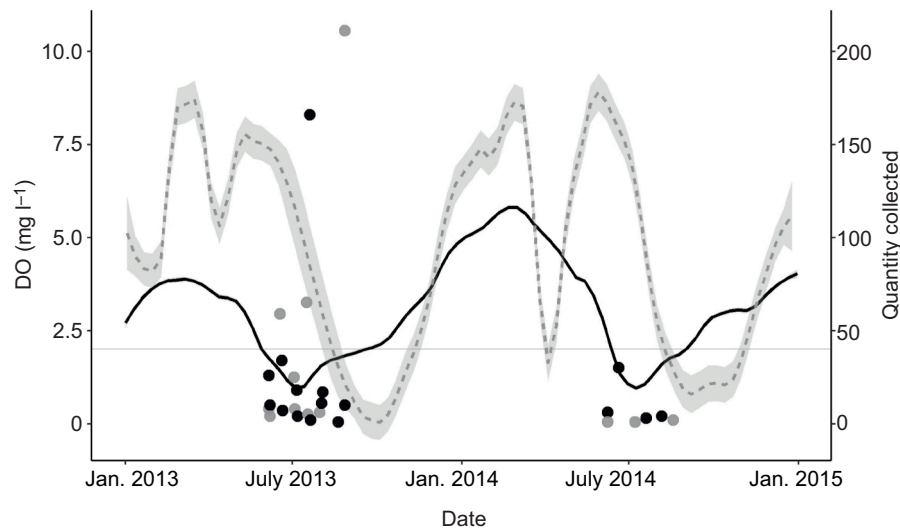


Fig. 4. Loess fits of DO concentrations. Data are plotted for wells HA10 (black) and HA12 (gray, dashed line) for the period between 1 January 2013 and 1 January 2015 and overlaid with the number of *P. frontalis* nymphs collected in each of those wells over the summer periods of insect sampling (May–September each year). Black dots are each sampling events in well HA10, and gray dots sampling events in HA12. Many nymphs were collected in hypoxic (indicated by horizontal line) to anoxic (DO=0) conditions.

(comparing individuals with holding times less than 12 days versus over 30 days).

RESULTS

Alluvial aquifer dissolved oxygen

Of the six RiverNet wells, HA10 and HA12 had consistent annual trends toward hypoxia that persisted through the late summer

(July and August) (Fig. 3). Well HA02, closest to the river channel, had the highest overall DO levels and most variability in DO patterns. Wells HA07, HA08 and HA15 all had annual DO patterns visible in the data, but tended to stay well oxygenated. Still, stonefly nymphs (mostly *P. frontalis*) were collected in wells HA10 and HA12 during and just prior to the onset of hypoxia (Fig. 4).

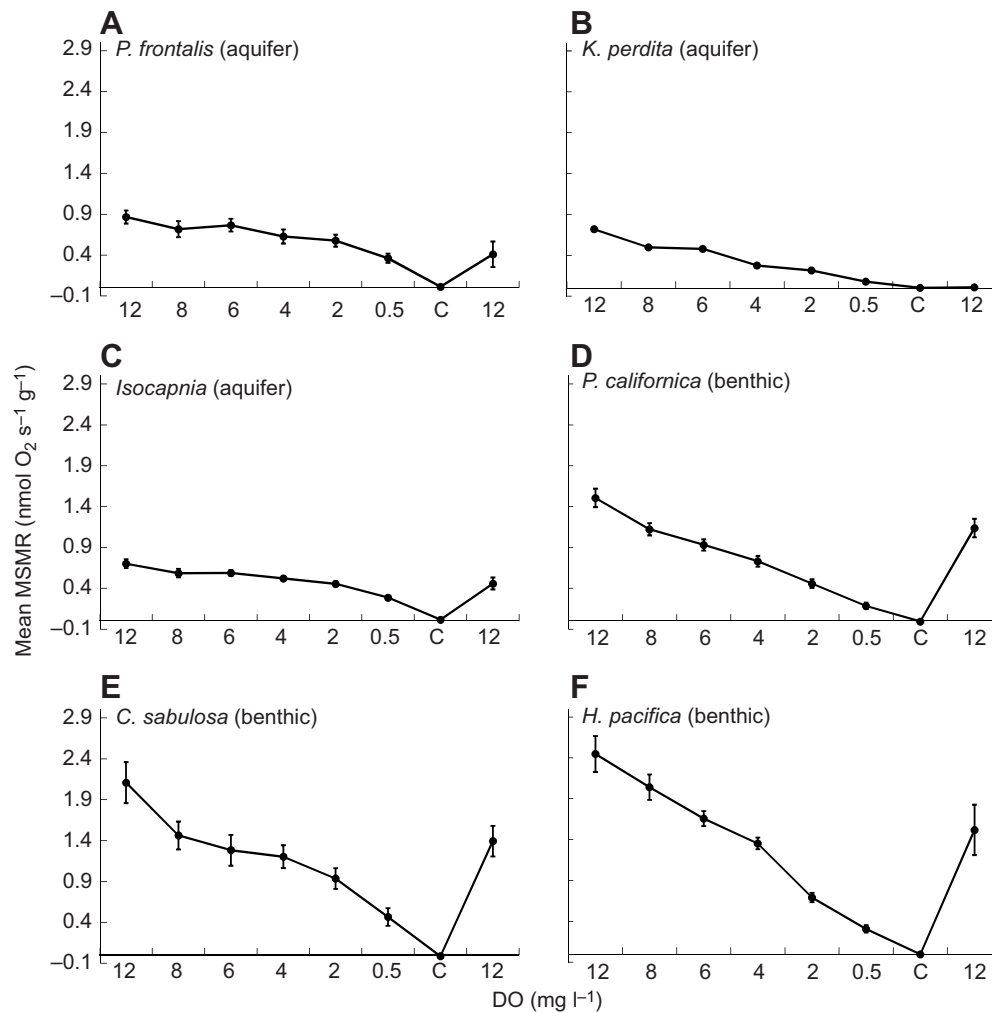


Fig. 5. Mass-specific metabolic rates (nmol O₂ s⁻¹ g⁻¹) for aquifer and benthic taxa during intermittent day-long respirometry trials (experiment 1). MSMRs were measured on individual stoneflies at 12, 8, 6, 4, 2 and 0.5 mg l⁻¹ before chambers were closed for the night for ~12 h (C indicates closed period) and then returned to normoxia for recovery measurements. Species measured include aquifer taxa (A) *Paraperla frontalis* (n=23), (B) *Kathroperla perditia* (n=1), (C) *Isocapnia* spp. (n=24) and benthic taxa (D) *Pteronarcys californica* (n=21), (E) *Claassenia sabulosa* (n=19), (F) *Hesperoperla pacifica* (n=23). Data are means±s.e.m.

Experiment 1: Respiratory rates during incremental depletion trials

Both aquifer and benthic taxa had high survival rates in day-long incremental depletion trials (97 ± 0.1 vs. $86 \pm 0.1\%$ survival, Chi square=2.402, d.f.=1, $P=0.1212$). Of benthic taxa, *P. californica* had the highest survival rate, which was most similar to aquifer species (96%), while *C. sabulosa* and *H. pacifica* had lower survival rates (79 and 84%).

Aquifer and benthic taxa had significantly different metabolic rates ($F_1=241.28$, $P<0.0001$). Metabolic rates of aquifer species decreased less than metabolic rates of benthic species as DO declined from 12 to 2 mg l⁻¹, dropping by a factor of 4.5 times compared with 7 times for the benthic species (Fig. 5). The interaction of oxygen level and taxa type was significant ($F_7=20.05$, $P<0.0001$). Aquifer taxa exhibited similar metabolic rates as oxygen levels dropped from 12 to 2 mg l⁻¹ (Table S1; Tukey's HSD, $t>1.028$, $P>0.437$; Fig. 6). At low oxygen levels, aquifer taxa exhibited significantly lower metabolic rates (both at 0.5 mg l⁻¹ and during the closed period vs. 12 mg l⁻¹; Tukey's HSD, $t>7.213$, $P<0.0001$; Fig. 6). Metabolic rates of aquifer taxa completely recovered since there was no difference in pre-exposure versus post-exposure rates (Tukey's HSD, $t=0.0706$, $P=0.0706$; Fig. 6). In contrast, metabolic rates of benthic taxa dropped significantly between each oxygen level from 12 mg l⁻¹ to the closed period (Tukey's HSD, $t>4.96$, $P<0.0001$; Fig. 6). Additionally, benthic taxa did not fully recover from hypoxia exposure; metabolic rates only recovered to levels measured at 8 mg l⁻¹ level (Tukey's HSD, $t=2.325$, $P=0.6049$; Fig. 6). See Table S1 for all pairwise comparisons for the interaction of taxa and oxygen level. Duration of holding time had no apparent effect on metabolic rates measured at 12 or 2 mg l⁻¹ for any of the species (*P. frontalis*, $t_{21}>-1.8792$, $P>0.0838$; *Isocapnia*, $t_{22}<1.4774$, $P>0.1537$; *P. californica*, $t_{19}<0.8619$, $P>0.3995$; *C. sabulosa*, $t_{17}<0.5758$, $P>0.7988$; *H. pacifica*, $t_{21}>-1.6083$, $P>0.1227$). At normoxia, mean MSMRs were almost 3 times lower for aquifer versus benthic taxa ($t_4=-4.4945$, $P=0.0109$).

Experiment 2: Respiratory rates during short-term (24 h) alternating normoxia and hypoxia

All *P. frontalis* and *Isocapnia* survived and fully recovered from repeated short-term exposure to hypoxia. Metabolic rates of *P. frontalis* declined rapidly in relation to declining oxygen over the 3 repeated exposures (on average 1.61 times lower at 2 vs. 12 mg l⁻¹, 1.69 times lower at 1 vs. 2 mg l⁻¹) in comparison to the metabolic rates of *Isocapnia* (on average 1.13 times lower at 2 vs. 12 mg l⁻¹, 1.19 times lower at 0.5–1 vs. 2 mg l⁻¹; Fig. 7).

Oxygen level had a significant effect on the metabolic rates of *P. frontalis* (Table 2), but the effect of repeated exposure period alone was not significant (Table 2), suggesting that overall metabolic rates were quite similar between repeated exposures. However, the interaction of oxygen and repeated exposure period was significant (Table 2), showing that metabolic rate response to dissolved oxygen level varied by the exposure level. We partitioned the dataset to test the response to repeated exposure at the 12 mg l⁻¹ DO concentration and the significant effect of repeated exposure was still present (Table 2), but the metabolic rates were clearly elevated in the first exposure. This suggests that *P. frontalis* might have exhibited a stress response from having recently been introduced into the chambers. Examination of just the response to repeated exposure at the 2 mg l⁻¹ DO concentration showed that rates were similar with repeated exposure (Table 2), but examination of the 0.5 mg l⁻¹ level alone showed that metabolic rates did vary, slightly rising with repeated exposure level (Table 2). Recovery rates were similar to metabolic rates measured at 12 mg l⁻¹ in repeat exposures 2 and 3 (Table 2).

Metabolic rates of *Isocapnia* were significantly different between oxygen levels, repeated exposure period and their interaction (Table 2). There was no significant difference in metabolic rates at the 12 mg l⁻¹ level, but rates were highest in the first exposure; recovery rates were similar to pre-exposure rates (Table 2). There were also no differences in metabolic rates at 2 or 0.5 mg l⁻¹ levels alone (Table 2).

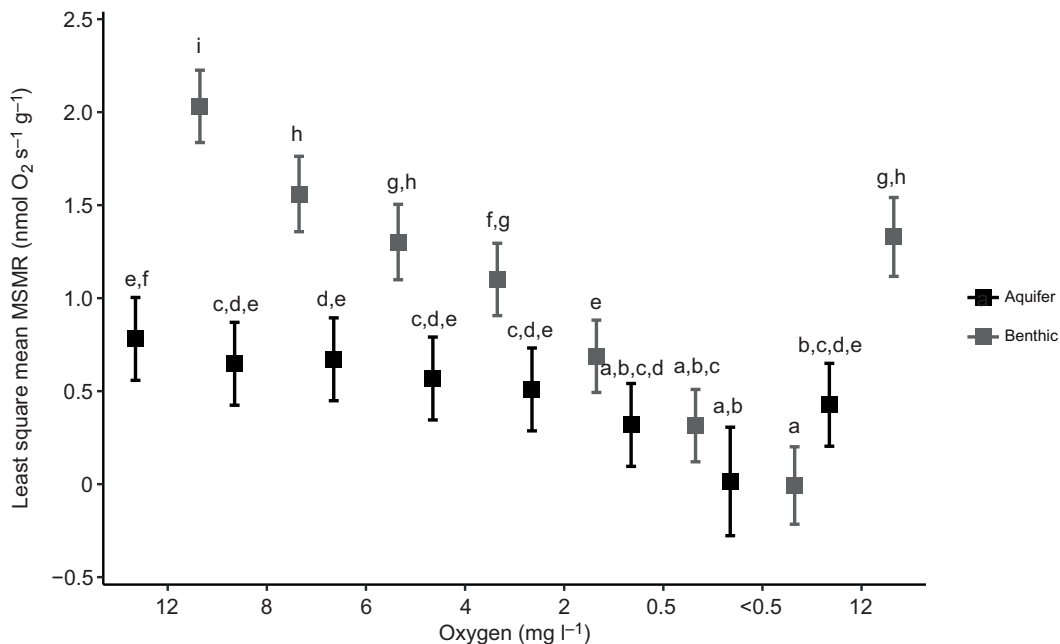


Fig. 6. Least square mean MSMRs for the interaction of oxygen and taxon type for individuals of aquifer (black, $n=48$) and benthic (gray, $n=63$) taxa. Significant mean separations for each group are available in Table S1. Significant differences ($P<0.05$) are indicated by different lowercase letters.

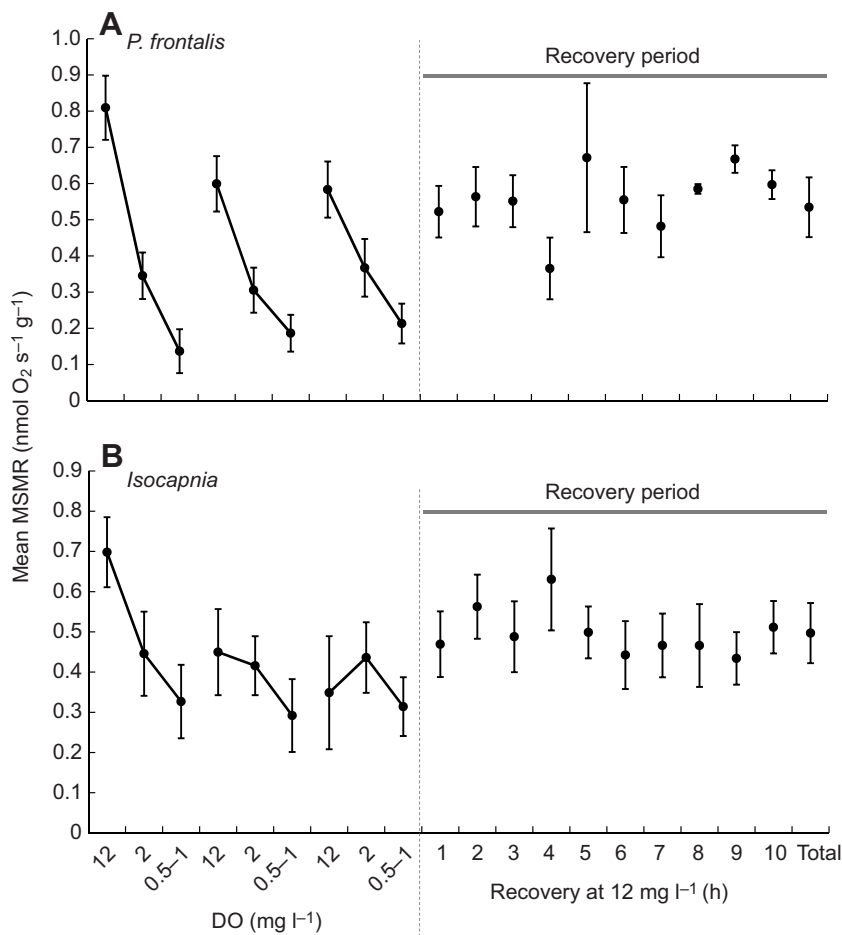


Fig. 7. MSMRs for short-term repeated normoxia-hypoxia trials (experiment 2). (A) *Paraperla frontalis* ($n=10$). (B) *Isocapnia* spp. ($n=10$). Metabolic rates were measured at 12, 2 and 0.5–1 mg l⁻¹ three times over the course of ~9 h prior to an 11 h recovery period during which metabolic rates were also measured. Each set of measurements at 12, 2 and 0.5–1 mg l⁻¹ are connected by lines. Mean±s.e.m. MSMRs were calculated for each hour of recovery and for the total recovery period.

Experiment 3: Respiratory rates during longer-term (week long) alternating normoxia and extreme hypoxia

Longer-term alternating normoxia and extreme hypoxia experiments resulted in greater mortality than the other experiments. *K. perditia* had the highest survival (91%), followed by *P. frontalis* (75%), and lastly *Isocapnia* spp. (38%). For the week-long repeated trials, metabolic rates of *P. frontalis* were significantly different by oxygen level and repeated exposure period (Table 2). The interaction of oxygen and repeated exposure period was also significant (Table 2; Fig. 8). There was a significant difference in metabolic rates between different exposures at the 12 mg l⁻¹ level (Table 2), and recovery rates were clearly lower than rates measured at the beginning of the experiment. Metabolic rates of *Isocapnia* were significantly different by oxygen level, repeated exposure period, and for their interaction (Table 2; Fig. 8). There was a significant difference in metabolic rates at the 12 mg l⁻¹ level (Table 2), and recovery rates were lower. Metabolic rates of *K. perditia* were also significantly different by oxygen level, repeated exposure period and for their interaction (Table 2; Fig. 8). There was a significant difference in metabolic rates at the 12 mg l⁻¹ level (Table 2), and recovery rates were lower. In contrast to the repeated short-term experiments, the metabolic rates measured in the first exposure period did not disproportionately affect the model fit for *P. frontalis*, *Isocapnia*, or *K. perditia* (model results were still significant for all effects for each species when repeated exposure period one was removed; Table 2). Duration of holding time had no apparent effect on metabolic rates measured at 12 or 2 mg l⁻¹ for replicate 1 or 5 for either species (*P. frontalis*, $t_6 > -1.673$, $P > 0.1453$; *Isocapnia*, $t_6 > -1.472$, $P > 0.1914$).

DISCUSSION

Compared with benthic species, aquifer stonefly species performed much better in hypoxia. They were able to sustain high rates of oxygen uptake even as oxygen levels declined to 0.5 mg l⁻¹. Once returned to normoxia, they rapidly re-established pre-exposure metabolic rates, unlike benthic taxa. Aquifer stoneflies also tolerated repeated short-term exposure to hypoxia with little effect on metabolic rates and/or survival. However, longer-term repeated exposure to low oxygen (with multiple >12 h periods of low oxygen) depressed metabolic rates, suggesting a limit in tolerance. Altogether, benthic species did not perform nearly as well in hypoxia and anoxia, reflecting their different evolutionary histories in more consistently flowing, well oxygenated water. Survival rates were likely more similar for aquifer and benthic taxa than expected because benthic taxa did not deplete oxygen levels quite as low as aquifer taxa during the closed period of these experiments. Benthic taxa clearly have lower survival rates than aquifer taxa when exposed to the same anoxic and hypoxic conditions (Malison et al., 2020). Numerous mechanisms could allow aquifer species to have higher hypoxia tolerance (see Malison et al., 2020). Here, we found that mean metabolic rates of aquifer taxa were lower than benthic taxa in normoxia and that metabolic rates of aquifer taxa remained more constant with declining oxygen compared to benthic taxa. This suggests that one mechanism behind aquifer stoneflies hypoxia tolerance may be to generally maintain lower metabolic rates like other groundwater taxa (Malard and Hervant, 1999).

Aquifer dissolved oxygen levels vary more than do well-mixed surface waters. Although the aquifer we studied is generally well

Table 2. ANOVA table listing linear mixed effect models and results for experiments 2 and 3

Model	Variable	Replicates	Oxygen level	num DF	den DF	F-value	P-value
Experiment 2							
<i>Paraperla frontalis</i>							
Full	Oxygen	1,2,3	All	1	69	68.82	<0.0001
Full	Replicate	1,2,3	All	1	69	0.91	0.3443
Full	Oxy:Rep	1,2,3	All	1	69	12.07	0.0009
Reduced1	Replicate	1,2,3,4	12 mg l ⁻¹	1	26	9.31	0.0052
Reduced2	Replicate	1,2,3	2 mg l ⁻¹	1	17	0.27	0.6096
Reduced3	Replicate	1,2,3	0.5 mg l ⁻¹	1	17	5.66	0.0293
Reduced4	Replicate	2,3,4	12 mg l ⁻¹	1	53	2.17	0.147
<i>Isocapnia</i>							
Full	Oxygen	1,2,3	All	1	77	4.65	0.0342
Full	Replicate	1,2,3	All	1	77	5.43	0.0224
Full	Oxy:Rep	1,2,3	All	1	77	8.86	0.0039
Reduced1	Replicate	1,2,3,4	12 mg l ⁻¹	1	29	3.17	0.0855
Reduced2	Replicate	1,2,3	2 mg l ⁻¹	1	19	0.06	0.8133
Reduced3	Replicate	1,2,3	0.5 mg l ⁻¹	1	19	0.07	0.7973
Experiment 3							
<i>Paraperla frontalis</i>							
Full	Oxygen	1,2,3,4,5	All	1	189	30.09	<0.0001
Full	Replicate	1,2,3,4,5	All	1	189	28.31	<0.0001
Full	Oxy:Rep	1,2,3,4,5	All	1	189	19.45	<0.0001
Reduced1	Replicate	1,2,3,4,5,6	12 mg l ⁻¹	1	39	20.12	0.0001
Reduced2	Oxygen	2,3,4,5,6	All	1	157	12.95	0.0004
Reduced2	Replicate	2,3,4,5,6	All	1	157	17.85	0.0001
Reduced2	Oxy:Rep	2,3,4,5,6	All	1	157	5.1	0.0253
<i>Isocapnia</i>							
Full	Oxygen	1,2,3,4,5	All	1	165	78.26	<0.0001
Full	Replicate	1,2,3,4,5	All	1	165	13.54	0.0003
Full	Oxy:Rep	1,2,3,4,5	All	1	165	16.92	0.0001
Reduced1	Replicate	1,2,3,4,5,6	12 mg l ⁻¹	1	34	12.98	0.001
Reduced2	Oxygen	2,3,4,5,6	All	1	137	25.56	<0.0001
Reduced2	Replicate	2,3,4,5,6	All	1	137	5.05	0.0262
Reduced2	Oxy:Rep	2,3,4,5,6	All	1	137	5.72	0.0182
<i>Kathroperla perditia</i>							
Full	Oxygen	1,2,3,4,5	All	1	261	43.38	<0.0001
Full	Replicate	1,2,3,4,5	All	1	261	4.79	0.0295
Full	Oxy:Rep	1,2,3,4,5	All	1	261	18.22	<0.0001
Reduced1	Replicate	1,2,3,4,5,6	12 mg l ⁻¹	1	54	12.63	0.0008
Reduced2	Oxygen	2,3,4,5,6	All	1	217	23.35	<0.0001
Reduced2	Replicate	2,3,4,5,6	All	1	217	6.32	0.0127
Reduced2	Oxy:Rep	2,3,4,5,6	All	1	217	11.94	0.0007

Repeated exposure periods (replicate) and oxygen levels included in each model are listed, as well as results for each variable in each model (num DF and den DF=numerator and denominator degrees of freedom, Oxy:Rep=interaction of oxygen and replicate).

oxygenated (Smith et al., 2011; Stanford and Ward, 1988), anoxia and hypoxia occurred every year at two of the sampling wells, with persistent hypoxia in late summer. Although only measured at a subset of the wells, hypoxic and anoxic zones are probably common in the aquifer. This is further suggested by the presence of methanogenic methane and obligate anaerobe methanogen taxa in the aquifer (DelVecchia et al., 2016, 2019). Furthermore, aquifer stoneflies clearly show adaptations to low oxygen conditions. Aquifer stoneflies are highly mobile, traveling kilometers underground away from the main channel after hatching from deposited eggs (Stanford et al., 1994). Because of their high mobility and ability to move in hypoxia and anoxia (Malison et al. 2020), it is unlikely that aquifer stoneflies are forced to use low oxygen environments, but rather that they have the ability to utilize and tolerate these habitats. The temporal and spatial scale of anoxia in the aquifer likely differs from the conditions experienced by stoneflies in our experiments, since they have the ability to move in and out of these zones in the aquifer. The short-term repeated hypoxia experiment may be a reasonable test of a time frame which stoneflies might be exposed to low oxygen if foraging in oxic–anoxic interfaces in the aquifer. The week-long repeated exposure experiment is likely to have exposed stoneflies to

much longer periods of anoxia than would be normally experienced and helps set the bounds on maximum extent of repeated anoxia exposure that may be possible for them to endure without facing fitness consequences.

Aquifer taxa recovered well from multiple short-term exposures to hypoxia, but there were some differences in how the species responded to repeated exposure. All individuals of both species survived and behaved normally following short-term repeated exposure to hypoxia. Although metabolic rates during recovery were lower than during pre-exposure for *P. frontalis*, rates may have been initially elevated from stress because of recent placement in experimental chambers. *P. frontalis* recovery rates were similar to metabolic rates measured at 12 mg l⁻¹ in the second and third repeated exposures. In contrast to *P. frontalis*, there was no significant difference in recovery versus pre-exposure rates for *Isocapnia* spp., probably because metabolic rates did not change as much between different oxygen levels and there was more variation in measured rates at each level. Previous work has found that *Isocapnia* tolerate anoxia better and survive longer periods of hypoxia and anoxia exposure (Malison et al., 2020). Metabolic rates of *Isocapnia* dropped initially and did not increase towards pre-

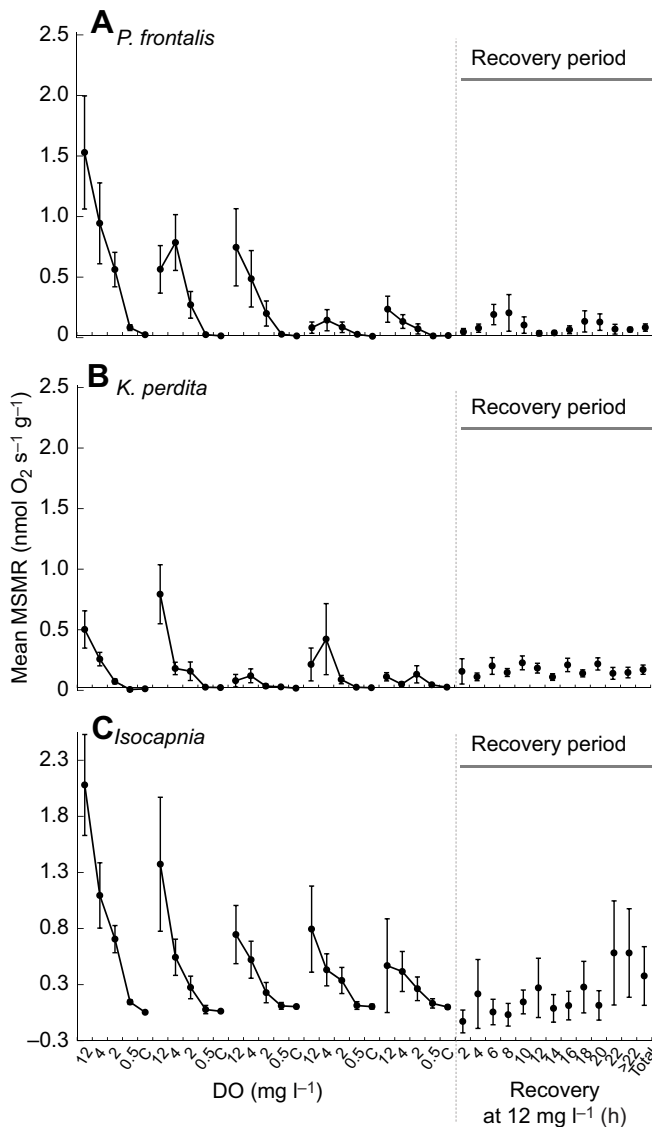


Fig. 8. MSMRs for week-long repeated normoxia and extreme hypoxia trials for aquifer species. (A) *P. frontalis* ($n=8$), (B) *K. perditia* ($n=11$) and (C) *Isocapnia* spp. ($n=8$). Metabolic rates were measured at 12, 4, 2 and 0.5 mg l⁻¹ prior to a closed period (C) during which organisms depleted oxygen overnight (to 0–0.7 mg l⁻¹ for ~12 h). This was repeated for 5 days and measurements for each day-long replicate are connected by lines. Mean \pm s.e.m. MSMRs were measured during the recovery period for each 2 h of recovery (up to 22 h), for the period greater than 22 h and for the total recovery period.

exposure levels until a few hours into the recovery period following repeated exposure. In contrast, the metabolic rates of *P. frontalis* varied more, returning towards pre-exposure levels multiple times as oxygen concentrations increased following each drawdown.

In contrast, longer-term (12 h) repeated exposure to hypoxia depressed survival and metabolic rates of all three aquifer taxa. *K. perditia* had the highest survival rates followed by *P. frontalis* and *Isocapnia*. *K. perditia* may have experienced less oxidative damage with each return to normoxia than the other two species because metabolic rates remained more depressed throughout the week-long experiment. In contrast, with each return to normoxia, metabolic rates of *Isocapnia* were 1.7–12.1 times higher than metabolic rates of *K. perditia*. Repeated recovery from anoxia results in a set of physiological challenges with each recovery (see Woods and Lane, 2016). Repeatedly reestablishing energy and ion homeostasis, and

repeated oxidative damage to cellular functions likely causes stress and lowers survival. These results parallel thermal literature showing insects that are adapted to withstand freezing have increased mortality when they experience multiple freeze–thaw cycles (Marshall and Sinclair, 2011).

The alluvial aquifer of the Nyack floodplain contains abundant populations of aquifer stoneflies that are top consumers of this underground food web. The aquifer stoneflies are found in hypoxic and anoxic zones in the alluvial aquifer, they have anoxia-tolerant phenotypes allowing them to tolerate short-term repeated exposure to hypoxia and also survive longer periods of anoxia. The ability to tolerate repeated short-term exposure to hypoxia provides another line of evidence that these taxa can exploit hot spots of productivity in alluvial aquifers. Aquifer stoneflies almost certainly eat at oxic–anoxic interfaces within aquifers. This idea is supported by previous observations that stonefly biomass appears to be derived from methane and that stonefly guts contain both anaerobic methanogenic and aerobic methanotrophic bacteria (DelVecchia et al., 2016, 2019). It is also supported by the data we present above, as well as other data on anoxia tolerance (Malison et al. 2020). These adaptations likely enable aquifer stoneflies to exploit the rich methane-derived carbon sources found in anoxic zones and help to explain how unconventional carbon sources in alluvial aquifers of river floodplains may be fundamental in supporting diverse and productive food webs.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: R.L.M., A.G.D., B.K.H., G.L., J.A.S.; Methodology: R.L.M., H.W.; Formal analysis: R.L.M., A.G.D., H.W.; Investigation: R.L.M.; Writing - original draft: R.L.M., A.G.D., H.W.; Writing - review & editing: R.L.M., A.G.D., H.W., B.K.H., G.L., J.A.S.; Funding acquisition: B.K.H., G.L., J.A.S.

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Supplementary information

Supplementary information available online at <https://jeb.biologists.org/lookup/doi/10.1242/jeb.225623.supplemental>

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Table S1: Pairwise comparison results for Two-way ANOVA for the interaction of taxa and oxygen level. Oxygen levels (mg/L) are represented by letters (A = 12, B = 8, C = 6, D = 4, E = 2, F = 0.5, G = <0.5 (closed period), and H = 12 (recovery period)). Significant p-values are in bold.

Contrast	Estimate	SE	df	t.ratio	p.value
A,Aquifer - B,Aquifer	0.13372	0.1066	824	1.255	0.9972
A,Aquifer - C,Aquifer	0.10954	0.1066	824	1.028	0.9997
A,Aquifer - D,Aquifer	0.21281	0.1066	824	1.997	0.8267
A,Aquifer - E,Aquifer	0.27159	0.1066	824	2.548	0.437
A,Aquifer - F,Aquifer	0.46244	0.1066	824	4.339	0.0017
A,Aquifer - G,Aquifer	0.76634	0.1242	824	6.172	<.0001
A,Aquifer - H,Aquifer	0.35426	0.1066	824	3.324	0.0706
A,Aquifer - A,Benthic	-1.2503	0.1	824	-12.499	<.0001
A,Aquifer - B,Benthic	-0.7791	0.1019	824	-7.647	<.0001
A,Aquifer - C,Benthic	-0.5209	0.1019	824	-5.113	<.0001
A,Aquifer - D,Benthic	-0.3197	0.1	824	-3.196	0.1022
A,Aquifer - E,Benthic	0.09336	0.1	824	0.933	0.9999
A,Aquifer - F,Benthic	0.46609	0.1	824	4.659	0.0004
A,Aquifer - G,Benthic	0.78823	0.1031	824	7.643	<.0001
A,Aquifer - H,Benthic	-0.5484	0.104	824	-5.271	<.0001
B,Aquifer - C,Aquifer	-0.0242	0.1066	824	-0.227	1
B,Aquifer - D,Aquifer	0.07909	0.1066	824	0.742	1
B,Aquifer - E,Aquifer	0.13787	0.1066	824	1.294	0.9961
B,Aquifer - F,Aquifer	0.32872	0.1066	824	3.084	0.1381
B,Aquifer - G,Aquifer	0.63262	0.1242	824	5.095	<.0001
B,Aquifer - H,Aquifer	0.22054	0.1066	824	2.069	0.7842
B,Aquifer - A,Benthic	-1.384	0.1	824	-13.836	<.0001
B,Aquifer - B,Benthic	-0.9128	0.1019	824	-8.96	<.0001
B,Aquifer - C,Benthic	-0.6546	0.1019	824	-6.426	<.0001
B,Aquifer - D,Benthic	-0.4534	0.1	824	-4.533	0.0007
B,Aquifer - E,Benthic	-0.0404	0.1	824	-0.403	1
B,Aquifer - F,Benthic	0.33237	0.1	824	3.323	0.0709
B,Aquifer - G,Benthic	0.65451	0.1031	824	6.346	<.0001
B,Aquifer - H,Benthic	-0.6821	0.104	824	-6.556	<.0001
C,Aquifer - D,Aquifer	0.10327	0.1066	824	0.969	0.9999
C,Aquifer - E,Aquifer	0.16205	0.1066	824	1.521	0.98

C,Aquifer - F,Aquifer	0.35289	0.1066	824	3.311	0.0734
C,Aquifer - G,Aquifer	0.6568	0.1242	824	5.29	<.0001
C,Aquifer - H,Aquifer	0.24472	0.1066	824	2.296	0.6267
C,Aquifer - A,Benthic	-1.3599	0.1	824	-13.594	<.0001
C,Aquifer - B,Benthic	-0.8886	0.1019	824	-8.722	<.0001
C,Aquifer - C,Benthic	-0.6305	0.1019	824	-6.188	<.0001
C,Aquifer - D,Benthic	-0.4292	0.1	824	-4.291	0.0021
C,Aquifer - E,Benthic	-0.0162	0.1	824	-0.162	1
C,Aquifer - F,Benthic	0.35655	0.1	824	3.564	0.0331
C,Aquifer - G,Benthic	0.67869	0.1031	824	6.581	<.0001
C,Aquifer - H,Benthic	-0.6579	0.104	824	-6.324	<.0001
D,Aquifer - E,Aquifer	0.05878	0.1066	824	0.552	1
D,Aquifer - F,Aquifer	0.24963	0.1066	824	2.342	0.592
D,Aquifer - G,Aquifer	0.55353	0.1242	824	4.458	0.001
D,Aquifer - H,Aquifer	0.14145	0.1066	824	1.327	0.9949
D,Aquifer - A,Benthic	-1.4631	0.1	824	-14.627	<.0001
D,Aquifer - B,Benthic	-0.9919	0.1019	824	-9.736	<.0001
D,Aquifer - C,Benthic	-0.7337	0.1019	824	-7.202	<.0001
D,Aquifer - D,Benthic	-0.5325	0.1	824	-5.323	<.0001
D,Aquifer - E,Benthic	-0.1195	0.1	824	-1.194	0.9984
D,Aquifer - F,Benthic	0.25328	0.1	824	2.532	0.4489
D,Aquifer - G,Benthic	0.57542	0.1031	824	5.58	<.0001
D,Aquifer - H,Benthic	-0.7612	0.104	824	-7.317	<.0001
E,Aquifer - F,Aquifer	0.19084	0.1066	824	1.791	0.9194
E,Aquifer - G,Aquifer	0.49475	0.1242	824	3.985	0.0073
E,Aquifer - H,Aquifer	0.08267	0.1066	824	0.776	1
E,Aquifer - A,Benthic	-1.5219	0.1	824	-15.214	<.0001
E,Aquifer - B,Benthic	-1.0507	0.1019	824	-10.313	<.0001
E,Aquifer - C,Benthic	-0.7925	0.1019	824	-7.779	<.0001
E,Aquifer - D,Benthic	-0.5913	0.1	824	-5.911	<.0001
E,Aquifer - E,Benthic	-0.1782	0.1	824	-1.782	0.9224
E,Aquifer - F,Benthic	0.1945	0.1	824	1.944	0.8544
E,Aquifer - G,Benthic	0.51664	0.1031	824	5.01	0.0001
E,Aquifer - H,Benthic	-0.82	0.104	824	-7.882	<.0001
F,Aquifer - G,Aquifer	0.3039	0.1242	824	2.448	0.5119
F,Aquifer - H,Aquifer	-0.1082	0.1066	824	-1.015	0.9998
F,Aquifer - A,Benthic	-1.7127	0.1	824	-17.122	<.0001
F,Aquifer - B,Benthic	-1.2415	0.1019	824	-12.186	<.0001
F,Aquifer - C,Benthic	-0.9834	0.1019	824	-9.652	<.0001
F,Aquifer - D,Benthic	-0.7821	0.1	824	-7.819	<.0001
F,Aquifer - E,Benthic	-0.3691	0.1	824	-3.69	0.0216

F,Aquifer - F,Benthic	0.00365	0.1	824	0.037	1
F,Aquifer - G,Benthic	0.32579	0.1031	824	3.159	0.1131
F,Aquifer - H,Benthic	-1.0108	0.104	824	-9.716	<.0001
G,Aquifer - H,Aquifer	-0.4121	0.1242	824	-3.319	0.0717
G,Aquifer - A,Benthic	-2.0167	0.1186	824	-17.006	<.0001
G,Aquifer - B,Benthic	-1.5454	0.1201	824	-12.862	<.0001
G,Aquifer - C,Benthic	-1.2873	0.1201	824	-10.714	<.0001
G,Aquifer - D,Benthic	-1.086	0.1186	824	-9.158	<.0001
G,Aquifer - E,Benthic	-0.673	0.1186	824	-5.675	<.0001
G,Aquifer - F,Benthic	-0.3003	0.1186	824	-2.532	0.449
G,Aquifer - G,Benthic	0.02189	0.1212	824	0.181	1
G,Aquifer - H,Benthic	-1.3147	0.122	824	-10.778	<.0001
H,Aquifer - A,Benthic	-1.6046	0.1	824	-16.041	<.0001
H,Aquifer - B,Benthic	-1.1333	0.1019	824	-11.124	<.0001
H,Aquifer - C,Benthic	-0.8752	0.1019	824	-8.59	<.0001
H,Aquifer - D,Benthic	-0.674	0.1	824	-6.737	<.0001
H,Aquifer - E,Benthic	-0.2609	0.1	824	-2.608	0.3942
H,Aquifer - F,Benthic	0.11183	0.1	824	1.118	0.9992
H,Aquifer - G,Benthic	0.43396	0.1031	824	4.208	0.003
H,Aquifer - H,Benthic	-0.9026	0.104	824	-8.676	<.0001
A,Benthic - B,Benthic	0.47125	0.095	824	4.96	0.0001
A,Benthic - C,Benthic	0.7294	0.095	824	7.677	<.0001
A,Benthic - D,Benthic	0.93062	0.093	824	10.004	<.0001
A,Benthic - E,Benthic	1.34367	0.093	824	14.444	<.0001
A,Benthic - F,Benthic	1.71639	0.093	824	18.451	<.0001
A,Benthic - G,Benthic	2.03853	0.0964	824	21.158	<.0001
A,Benthic - H,Benthic	0.70194	0.0973	824	7.213	<.0001
B,Benthic - C,Benthic	0.25815	0.097	824	2.663	0.3568
B,Benthic - D,Benthic	0.45938	0.095	824	4.835	0.0002
B,Benthic - E,Benthic	0.87243	0.095	824	9.182	<.0001
B,Benthic - F,Benthic	1.24515	0.095	824	13.105	<.0001
B,Benthic - G,Benthic	1.56729	0.0983	824	15.949	<.0001
B,Benthic - H,Benthic	0.2307	0.0992	824	2.325	0.6049
C,Benthic - D,Benthic	0.20123	0.095	824	2.118	0.7532
C,Benthic - E,Benthic	0.61428	0.095	824	6.465	<.0001
C,Benthic - F,Benthic	0.987	0.095	824	10.388	<.0001
C,Benthic - G,Benthic	1.30914	0.0983	824	13.322	<.0001
C,Benthic - H,Benthic	-0.0275	0.0992	824	-0.277	1
D,Benthic - E,Benthic	0.41305	0.093	824	4.44	0.0011
D,Benthic - F,Benthic	0.78577	0.093	824	8.447	<.0001
D,Benthic - G,Benthic	1.10791	0.0964	824	11.499	<.0001

D,Benthic - H,Benthic	-0.2287	0.0973	824	-2.35	0.5863
E,Benthic - F,Benthic	0.37272	0.093	824	4.007	0.0067
E,Benthic - G,Benthic	0.69486	0.0964	824	7.212	<.0001
E,Benthic - H,Benthic	-0.6417	0.0973	824	-6.594	<.0001
F,Benthic - G,Benthic	0.32214	0.0964	824	3.343	0.0666
F,Benthic - H,Benthic	-1.0145	0.0973	824	-10.424	<.0001
G,Benthic - H,Benthic	-1.3366	0.1005	824	-13.3	<.0001