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Winter Survival, Habitat Use, and Hypoxia Tolerance of Montana Arctic Grayling in a Winterkill-Prone Lake

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**Abstract.**- Winter hypoxia in shallow, ice-covered lakes can be a significant limiting factor for overwintering fish populations. In this study we tested the hypothesis that low overwinter survival due to winter hypoxia is a limiting factor for a rare, adfluvial population of native Arctic Grayling (*Thymallus arcticus*) inhabiting Upper Red Rock Lake, Montana. We used a combined laboratory and telemetry study to document extent of hypoxia over two winters and assess the physiological tolerance, behavioral response, and winter survival in relation to hypoxia. In the laboratory, we observed a significant behavioral and physiological response to dissolved oxygen (DO) levels  $\leq 4$  mg/L and determined acute 24-hr LD<sub>50</sub> values of 0.75 mg/L for adults and 1.50 – 1.96 mg/L for juveniles at temperatures of 1 – 3°C. In the field study we observed dynamic DO concentrations (DO  $< 1$  -10 mg/L) during winter ice cover, ranging from persistent near-anoxic conditions near the bottom to DO concentrations  $> 4$  mg/L in the epilimnion. Radiotelemetry indicated adult winter survival rate was high (0.97 in 2014, 0.95 in 2015) and that telemetered fish selected deeper ( $> 1$ m), more oxygenated habitat during ice cover. Our study demonstrated that Arctic Grayling have a high tolerance to acute hypoxia exposure and exhibit a physiological and behavioral stress response to DO concentrations  $\leq 4$  mg/L. Although hypoxia was present in parts of the lake, sufficient suitable habitat  $> 4$  mg/L was available in the lake epilimnion in both study winters. However, winter conditions more severe than those observed during our two-year study occur periodically in the lake and thus winterkill could still be a limiting factor for the population.

## Introduction

Ice-covered lakes can exhibit pronounced spatial and temporal variability in dissolved oxygen (DO) availability (Greenbank 1945; Agbeti & Smol 1995; Hasler et al. 2009; Leppi et al. 2016). A condition of low oxygen, or hypoxia, in winter is a common phenomenon in these systems and results from persistent snow and ice cover, which prevents or greatly limits atmospheric gas exchange and photosynthetic production (Mathias and Barica 1980). If ice cover persists for a sufficient duration, the consumption of DO by microbial decomposition of organic matter may deplete DO to the point of hypoxia (Mathias and Barica 1980; Bertilsson et al. 2013). Lake depth, duration and thickness of ice cover, macrophyte abundance, and presence or absence of inlet streams have been shown to

influence the degree of winter hypoxia (Mathias and Barica 1980; Guenther and Hubert 1991; Leppi et al. 2016). In many northern temperate lakes, the oxygen-limiting period only ends with the initiation of atmospheric re-aeration and photosynthetic DO production associated with ice break-up in spring (Barica & Mathias 1979).

Obtaining oxygen during winter hypoxia can place high demands on the physiology of overwintering fishes. Severe or prolonged hypoxia can be a major cause of winter mortality (Greenbank 1945; Hughes 1973), which in turn, can be a major factor limiting species abundance and persistence in ice-covered lakes (Danylchuk & Tonn 2003). Fish may respond to hypoxia by avoiding hypoxic areas (Whitmore et al. 1960; Skjaeraasen et al. 2008; Poulsen et al. 2011), resulting in vertical or horizontal shifts in distribution (Magnuson et al. 1985; Kramer 1987; Eby and Crowder 2002; Hasler et al. 2009), or migration out of lakes into comparatively well-oxygenated streams for the winter (Magnuson et al. 1985). Fish also exhibit a physiological response to winter hypoxia by increasing oxygen uptake via mechanisms such as increased ventilation rate and increased blood perfusion of the gills (Booth 1979; Nilsson 2007), or by reducing oxygen demand via reduced activity (Kramer 1987). Winter hypoxia tolerance varies widely among species (Davis 1975; Landman et al. 2005), which can result in differences in species composition in lakes with high winter hypoxia risk (Tonn & Magnuson 1982).

Past studies on the effects of winter hypoxia have generally been either laboratory hypoxia tolerance studies (Petrosky & Magnuson 1973; Furimsky et al. 2003; Landman et al. 2005; Poulsen et al. 2011) or field studies examining changes in distribution and survival in relation to DO availability (Magnuson et al. 1985; Danylchuk & Tonn 2003). Laboratory studies have focused primarily on acute response to hypoxia and estimation of hypoxia thresholds, whereas field studies have focused on characterizing the extent and severity of hypoxia in the wild. A combined laboratory and field study design can be a powerful tool for identifying acute responses to winter hypoxia, its spatial and temporal extent within a system, and associated fish behavioral and survival responses to low DO during winter in natural conditions (Hasler et al. 2009).

The purpose of this study was to evaluate the response of Arctic Grayling (*Thymallus arcticus*) to winter hypoxia in Upper Red Rock Lake, Montana. Montana Arctic Grayling are a now rare, glacial relict species occupying only a fraction of their historic range (Liknes & Gould 1987; Kaya 1992). The population in Upper Red Rock Lake is the only significant remaining native, adfluvial population, is genetically distinct from other Montana Arctic Grayling (Peterson & Ardren 2009), and has undergone significant declines in abundance and distribution (Nelson 1954; Kaya 1992; Warren et al. 2017). Previous studies have documented instances of very low winter DO in the lake and poor overwinter survival due to winter hypoxia has been hypothesized as a potential limiting factor for this population (Vincent 1962; Mogen 1996; Gangloff 1996). In this study, we used complementary laboratory and field investigations (*sensu* Hasler et al. 2009) to test the winter hypoxia hypothesis for Arctic Grayling. We used laboratory experiments to define stressful and lethal DO concentrations and field measurements to estimate overwinter survival, characterize winter habitat use, and examine the extent, timing, and severity of hypoxia in Upper Red Rock Lake.

## Methods

### Laboratory Study

Hypoxia tolerance of Arctic Grayling was tested in both adults and juveniles to determine life stage-specific stressful and lethal DO thresholds. Adult Arctic Grayling (n = 50, 246 – 324 mm total length; 201 – 328 g) were collected from Axolotl Lake, near Ennis, Montana (45°14' 2" N, 111° 52' 24" W), using short-term (~ 1 h) gill net sets. Axolotl Lake Grayling are a broodstock population of fluvial Big Hole River origin, and were used in place of Red Rock grayling since the size of the Upper Red Rock Lake population was insufficient to allow for collection of adults. The Big Hole River is in the same upper Missouri River drainage as Upper Red Rock Lake (Kaya 1992; Peterson and Ardren 2009). Adult Arctic Grayling were transported to the Bozeman Fish Technology Center and held at 8°C for a minimum of 2 months prior to testing in a 3.0 m x 1.0 m x 1.0 m flow-through tank supplied by well water and fed daily via a belt feeder with 3.5 mm floating Classic Trout diet (Skretting, Tooele, UT). Juvenile Arctic Grayling used in testing (age 0, 147 – 162 mm) were obtained as embryos in May 2014 from adults spawning in Red Rock Creek, a main spawning tributary for Arctic Grayling from Upper Red Rock Lake. Fertilized embryos were hatched in a flow-through vertical incubator (MariSource, Fife, WA), transferred to a flow-through, 150 L round tank after hatching and supplied with 10 – 12°C well water to enhance growth until the desired test size was achieved. Fry were initially fed a starter diet (Otohime Beta, Nisshin Feed Co. Ltd. Tokyo), followed by pelleted trout feed. Fry were transferred to a 3.0 m x 1.0 m x 1.0 m flow-through tank at 8°C after reaching 60 – 70 mm and acclimated at these conditions for ~2 mo prior to testing.

*Test apparatus and procedure.*- A specialized apparatus for chilling and deoxygenating recirculating water was constructed to assess behavioral and physiological response to hypoxia and determine lethal DO thresholds under simulated winter conditions. Deoxygenated water was attained by circulating water through a custom-built, sealed, packed-column cylinder (30.5 x 91.4 cm) filled with 5-cm-diameter plastic random packing (Koch-Glitsch, Wichita, KS). DO was removed from cascading water by exposure to a high vacuum from a vacuum generator (Air-Vac, Seymour, CT) attached to the packed column. Desired DO test concentrations were achieved by adjusting the vacuum pressure via a bleeder valve on the column, as determined from pre-trial measurements. Degassing using a vacuum column is a common treatment of hatchery well water at risk of gas bubble disease from nitrogen supersaturation (Marking 1987). We used this approach to deoxygenate water in our study so as to reduce the possibility of an additive effect of nitrogen supersaturation during testing of responses to hypoxia, which might be present when the more common nitrogen infiltration method of deoxygenation is used in hypoxia experiments (see also Landman and van den Heuvel 2003; Landman et al. 2005). Water temperatures as low as 1°C were achieved by pumping deoxygenated water through a stainless steel heat exchanger coil submerged in chilled brine solution inside an insulated reservoir. The brine solution was chilled by a 1.5 hp Deltastar chiller (Aqualogic, San Diego, CA), optimally tuned for performance at low water temperatures. A 1.5 hp external pump (Goulds jet pump; Xylem, Rye Brook, NY) was used to recirculate water through the system. During trials, chilled, deoxygenated water was recirculated to six circular 100 L insulated test tanks (45 cm deep x 60 cm diameter). Clear, rigid 0.3-cm-thick polycarbonate covers were positioned just below the water surface to simulate lake ice and prevent test fish from jumping out of the tank. Tanks were also covered with a one-way mirror, positioned slightly off horizontal, to shield fish from observers; fish did not react to observer presence when mirrors were in place. The DO concentration in the test tanks was monitored with two electronic DO probes (YSI Professional Plus, Yellow Spring, OH), one

positioned in the line supplying water to the tanks, the other placed in the line returning water from the tanks to the chiller/vacuum column assembly. During all tests, inflow and outflow DO concentrations remained within 0.1 mg/L of target concentration. Water temperature was adjusted by regulating chiller output using a digital temperature controller (Aqualogic, San Diego, CA). The apparatus delivered a range of temperatures (1-7°C) and DO concentrations (0.5 - 10 mg/L) with a high level of precision ( $\pm 0.1^\circ\text{C}$ ,  $\pm 0.10$  mg/L DO), enabling us to simulate the low DO and low temperature environmental conditions present in Upper Red Rock Lake during the winter. However, a limitation of the test apparatus was that the combination of very low DO and temperature could only be maintained for relatively short periods as initial testing showed a high risk of pump failure from overheating and for ice build-up on the chiller unit (reducing efficiency) when test periods extended beyond a few days. Thus, we were limited to testing for short-term acute responses to hypoxia with relatively short acclimation periods.

Behavioral and physiological responses to progressive hypoxia in juvenile and adult Arctic Grayling were tested at two common winter temperatures of 1°C and 3°C. At the start of a trial, fish were netted haphazardly from holding tanks (8°C) and placed individually into the 6 test tanks. For the 1°C trials, only 3 tanks were tested at a time since a reduced volume of water was required to maintain such a low test temperature. At 3°C, we performed 4 trials with adults ( $n = 6$  per trial  $\times$  4 trials = 24 fish tested) and 6 trials with juveniles ( $n = 36$ ). At 1°C, we performed 4 trials with adults ( $n = 12$ ) and 4 trials with juveniles ( $n = 12$ ). After introduction of test fish to test tanks, water temperatures were reduced by 1°C/h by adjusting the volume of water circulating through the chiller until the 1°C or 3°C test temperature was achieved. Once the test temperature was reached, fish were maintained at the final test temperature for a 24-h holding period. At the end of acclimation, progressive hypoxia was then induced via a stepwise reduction in DO over a 9 – 11 h test period, depending on final DO level. Stepwise reduction in DO was accomplished using a series of 2-h-long DO reduction periods. For the first hour, DO was reduced gradually to the next lowest target level by increasing the vacuum within the packed column; in the second hour, DO was held constant. Stepwise reduction occurred at each of progressively lower DO levels of 10.0, 6.0, 4.0, 2.0, 1.0 and, in adult trials only, 0.5 mg/L ( $\pm 0.1$  mg/L).

Physiological response to DO reduction was measured by counting ventilation rate (opercular beats/min; Flint et al. 2015) and behavioral response was assessed by recording whether the majority of the observation time was spent above the midline of the tank or below (coded 0 or 1). Both measurements were made during a 2-minute observation period for each test fish at the conclusion of each 2-hr exposure period. Increased ventilation rate and movement to the water surface in search of higher DO levels are common responses to hypoxia among fishes (Hasler et al. 2009; Barreto & Volpato 2011). At the end of the stepwise DO reduction period of each trial, fish were then held at the target DO concentration for 24 h to determine the acute lethal threshold. Test concentrations included the range of DO levels bracketing a lethal threshold between 0% and 100% mortality (actual mortality or loss of equilibrium) observed in initial, short-term trials. For adults, 24-hr exposures were conducted at 1.0 and 0.5 mg/L at both test temperatures. For juveniles, 24-hr exposures were conducted at 2.0 and 1.0 mg/L at both test temperatures and also at 4.0 mg/L at 3°C

only. The additional 4.0 mg/L – 3°C treatment for juveniles was necessary to achieve a 100% survival level for calculation of the acute lethal threshold. Test fish were used only once in trials. Chronic lethal DO threshold for adults was also determined in separate 96-hr exposure trials at 3°C and 1.0 mg/L (2 trials,  $n = 12$ ). For the 96-hr chronic test, the same stepwise DO reduction protocol describe above was used, except that the final exposure period was lengthened from 24 to 96 h. Juvenile trials were conducted during winter (December to March) and adult trials during spring (May to June) using the natural, seasonal photoperiod present at the time of the trials.

*Data analysis.*- Ventilation rate data were first assessed for normality, with adult data exhibiting a normal distribution and juvenile data requiring rank transformation; thus, adult and juvenile ventilation rate data were examined separately. Adult ventilation rate response to all combinations of DO concentration and temperature level was assessed using mixed effects models with individual fish ventilation rate as the random effect, and DO and water temperature as fixed effects. For adult ventilation data, at each test temperature we used ANOVA with orthogonal contrasts and identified the DO concentrations at which adult Arctic Grayling exhibited a significant difference ( $p \leq 0.05$ ) in mean ventilation rate compared to the control DO level (10 mg/L). For juvenile ventilation data, the rank transformation precluded use of mixed effects models. Instead, a one-way repeated measures test including DO, water temperature, and the interaction of DO and water temperature was applied. Surfacing time data was examined using mixed effects logistic regression with individual as the random effect, and DO, water temperature, and life stage (adult vs. juvenile) as fixed effects. Comparison of all models was performed using Akaike's information criterion adjusted for small sample size (*AICc*; Burnham & Anderson 2002; Compton et al. 2002). We considered the model with the lowest value to be the best and evaluated the plausibility of other models based on the difference between the information criteria value for the top model and that of every other model ( $\Delta AICc$ ). Following recommendations of Burnham and Anderson (2002), models with  $\Delta AICc$  values  $<2$ ,  $2-10$ , and  $>10$ , were considered to have substantial, some, and essentially no evidence of support, respectively, as a plausible model (Burnham & Anderson 2002).

We applied generalized linear models (Kerr & Meador 1996) to the survival data to calculate a) the acute lethal DO concentration ( $LC_{50}$ ) (Davison et al. 1959; Wagner et al. 2001) for juvenile and adult Arctic Grayling at each test temperature, and b) the chronic exposure threshold time (time to 50% mortality,  $LT_{50}$ ) for adults during the 96-h trials. All data analyses were conducted using program R version 3.0.2 (R Development Core Team 2014).

#### Field Study

*Study site.*- Upper Red Rock Lake lies in the high elevation (2,030 m) Centennial Valley in the Upper Missouri River Basin of southwest Montana (Figure 1). The 893 ha lake is surrounded by extensive wetlands with predominantly emergent sedge (*Carex*) lining wetland margins, and lies within Red Rock Lakes National Wildlife Refuge. The lake is fed by five streams, with Red Rock Creek contributing the largest volume of surface water (Gangloff 1996; Warren et al. 2017). Flows exit the lake through a single outlet that connects Upper Red Rock Lake to the much shallower Lower Red Rock Lake and Red Rock River. Grayling are not known to inhabit the lower lake during winter. Upper Red Rock Lake exhibits many characteristics typical of a winterkill-prone lake. The lake experiences long winters, typically remaining ice-covered from October to April. Depth is shallow and rather

uniform, with a maximum depth of only 2 m. There are abundant rooted macrophytes (*Potamogeton*), on a uniform bottom comprised of mud, peat, and detritus (Gangloff 1996; Mogen 1996). The lake supports an abundant fish assemblage, including native Arctic Grayling, Burbot (*Lota lota*), White Sucker (*Catostomus commersonii*), Longnose Sucker (*Catostomus catostomus*), Longnose Dace (*Rhinichthys cataractae*), and Mottled Sculpin (*Cottus bairdi*), and nonnative Brook Trout (*Salvelinus fontinalis*), and Cutthroat-Rainbow Trout hybrids (*Oncorhynchus clarkii bouvieri* x *Oncorhynchus mykiss*) (Gangloff 1996).

**Lake Sampling.**- To assess the extent, duration, and annual variability in winter hypoxia, DO measurements were made every 2-4 weeks during the winters of 2013-14 and 2014-15 from before ice-up (October) until travel on lake ice became unsafe prior to break up (April). This resulted in 14 sampling occasions spread across two separate winters, 8 occasions in 2013-14 and 6 occasions in 2014-15. Fifteen sample sites were generated randomly for each sampling occasion using the Create Random Points tool in ArcGIS (ver. 10.2; ESRI, Redlands, CA) and included both mid-lake (10 sites) and stream mouth (5 sites) limnetic types. Sample locations were stratified to detect anticipated differences between DO concentration in the lake and in areas  $\leq 300$  m of stream mouths (Gangloff 1996). Sample size was determined by the maximum number of sites that could be reliably and safely sampled in a day, given frequent severe winter conditions. Holes were drilled in the ice with a 10-cm-diameter hand auger at each sample location and DO (mg/L) and water temperature (to nearest  $\pm 0.1^\circ\text{C}$ ) were measured at 1-m intervals from the ice-water interface to the bottom using an electronic DO probe (YSI Professional Plus, Yellow Spring, OH). The DO probe was calibrated at the start of each sampling day using the recommended procedure with water-saturated air. Lake habitat covariates of ice thickness, snow depth, and water depth were also measured at each sample site using a 3-m-long extendable measuring rod. A high-resolution bathymetric map of the lake was created by measuring depths prior to winter with a boat-mounted Intelliducer transom mount sensor (Garmin, Olathe, KS) along transects spaced 150 m apart.

**Radiotelemetry.**- Survival and distribution of Arctic Grayling during winter ice cover was measured with radiotelemetry. In September of each study year  $\sim 1$  month prior to surface ice formation, 49 adult Arctic Grayling (261 – 431mm; 220 – 765 g) were captured using short-term ( $\sim 1$  h) gillnet sets and implanted with radio transmitters fitted with mortality (motion) sensors and external antennas (Lotek MCFT2-3BM, 11 x 43mm, Newmarket, Ontario, Canada). Tag weight (8 g) was 1 – 3.6 % of body weights. During implantation, fish were anesthetized with MS-222, and tags positioned anterior to the pelvic girdle using the shielded needle technique (Ross & Kleiner 1982). Following recovery in holding pens ( $\sim 30$  min), tagged fish were released back into the lake.

Telemetry surveys coincided with lakewide DO sampling, occurring every 2 – 4 weeks during winter ice cover. To locate fish, two surveyors, each with a telemetry receiver, skied 100 – 300 m apart during daylight hours on the ice in north-south transects across the lake until the entire lake was sampled. Fish were located to within 10 m (confirmed by relocation of test transmitters under the ice), their live/dead status noted via telemetry signal, and coordinates recorded using handheld GPS

(Garmin GPSmap 60csx, Olathe, KS). DO, water temperature, and habitat covariates were measured using the same protocol as described above at a random subset of 10 fish relocations during each sampling occasion (~ one-third of the total number of fish relocations per sample period) for use in assessing habitat selection.

*Data analysis.*- At each sample site, DO at 0 and 1 m were averaged and input into ArcGIS. Only data from the upper 1 m of the water column were used as it was the only well-oxygenated zone in the lake in both winters. The lower meter of the water column was consistently hypoxic soon after complete ice cover (mean DO < 2.0 mg/L in 2013-14, < 3.0 mg/L in 2014-15). DO levels in this zone coincided with levels that elicited physiological stress and behavioral avoidance in our laboratory studies and we therefore assumed they would not be likely to be selected by Arctic Grayling during winter ice cover. Maps of spatial distribution of DO concentration in the upper meter were generated for each sampling occasion during the ice cover period using the inverse distance weighted interpolation (IDW) tool in ArcMap Spatial Analyst based on interpolated raster datasets of 20 m x 20 m grid cells of DO concentration. Accuracy of predicted DO from spatial mapping was assessed by comparing predicted DO to an independent set of DO measurements conducted 1 – 2 days after initial field measurements (10 sites per sample date, ranging from 0.5 – 10.8 mg/L DO) using simple linear regression. There was a strong association between predicted and measured DO values ( $r = 0.85$ ,  $n = 120$ ;  $p < 0.01$ ), indicating that maps generated using IDW provided good approximations of the spatial variation in DO present in the lake at each sampling occasion.

Overwinter survival for each of the two study years was estimated using Kaplan – Meier survivorship curves (Kaplan & Meier 1958) using bimonthly data on the number of tagged fish alive or dead. We focused our analysis on the period from date of complete ice cover to lake-wide DO rebound to determine the potential effect of hypoxia and winter conditions on survival and to allow for a post-surgery recovery period (~ 1 mo. prior to lake ice formation). Fish were censored from analysis if their transmitter signal went undetected and was not detected on subsequent sampling occasions (Pollock et al. 1989).

Winter habitat selection was assessed in two ways. First, paired logistic regression was used to compare habitat covariates between fish locations and randomly generated locations on each sampling occasion. For both fish and random locations, habitat covariate measurements from the nearest lake sampling point were used to assign habitat covariate values (Breslow & Day 1980; Hosmer & Lemeshow 1989). Comparisons of the different paired logistic regression models of habitat selection were performed using AIC.

We further analyzed winter habitat selection of Arctic Grayling by assessing if fish used habitat in proportion to availability for two different categories of DO and two different categories of lake depth for each sampling occasion during the midwinter DO minima of both winters. For DO, DO measurements were categorized as either unsuitable (0 - 4 mg/L) or suitable (> 4.0 mg/L) winter habitat. These categories were chosen based on results of the laboratory study that demonstrated

increased stress at DO levels below 4 mg/L DO (see below). The proportional area of each DO category was quantified for each sampling occasion using ArcGIS. Fish relocations at each sampling occasion during the midwinter DO minima were then plotted on maps of DO availability and habitat use calculated as the proportion of relocations made within each DO category (Manly et al. 2002). For depth, we first generated a lake depth raster using the Triangular Irregular Network tool in ArcGIS. We then divided available lake depths into categories (0-1 m and > 1 m) and plotted fish locations and compared use versus availability using the aforementioned protocol. Chi-square tests were used to test whether use was in proportion to availability for DO and depth categories for each sampling occasion (Zar 1999). Standardized selection indices with 95% Bonferroni confidence intervals were also calculated to show the level of selection for each DO category and depth category by each sample date, with indices >1 indicating preference, and indices <1 indicating avoidance, by telemetered fish (Manly et al. 2002).

## Results

### Laboratory Study

*Physiological and Behavioral Response to Hypoxia.*- At 3°C, ventilation rate of adult Arctic Grayling averaged 50 beats/min at the control DO level of 10.0 mg/L. Ventilation rate was similar at 6 mg/L but increased by ~10% at 4 mg/L. At 2 mg/L, mean ventilation rate increased markedly to 66 beats/min (+34% increase from control), and increased further to 75 beats/min at 1 mg/L (Figure 2). At 1°C, adult ventilation rate was ~12% higher than at 3°C at the control DO. As at 3°C, ventilation rate at 1°C was similar at 10 and 6 mg/L but increased 20% at 4 mg/L compared to control levels, and increased further at 2 mg/L. Within the two temperature groups, ventilation rate of adult Arctic Grayling was significantly higher at 4 and 2 mg/L compared to control levels. Similar to adults, juvenile ventilation rate at 3°C increased from control levels (~ 7%) at 4.0 mg/L. At 1°C, ventilation rate at 10.0 mg/L was ~ 22% lower for juveniles than at 3°C, and increased significantly to 81 and 87 beats/min, respectively (8 and 16% increase from control), at 4.0 and 2.0 mg/L. Comparison of all mixed effects linear models of adult ventilation rate indicated that the model including DO and water temperature as fixed effects best fit the data (all other models had  $\Delta AICc > 10$ ), with ventilation rate negatively correlated with both DO and water temperature. Water temperature and the interaction between DO and water temperature significantly influenced juvenile ventilation rate.

Adult Arctic Grayling stayed below the tank midline during 3°C trials except at 1 mg/L when fish averaged 28 of 120 s in the upper half of test tanks. However, during 1°C trials, there was a sharp increase in mean time above the midline at 4 mg/L (114 sec). For juvenile Arctic Grayling, time above the midline at 3°C was ~50 sec (42% of observation period) at 10 and 6 mg/L and decreased slightly at <4 mg/L. In contrast, juvenile surfacing behavior was much more pronounced at 1°C, increasing from about 40 sec (33% of observation period) at 10 and 6 mg/L to ~100 sec (83% of observation period) at 4.0 mg/L. Comparison of all mixed effects logistic regression models of surfacing behavior indicated the model with both water temperature and life stage was most plausible, with all other models having low support ( $\Delta AICc > 10$ ). The odds of rising to the surface for a majority of the observation time was positively correlated with the juvenile life stage and negatively correlated with

water temperature.

*Lethal DO thresholds.*- Survival of adult Arctic Grayling at 1 and 3°C was 100% at DO concentrations of 10 to 1 mg/L, however 0% of fish survived at DO concentrations of 0.5 mg/L at either temperature. The calculated LC<sub>50</sub> for both temperatures (1 and 3°C) was 0.75 mg/L. In contrast, juvenile Arctic Grayling had a higher lethal DO concentration than adults and the lethal response varied with temperature. Juvenile survival at 1 and 3°C was 100% at DO concentrations of 10 to 4 mg/L and 0% at 1 mg/L. Juveniles at 3°C had a slightly lower survival at 2 mg/L than those tested at 1°C (75% vs. 100%). Juvenile LC<sub>50</sub> was 1.96 mg/L at 3°C and 1.50 mg/L at 1°C. Juvenile LC<sub>50</sub> ranged from 0.75 – 1.21 mg/L higher than the lethal level calculated for adults. For the chronic exposure test, adults held at 1 mg/L at 3°C had 100% survival at 24 h, 58% survival at 48 h and 0% survival after 72 h exposure; the calculated LT<sub>50</sub> was 48.3 hours.

#### Field Study

*Extent and timing of hypoxia.* Complete ice cover formed on the lake by November 15 (2013) and by November 25 (2014) and grew to ~ 0.5 m thick in both winters. Ice cover was uniform across the lake throughout the winter except for intermittent, small ice-free areas of open water ( $\leq 100 \text{ m}^2$ ) present near the mouths of Elk Springs, Red Rock, Shambow and Grayling creeks during many sampling occasions. Ice break up began in the first week of April in 2014 and the last week of March in 2015 and ice cover was absent by mid-April of both years.

Lake-wide average DO concentration in October prior to ice formation was 9.0 mg/L and 10.2 mg/L in 2013 and 2014, respectively, and was uniform throughout the lake with little variation with depth and across sample sites. Following ice formation, lake-wide average DO declined to minimum levels of 4.3 mg/L in the winter of 2013-14 and 5.9 mg/L in 2014-15 within 40-50 days after onset of lake ice. DO concentrations were often hypoxic (0-4 mg/L) in the hypolimnetic layer below 1 m throughout the ice cover period. DO concentrations in the > 1 m deep hypolimnetic layer began to increase beginning March 27 in 2014 and February 16 in 2015, about a month prior to ice off in both winters.

DO interpolation maps showed the expansion and contraction of hypoxic areas as well as differences in DO concentration between the two study winters (Figures 3, 4). During the midwinter DO minima period, low DO areas were concentrated, and persistently so, in the shallow northwest and southern parts of the lake, particularly in 2014, when overall DO concentrations were significantly lower than in 2015 ( $p < 0.01$ ). In contrast, in both years, areas near inlet streams and the upper 1 m of the water column remained relatively well-oxygenated during the winter, even during the January-February DO minima period. In winter 2014, the proportional volume of the hypoxic ( $\leq 4 \text{ mg/L}$ ) zone in the upper 1 m of the water column under the ice steadily expanded from 7% in early January to its peak of 31% in late February, followed by a decline to 0% by the end of March. In contrast, the proportional volume of hypoxic areas was ~ 1% in winter 2015, and high DO levels were present in the upper 1 m under the ice throughout the lake by mid-February.

*Fish distribution.*- Generally, Arctic Grayling were widely distributed across the lake in both winters (Figures 3, 4). However, across all sampling occasions, very few fish were relocated near the lake outlet in the northwest corner of the lake. This lake section was characterized by shallow depths and generally had the lowest DO concentrations. Repeated relocations of individual fish during a sampling day and between sampling occasions indicated fish did not remain in fixed locations but moved frequently and over substantial (> 2 km) distances. Fish tended to aggregate near the mouths of inlet tributaries, especially Elk Springs Creek. During the last two sampling occasions in winter 2015, fish were notably absent from the southwest corner of the lake and were concentrated in the northeast corner and its associated inlet tributaries.

*Overwinter survival.*- Kaplan-Meier survival estimates for the overwinter period were calculated using data from individuals alive at the time of complete ice cover each year, 35 in winter 2014 and 38 in winter 2015. We relocated 89 – 100 % of telemetered fish at each sampling occasion and made unsuccessful attempts to relocate the few censored fish (1 in 2014, 2 in 2015) by conducting occasional telemetry surveys in tributary streams within ~ 500 m upstream of the lake. Nearly all fish survived the approximate 5 month overwinter period of ice cover and hypoxia: 0.97 (95% CI: 0.92 – 1.0) in winter 2014 and 0.95 (95% CI: 0.88 – 1.0) in winter 2015 (Figure 5).

*Habitat use.*- AICc model comparison yielded four models with  $\Delta\text{AICc}$  values < 10, indicating these were the only models with strong or some support as plausible models (Table 1). A combined DO and depth model ( $\Delta\text{AICc} = 1.55$ ) was considered the most plausible as it had the lowest  $\Delta\text{AICc}$  value among models in which all coefficients were significant (Compton et al. 2002). All models that received support included both DO and depth as covariates. Coefficients for DO and depth were positive and significant, indicating Arctic Grayling selected deeper, more oxygenated areas within the lake. Depth and DO were not collinear ( $r = -0.15$ ,  $n = 105$ ;  $p < 0.01$ ), indicating an important role of both depth and DO in habitat selection. Odds ratios indicated that every 1.0 mg/L increase in DO resulted in a 30% increase in selection, while every 0.5 m increase in depth resulted in a 63% increase in selection (Table 2).

Arctic Grayling used DO categories of  $\leq 4$  and  $> 4.0$  mg/L in proportion to availability ( $\chi^2 = 0.03 - 1.73$ ,  $p = 0.19 - 0.87$ ), except on the February 27, 2014 sampling occasion when the observed number of fish in each DO category was significantly different from expected ( $\chi^2 = 6.09$ ,  $p = 0.01$ ), and fish exhibited significant selection for DO  $> 4.0$  mg/L (Figure 6). On this occasion, DO  $> 4$  mg/L comprised 69% of available habitat in the upper 1 m, the lowest proportion observed during the two study winters, whereas other sampling occasions had a much higher proportion of lake area  $> 4$  mg/L habitat available. Grayling showed significant selection for areas of the lake with maximum depths  $> 1.0$  m deep on all sampling occasions ( $\chi^2 = 5.25 - 22.91$ ,  $p < 0.01 - 0.02$ ), except on the February 27, 2014 sampling occasion ( $\chi^2 = 0.85$ ,  $p = 0.4$ ) when the observed number of fish in each depth category was not significantly different from expected (Figure 6).

## Discussion

We found that hypoxic winter DO concentrations developed in Upper Red Rock Lake, but substantial

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areas of high DO persisted even under complete ice cover. Contrary to expectations, we observed unexpectedly high winter survival of adult Arctic Grayling ( $\geq 95\%$  of tagged individuals) over the two study winters. We also found no evidence that Arctic Grayling emigrated from the lake in large numbers to seek winter refuge in tributaries. Study results further indicated that a high tolerance to hypoxia and the ability to preferentially select oxygen-rich winter habitat may be important contributing factors enabling overwinter survival of Arctic Grayling.

Laboratory results showed that Arctic Grayling have a high tolerance to acute hypoxia. The  $LC_{50}$  of 0.75 mg/L at 1 and 3°C for adult Arctic Grayling is slightly lower than the critical oxygen minima of 1.3 mg/L at 6°C established by Feldmeth and Eriksen (1978) and is lower than the commonly reported 24-hr lethal oxygen threshold of 1.5 – 2.0 mg/L for salmonids at acclimation temperatures of 10 – 20°C (Doudoroff & Shumway 1970; Alabaster & Lloyd 1982). The  $LC_{50}$  of 1.50 – 1.96 mg/L at 1 – 3°C for juvenile Arctic Grayling in our study is similar to the lethal threshold (1.5 mg/L at 5°C) identified by McLeay et al. (1983) and similar to that of juvenile rainbow trout (Landman et al. 2005). Possible explanations for why our  $LC_{50}$  values are lower than the lethal DO thresholds for most other adult salmonids include: a) the extremely low water temperatures used in our experiments, b) differences in hypoxia tolerance between Arctic Grayling and other salmonid species, and c) differences in experimental methods. Most past studies of hypoxia tolerance have conducted experiments at water temperatures  $> 10^\circ\text{C}$  (Davis 1975; Landman et al. 2005), considerably higher than the 1 and 3°C used in this study. The low water temperatures used in this study, which more closely simulated winter conditions, likely reduced metabolic oxygen demand compared to higher test temperatures (Clarke & Johnston 1999). Observed differences in lethal thresholds could also reflect species differences in hypoxia tolerance. The Holarctic distribution of Arctic Grayling suggests they may be better adapted to the winter hypoxia common to high latitude Arctic systems (Stamford & Taylor 2004). Lastly, the discrepancy in lethal DO thresholds could be the result of differences in experimental methods, especially exposure time used to define the acute lethal threshold. Studies using a  $> 24\text{-h}$  exposure time (Downing & Merckens 1957; Doudoroff & Shumway 1970; Landman et al. 2005) are likely to indicate a higher lethal threshold than the 24-h exposure time in our study. This difference is illustrated by the higher lethal threshold in our 96-h exposure test (100% mortality at 1.0 mg/L) compared to our 24-h exposure test (100% mortality at 0.5 mg/L). Furthermore, our study demonstrated that adult Arctic Grayling have a higher tolerance to severe hypoxia than juveniles, supporting the theory that a large body size results in a greater tolerance to hypoxia in fishes (e.g., Nilsson & Ostlund-Nilsson 2008).

A limitation of our laboratory survival tests was that we only evaluated acute responses to winter hypoxia, and how Arctic Grayling respond to long term, chronic exposure to low DO was not determined. We also used relatively short acclimation periods, which could have also influenced results. Full acclimation to low temperatures can be a slow process, taking up to 20 d or longer (Cossins et al. 1977), and thus our test fish were likely not fully acclimated to the very low winter temperatures when tested, though they had been acclimated to 8°C for at least 2 mo prior to testing. Winter photoperiod has been shown to reduce metabolic oxygen demands of fish during winter independent of temperature (Evans 1984), and our adult trials were run in the spring during non-winter photoperiods, which could therefore have also influenced our estimates of minimum lethal DO concentration for this life stage.

Our laboratory testing revealed Arctic Grayling exhibit stress responses to low DO at concentrations

well above minimum lethal levels. As DO concentrations dropped to 4 mg/L, adult and juvenile adult Arctic Grayling exhibited both increased ventilation rate and increased incidence of surface movement. Such increases in ventilation rate signal the onset of the physiological stress response as individuals attempt to extract more oxygen from the hypoxic environment (Kramer 1987), while increased surfacing time marks the initiation of behavioral avoidance to low DO (Hasler et al. 2009). Environmental conditions that cause significant departures from baseline physiological and behavioral states results in a reduced probability of survival (Davis 1975; Schreck et al. 1997), indicating that DO concentration of 4 mg/L should be considered a critical threshold for winter survival of Arctic Grayling. We also observed a rather unexpected depression in ventilation rate near 1.0 mg/L, especially in juveniles, which contrasted with the more common trend of increasing ventilation rate with decreasing DO. We suggest this reflected an inability to cope with the physiological consequences of sustained stress under extreme hypoxia exposure (Schreck 2010), rather than hypoxia acclimation, since most fish exhibiting this response lost equilibrium soon thereafter. Moreover, the interaction between low temperature and DO suggests that juvenile survival may be more sensitive than adults to the combination of very low DO and very low water temperature in winterkill-prone conditions. Further exploration of juvenile distribution and survival in relation to winter hypoxia would be a fruitful area for additional research.

Overwintering Arctic Grayling displayed a preference for more oxygen-rich habitat in the lake, an additional behavioral response that likely aids survival under winter hypoxia. Arctic Grayling had access to a wide range of available DO (0 – 10 mg/L), however, an abundance of available habitat with DO > 4 mg/L made it the most commonly utilized DO range by transmittered fish. Arctic Grayling exhibited significant selection for DO > 4 mg/L when extent of hypoxia was at its greatest, suggesting that preference for DO > 4 mg/L became more pronounced when DO was most limiting. In contrast to DO, Arctic Grayling had access to a narrow range of available lake depths (maximum depth of 2 m), yet exhibited significant selection for sites with lake depths > 1 m. We hypothesize that depth is an important habitat feature for overwintering Arctic Grayling since the thickness of the well-oxygenated zone along the ice-water interface was greatest in areas > 1 m deep. We did not assess habitat selection of juvenile Arctic Grayling in this study, but laboratory results suggest they may similarly avoid DO  $\leq$  4 mg/L.

The very high overwinter survival we observed in Arctic Grayling in Upper Red Rock Lake did not appear to be a direct result of their high tolerance to low DO. Rather, our results suggest that the high survival rate was a result of a high availability of suitable DO levels > 4 mg/L throughout the winter period. Despite the presence of hypoxic conditions along the lake bottom and considerable spatial variability in DO, the large proportional volume (69 -100%) and persistence of DO > 4 mg/L in the upper meter of the water column throughout both study winters indicated that winterkill risk was low during the two study years. The lack of extensive low DO was surprising given that Upper Red Rock Lake has all the common attributes of high winterkill risk (shallow, low volume, highly eutrophic, long period of ice cover; Barica & Mathias 1979; Mathias & Barica 1980; Babin & Prepas 1985; Terzhevik et al. 2009), and that very low DO concentrations have been regularly recorded in parts of the lake in past winters (Gangloff 1996). We hypothesize that some portions of the upper layer of the lake remained well oxygenated due to input of oxygen-rich stream water, given that stream mouths were often ice-free and always well-oxygenated, and that the comparatively lower density and higher temperature of this input would have allowed this oxygen-rich water to spread out under the ice-water interface without mixing with bottom layers (Bergmann & Welch 1985;

Bertilsson et al. 2013). Similarly, Guenther and Hubert (1991) found a lower risk of winter DO depletion in small, high elevation Wyoming reservoirs when inflowing water was present. However, climatic conditions at Upper Red Rock Lake show a high degree of interannual variability and the possibility of high winterkill risk is likely to periodically occur (Davis 2016). DO surveys during a much colder winter (2015-16) than our two study winters (2013-14 and 2014-15) found much more extensive epilimnetic oxygen depletion than we observed in our study (Warren et al. 2017).

The coupled study design of laboratory and field investigations proved to be an effective approach for assessing risk posed by an environmental stressor. We were able to identify minimum winter DO requirements for Arctic Grayling in our laboratory experiments and directly evaluate the effect of DO levels on overwinter survival and habitat use in field investigations. Our results demonstrated that exposure to DO levels  $\leq 4$  mg/L are likely to result in a reduced probability of winter survival for Arctic Grayling. We also identified key habitat variables, emphasizing the importance of both adequate depth and presence of highly-oxygenated areas to overwintering Arctic Grayling in Upper Red Rock Lake. The method of quantification of the dynamic expansion and contraction of the hypoxic zone used in our study could be employed in other winterkill-prone lakes as a means to readily measure hypoxic zone development and assess the need for employing preemptive actions to increase DO levels under surface ice to prevent extensive winterkill (Barica & Mathias 1979; Barica et al. 1983; Prowse & Stephenson 1986).

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## Tables

Table 1. AICc,  $\Delta$ AICc and model coefficients for paired logistic regression models of Arctic Grayling habitat selection. Model variables include dissolved oxygen (*DO*), lake depth (*depth*), the combined thickness of ice and snow (*icesnow*) and water temperature (*temp*).

Model	AICc	$\Delta$ AICc	<i>DO</i>	<i>depth</i>	<i>icesnow</i>	<i>temp</i>
<i>DO + depth + temp</i>	543.4767	0.0000	0.3705	0.0219		0.3957
<i>DO + depth + icesnow + temp</i>	544.8249	1.3482	0.3755	0.0211	0.0064	0.3638
<i>DO + depth</i>	545.0293	1.5526	0.3591	0.0201		
<i>DO + depth + icesnow</i>	545.7200	2.2433	0.3673	0.0193	0.0088	

Table 2. Coefficients and odds ratios for best-supported paired logistic regression model of Arctic Grayling habitat selection. Values in parentheses are the *DO* and *depth* increments for which odds ratios were calculated.

Variable	Coefficient	Odds ratio	95% CI
<i>DO</i>	0.3591	1.432 (1 mg/L)	1.294 – 1.585
<i>depth</i>	0.0201	2.732 (0.5 m)	1.908 – 3.789

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Figure 4. Interpolated maps of dissolved oxygen concentrations and observed fish locations by sampling date in Upper Red Rock Lake, winter 2014-15.

Figure 5. Kaplan-Meier survival probabilities with 95% confidence intervals for radio-tagged Arctic Grayling in winter 2013-14 and 2014-15. Cross hatches indicate censored individuals (1 fish in 2013-14; 2 fish in 2014-15).

Figure 6. Standardized selection indices for two levels of DO (< 4 and > 4 mg/L) and depth (< 1 m and > 1 m) for each sampling date for winters 2013-14 and 2014-15. Dotted line indicates no selection.

Fig. 1

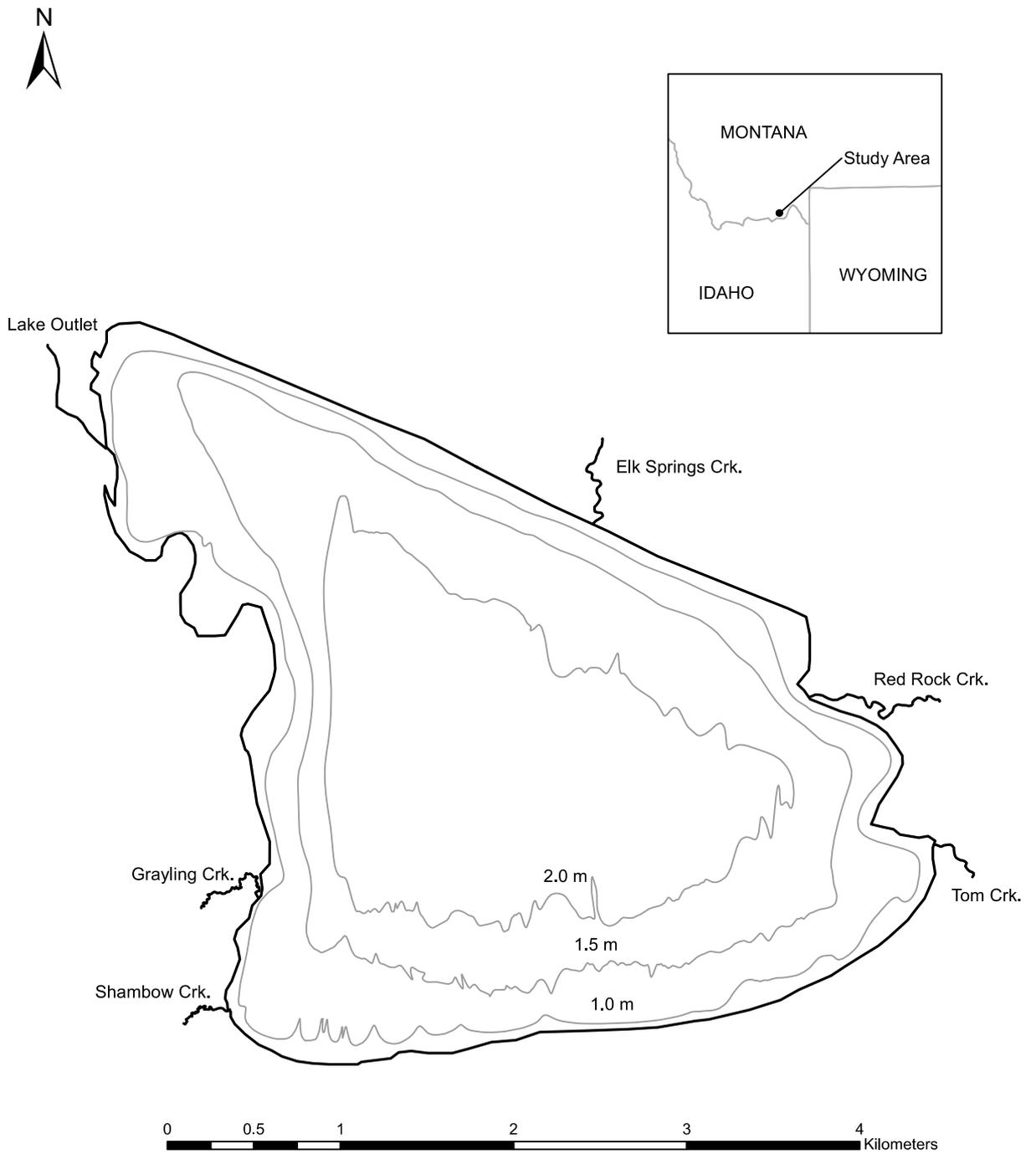


Fig. 2

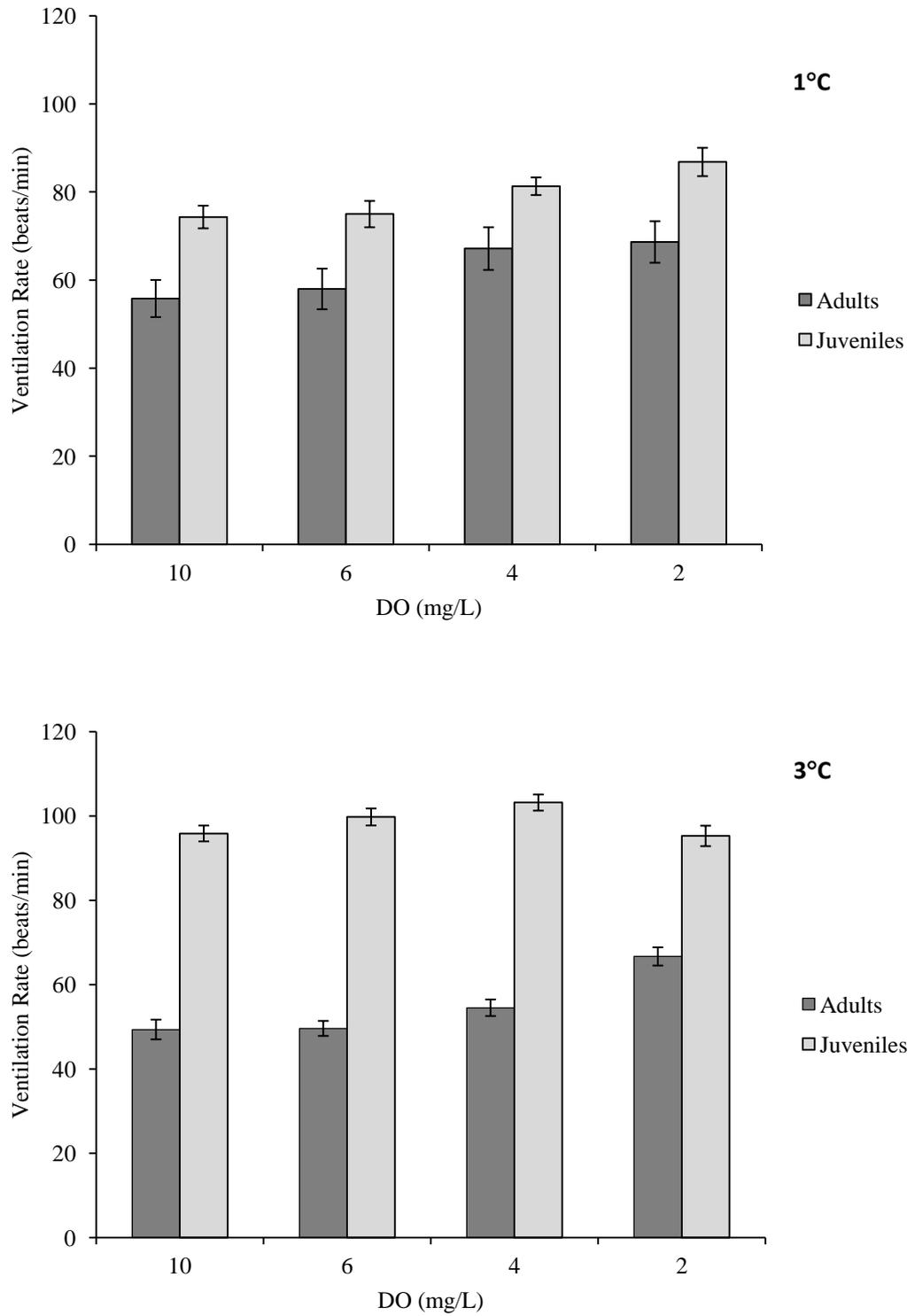


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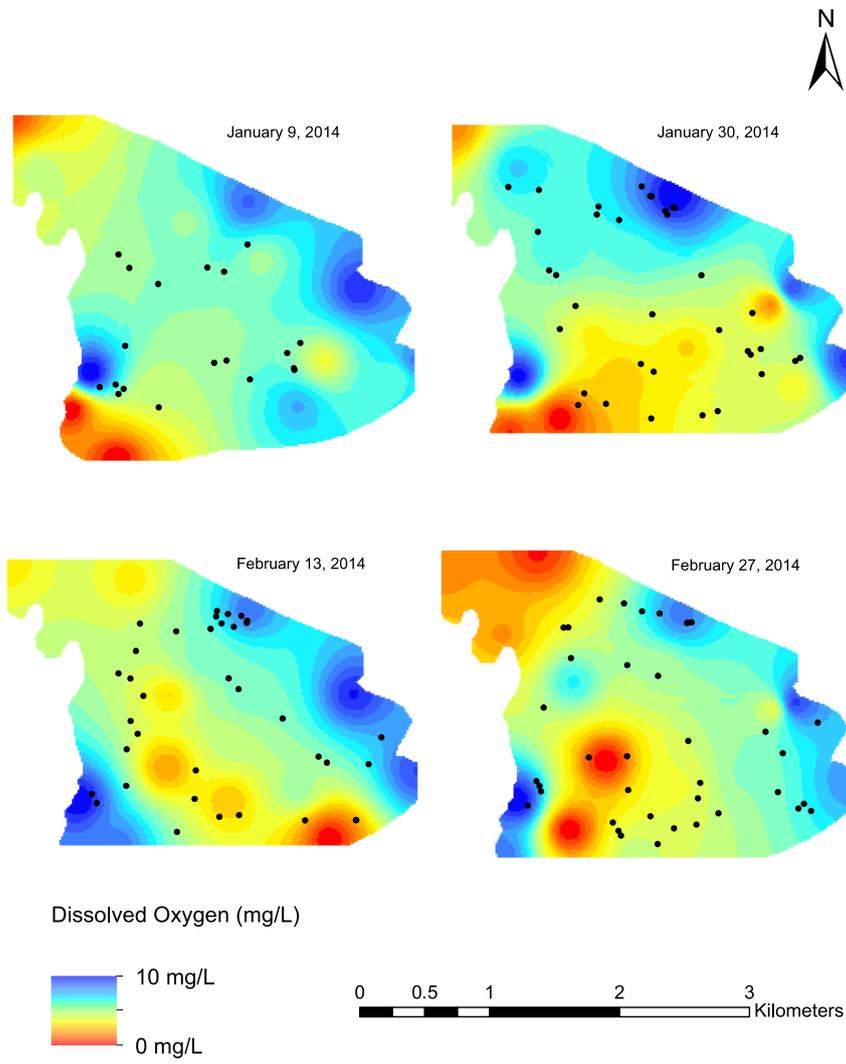


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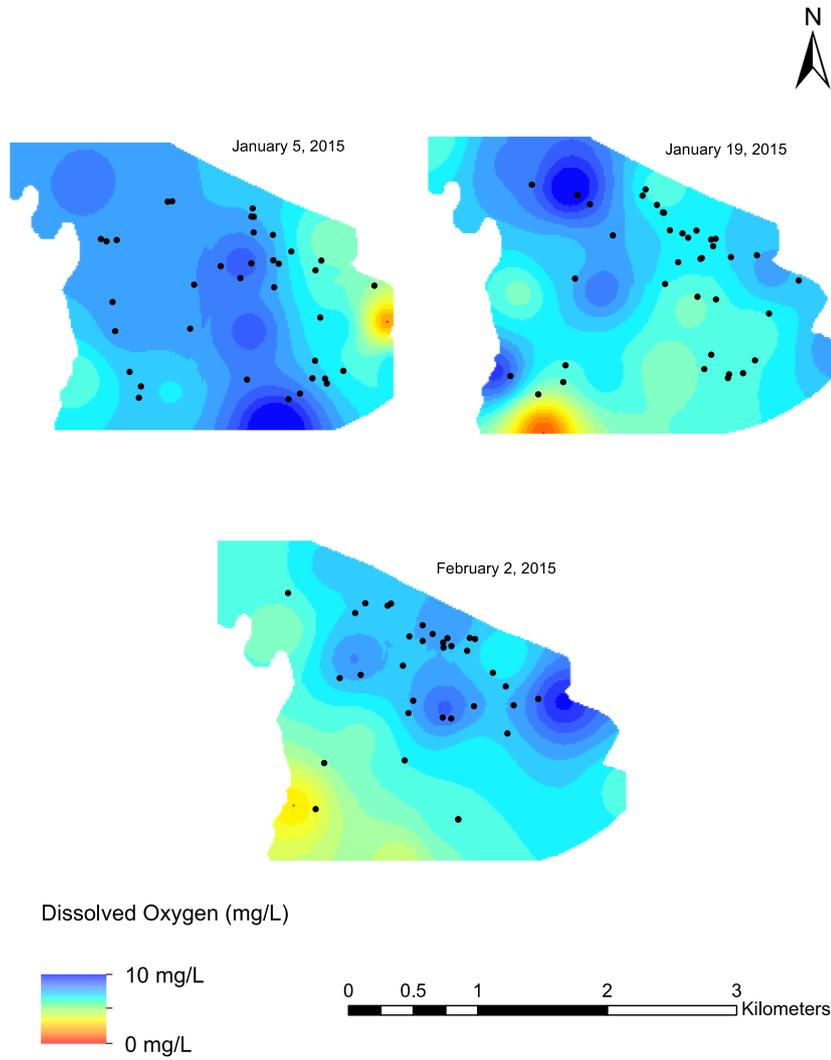


Fig. 5

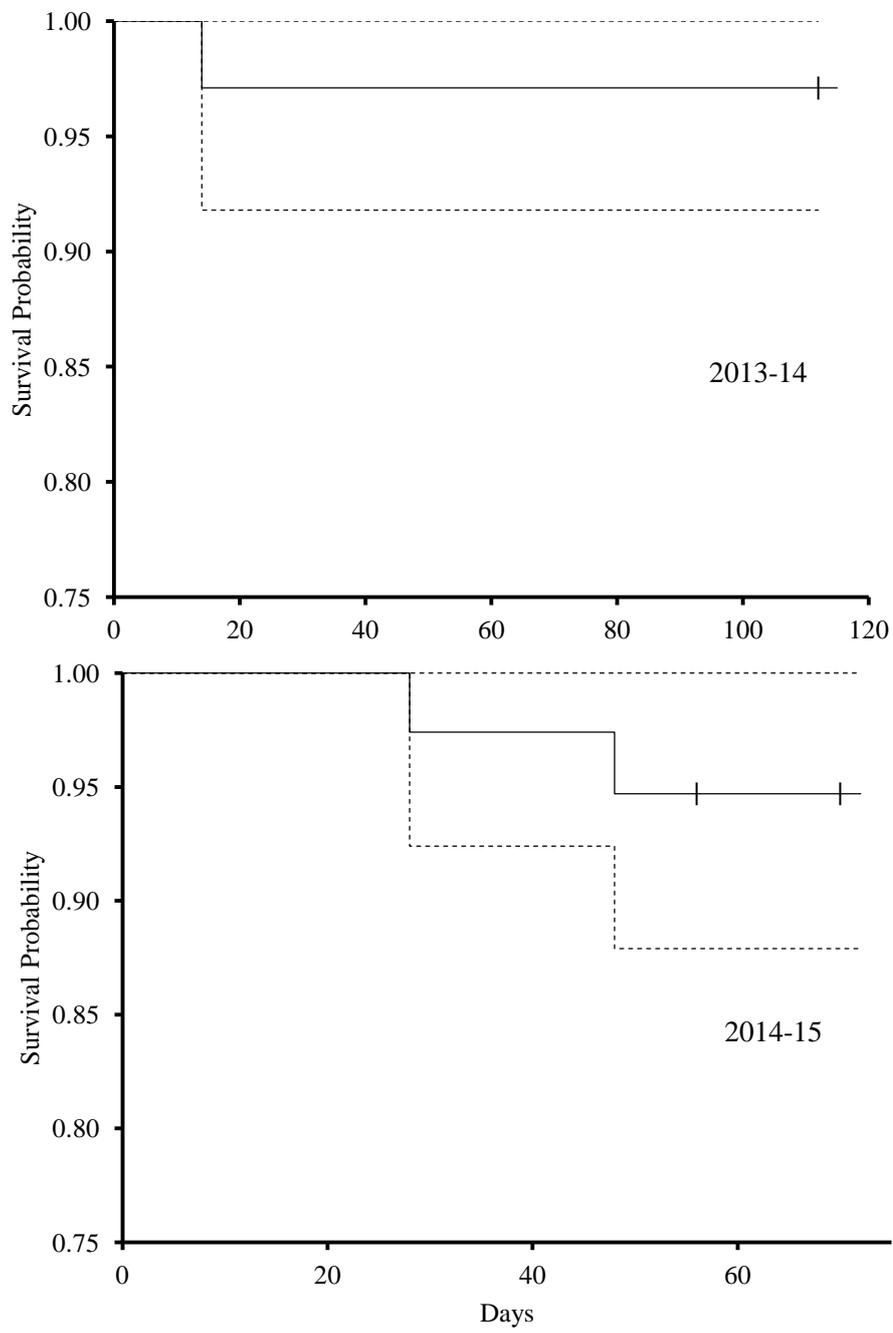


Fig. 6

