

Hypoxia tolerance of the invasive zooplankton
Bythotrephes longimanus and the native zooplankton
Leptodora kindtii

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ABSTRACT

A predatory species of Eurasian zooplankton, *Bythotrephes longimanus* (spiny water flea), has been invading lakes and damaging food webs across the central part of North America since the early 1980s. In an effort to understand its niche, I investigated its short term survival under low dissolved oxygen (D.O.) concentrations in the laboratory and compared it to the native predatory zooplankton *Leptodora kindtii*. Both species are large-bodied, strongly selected by zooplanktivorous fish, and known to commonly engage in diel vertical migration. Trial conditions were chosen to mimic the hypolimnion of a eutrophic temperate zone lake during late summer. Tested *B. longimanus* (n=690) exhibited a pooled hypoxia tolerance limit (LC₅₀) of 1.65 mg L⁻¹ D.O. (95% confidence interval: 1.59, 1.72), while that of *L. kindtii* (n=380) was 0.58 mg L⁻¹ D.O. (0.51, 0.65). Both species were more tolerant of hypoxia than zooplanktivorous fish, which cannot generally withstand prolonged exposure to D.O. concentrations lower than 2 mg L⁻¹. However, *B. longimanus* was significantly less tolerant than *L. kindtii*, and may exhibit greater spatial overlap with zooplanktivorous fish in seasonally-hypoxic lakes. These lab-based physiological results are consistent with landscape-scale observations that *B. longimanus* successfully invades primarily mesotrophic and oligotrophic lakes while *L. kindtii* thrives across a wider range of lakes, including eutrophic ones. The combined effects of climate change and cultural eutrophication throughout the 21st Century will likely increase the occurrence of temperate lake hypoxia, and provide a barrier to *B. longimanus* invasion of North American lakes.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	i
ABSTRACT.....	ii
TABLE OF CONTENTS.....	iii
LIST OF TABLES.....	iv
LIST OF FIGURES.....	vii
INTRODUCTION.....	1
METHODS.....	8
RESULTS.....	15
DISCUSSION.....	21
TABLES.....	32
FIGURES.....	49
REFERENCES.....	58
APPENDIX A: LOGISTIC REGRESSION R CODE.....	68

LIST OF TABLES

Table 1. Dates and locations of zooplankton collections for 10°C hypoxia survival trials ordered by date for <i>B. longimanus</i> and then <i>L. kindtii</i>	32
Table 2. Summary of <i>B. longimanus</i> hypoxia survival trials conducted at 10°C including source lake, date of experiment, number of individuals per trial (n), proportions of animals in each developmental stage comprising a trial, and the mean dry weight of all animals in a trial, χ^2 (df = 1) goodness of fit statistic, and χ^2 goodness of fit p-value. A 95% confidence interval could not be calculated for the 17 September 2014 trial due to the small number of tested individuals (n = 40).....	33
Table 3. Summary of <i>L. kindtii</i> hypoxia survival trials conducted at 10°C including source lake, date of experiment, number of individuals in a trial (n), mean dry weight of all animals in a trial, χ^2 (df = 1) goodness of fit statistic, and χ^2 goodness of fit p-value. <i>L. kindtii</i> from 20 August 2014 were not saved and were not included in any length or weight-based analyses.....	34
Table 4. Pooled results of all <i>B. longimanus</i> and <i>L. kindtii</i> hypoxia trials conducted at 10°C during the summers of 2014 and 2015. The results from both study lakes were included for both species. B ₀ and B ₁ represent the parameter estimates of the logistic regression. χ^2 indicates the logistic regression model goodness of fit statistic (df = 1), followed by the goodness of fit p-value.....	35
Table 5. Pooled results of <i>B. longimanus</i> developmental stage analysis. All <i>B. longimanus</i> tested at 10°C from both lakes (Island and Mille Lacs) were used for this analysis. B ₀ and B ₁ represent the parameter estimates of the logistic regression. χ^2 indicates the logistic regression model goodness of fit statistic (df = 1), followed by the goodness of fit p-value.....	36

Table 6. Pooled results of *B. longimanus* weight class analysis. *B. longimanus* dry weights were separated into eight even-width percentile ranges, and an LC₅₀ estimate was calculated for each. The proportion of each developmental stage in each weight class is noted. All *B. longimanus* tested at 10°C from both lakes (Island and Mille Lacs) were used for this analysis. B₀ and B₁ represent the parameter estimates of the logistic regression. χ^2 indicates the logistic regression model goodness of fit statistic (df = 1), followed by the goodness of fit p-value. Superscript numbers on the 95% confidence intervals indicate significant difference at a level of p = 0.05. Lack of common superscripts indicates significant difference.....37

Table 7. Pooled results of *L. kindtii* weight class analysis including four quartiles, the number of individuals per quartile, the mean weight and standard deviation of each quartile, and the LC₅₀ estimate. None of the weight classes were different from one another at a level of p = 0.05. All *L. kindtii* tested at 10°C from both lakes (Island and Caribou) were used for this analysis. B₀ and B₁ represent the parameter estimates of the logistic regression. χ^2 indicates the logistic regression model goodness of fit statistic (df = 1), followed by the goodness of fit p-value.....38

Table 8. Weight class linear regression results. The mean weight of each *B. longimanus* and *L. kindtii* weight class was used to predict dissolved oxygen LC₅₀. The results from both study lakes were included for both species. *a* represents the slope coefficient of the linear regression.....39

Table 9. Weight class power law regression results. The mean weight of each *B. longimanus* and *L. kindtii* weight class was used to predict dissolved oxygen LC₅₀. The results from both study lakes were included for both species. *b* represents the slope coefficient of the linear regression.....40

Table 10. Comparison of linear and power law models used for predicting *B. longimanus* (n=690) dissolved oxygen LC₅₀ based on mean dry weight of eight percentile-based weight classes. *W* in each equation represents mean dry weight.....41

Table 11. Inter-lake results of *B. longimanus* hypoxia survival trials including lake of collection, dates of trial, total number of individuals (n) tested, and dissolved oxygen LC₅₀ estimate. χ^2 indicates the logistic regression model goodness of fit statistic (df = 1), followed by the goodness of fit p-value.....42

Table 12. Inter-lake results of *L. kindtii* hypoxia survival trials including lake of collection, dates of trial, total number of individuals (n) tested, LC₅₀ estimate, and dissolved oxygen LC₅₀ estimate. χ^2 indicates the logistic regression model goodness of fit statistic (df = 1), followed by the goodness of fit p-value.....43

Table 13. Pooled results of *B. longimanus* developmental stage analysis for Island Lake hypoxia survival trials only. B₀ and B₁ represent the parameter estimates of the logistic regression. χ^2 indicates the logistic regression model goodness of fit statistic (df = 1), followed by the goodness of fit p-value.....44

Table 14. Calculation of simulated *B. longimanus* LC₅₀ based on the proportion of each developmental stage found in the Mille Lacs Lake trial. The value in Island Lake trials column was multiplied by the proportion of Mille Lacs Lake trial column to produce a stage estimate. The sum of the stage estimate values for each developmental stage produced an LC₅₀ and associated confidence interval, 1.59 mg L⁻¹ (1.39, 1.75).....45

Table 15. Results of simulated *B. longimanus* hypoxia survival trial comparing the Island Lake and Mille Lacs Lake populations of *B. longimanus*.....46

Table 16. *B. longimanus* temperature test on 2-3 July 2015. Superscript numbers on the 95% confidence intervals indicate significant difference at a level of p = 0.05. Lack of common superscripts indicates significant difference. χ^2 indicates the logistic regression model goodness of fit statistic (df = 1), followed by the goodness of fit p-value.....47

Table 17. *B. longimanus* temperature test treatments on 2-3 July 2015. Five treatment D.O. concentrations with ten replicates each were tested at each temperature.....48

LIST OF FIGURES

- Figure 1.** Sample result of a 10°C *B. longimanus* hypoxia trial from 19 June 2015. A typical trial consisted of five D.O. treatment levels, one of which served as a control and did not involve any nitrogen sparging. Ideally, the D.O. treatment levels would produce a range of survival proportions, both above and below 0.5. For this particular trial, logistic regression produced an LC₅₀ estimate of 1.52 mg L⁻¹ (1.33, 1.67).....49
- Figure 2.** Hypoxia tolerance curves and raw data of *B. longimanus* (n=690) (panel a) and *L. kindtii* (n=380) (panel b), and the curves only for both species (panel c) based on the pooled results of all experiments conducted at 10°C during 2014 and 2015. The thin solid lines represent 95% confidence limits.....50
- Figure 3.** Length distribution of all *B. longimanus* (n=690) subjected to hypoxia trials during 2014 and 2015. All *B. longimanus* tested at 10°C from both lakes (Island and Mille Lacs) are shown.....51
- Figure 4.** Weight distribution of all *B. longimanus* (n=690) subjected to 10°C hypoxia trials during 2014 and 2015. All *B. longimanus* tested at 10°C from both lakes (Island and Mille Lacs) are shown.....52
- Figure 5.** Length distribution of most *L. kindtii* (n=300) subjected to 10°C hypoxia trials during 2014 and 2015. The total length of *L. kindtii* spans from the center of the compound eye to the base of the caudal stylets. *L. kindtii* from 20 August 2014 were not saved and were not included in this analysis.....53
- Figure 6.** Weight distribution of most *L. kindtii* (n=300) subjected to hypoxia trials during 2014 and 2015. *L. kindtii* from 20 August 2014 were not saved and were not included in this analysis.....54
- Figure 7.** Hypoxia tolerance curves and raw data for *B. longimanus* stage 1 (panel a), stage 2 (panel b), and stage 3 (panel c), and the curves only for all stages (panel d) based on the pooled results of all 10°C experiments conducted during 2014 and 2015. The 95% confidence limits in panel d were excluded in order to see the regression lines. The LC₅₀ estimates of all three stages were significantly different at a level of p = 0.05.....55

Figure 8. Linear regression using mean weight as a predictor of dissolved oxygen LC₅₀. *B. longimanus* (n=690) dry weights were apportioned into eight weight classes at 12.5 percentile intervals, while *L. kindtii* (n=300) dry weights were split into quartiles. Each circle represents the mean weight of a *B. longimanus* weight class, while each square represents the mean weight of a *L. kindtii* quartile. The slope of the *B. longimanus* line was significantly different from zero (ANOVA, $F_{1,6} = 52.5$, $p < 0.001$), while the slope of the *L. kindtii* line was not (ANOVA, $F_{1,2} = 2.7$, $p = 0.24$).....56

Figure 9. Implications of hypoxia tolerance for deep water refuge of *B. longimanus* and *L. kindtii* in a eutrophic lake. Many fish cannot withstand hypoxia below 2 mg/L (Vanderploeg et al. 2009), while the hypoxia limits (12 hour LC₅₀ at 10°C) for *B. longimanus* and *L. kindtii* are 1.65 mg/L and 0.58 mg/L, respectively.....57

INTRODUCTION

Invasive, nonnative species are responsible for more than \$120 billion yearly in environmental damages in the United States (Pimentel et al. 2005). Aquatic invasive species, in particular, pose a major threat to the environment and economy of Minnesota, given the state's abundance of waterbodies. As of 2014, 690 occurrences of aquatic invasive species had been documented across Minnesota (Minnesota Department of Natural Resources). Many of the state's infested lakes, such as Mille Lacs Lake, represent key features of Minnesota's tourism industry.

One nonnative, invasive species of contemporary concern in Minnesota is the spiny water flea, *Bythotrephes longimanus* (Cladocera: Onychopoda). *B. longimanus* is a predatory species of crustacean zooplankton that is native to Eurasia (Sprules et al. 1990, Straile and Halbich 2000). Since its introduction to North America via the Laurentian Great Lakes in the early 1980s, *B. longimanus* has spread to over 130 inland lakes (Brown et al. 2012). *B. longimanus* can occur at densities greater than 100 individuals m⁻³ and has the potential to alter native zooplankton communities, change fish diets, and interfere with fishing and recreational equipment (Berg and Garton 1988, Coulas et al. 1998, Yan and Pawson 1997, Schulz and Yurista 1999, Brown et al. 2012). *B. longimanus* is easily recognizable due to its large compound eye and stiff caudal spine that makes up about 80% of its approximately 1 cm adult length.

Leptodora kindtii (Cladocera: Haplopoda) is a species of predatory crustacean zooplankton that, unlike *B. longimanus*, is native to North America (Garton et al. 1990). Like *B. longimanus*, *L. kindtii* grows to an adult length of approximately 1 cm. However,

it does not possess a large caudal spine and instead has two small stylets at the caudal end. *L. kindtii* uses extreme body transparency, a precontact defense, as a means of deterring fish predation, while *B. longimanus* relies on the use of its caudal spine, a postcontact defense (Balcer et al. 1984, Branstrator et al. 2005). The caudal spine clogs the buccal cavity of fishes that attempt to consume it and has been shown to protect *B. longimanus* from fishes less than about 10 cm length (Barnhisel 1991, Compton and Kerfoot 2004).

As predatory zooplankton in the pelagic zone of lakes, *B. longimanus* and *L. kindtii* occupy the same trophic level and can compete with one another where they coexist (Branstrator 1995). Both species target small-bodied cladoceran zooplankton (e.g., bosminids, daphnids) as preferred prey (Branstrator and Lehman 1991, Branstrator 1995, Branstrator 1998). Several studies have documented declines in *L. kindtii* abundance during and after the invasion of *B. longimanus* in North American lakes (Branstrator 1995, Yan and Pawson 1997, Hessen et al. 2011, Foster et al. 2012). This decline is likely a result of competition for prey and direct predation by *B. longimanus* on *L. kindtii*.

While the geographic distributions of *B. longimanus* and *L. kindtii* overlap in some instances, the two species often inhabit different types of lakes. *B. longimanus* tends to be found in oligotrophic and mesotrophic lakes, while *L. kindtii* more commonly inhabits eutrophic lakes (MacIsaac et al. 2000, Palmer et al. 2001, Wetzel 2001). The reason for the distribution distinction between *B. longimanus* and *L. kindtii* is not known but provides a template for predicting the suitability of ecosystems to invasion by *B.*

longimanus. MacIsaac et al. (2000) argued that a lack of *B. longimanus* in eutrophic lakes in North America and Eurasia could be the result of strong top down control by fishes large enough to eat them. On the other hand, Branstrator et al. (2006) argued that a low-light (deep water) daytime refuge for *B. longimanus* could be important as well. Additionally, Branstrator et al. (2006) speculated that oxygen availability may be a key factor determining suitable daytime refuge.

Dissolved oxygen concentration is one of the principle factors determining suitable biological habitat in freshwaters (Wetzel 2001). Dissolved oxygen concentrations can influence zooplankton growth rates and physiological processes as well as spatial distributions (Homer and Waller 1983, Pothoven et al. 2012). Dissolved oxygen, like temperature and light, is a complex limnological parameter because it varies spatially and temporally between lakes and within a single lake (Davis 1975, Wetzel 2001). It is possible that *B. longimanus* and *L. kindtii* have different dissolved oxygen preferences, allowing them to thrive in their respective habitats.

Oligotrophic and mesotrophic lakes, the types inhabited by *B. longimanus*, have a vertical oxygen profile during summer stratified months that is largely driven by physical processes. In such lakes, the concentration of dissolved oxygen is lowest in the warm epilimnion or metalimnion and highest in the cool hypolimnion. This contrasts with eutrophic lakes more commonly inhabited by *L. kindtii* which are highly productive and often have high epilimnetic oxygen concentrations but depressed hypolimnetic oxygen concentrations in late-summer months (Wetzel 2001).

The concentration of dissolved oxygen in the hypolimnion of a lake is an important determinant of zooplankton habitat since many species change their vertical position in the water column throughout a 24 hour period. Such behavior, termed diel vertical migration (DVM), often results in zooplankton taking advantage of the dark hypolimnion during the day to escape fish predation and rising to the epilimnion at night for feeding and developmental benefits (Lampert 1989). The magnitude of DVM behavior can be influenced by local dissolved oxygen gradients as well as other factors such as predation (LaRow 1970, Young and Yan 2008, Stratton and Kesler 2011, Bowersox et al. 2013). Diel vertical migration has been documented in both *B. longimanus* and *L. kindtii*, however little is known about the hypoxia tolerance limits of these species (Chang and Hanazato 2004, Brown et al. 2012, Vogt et al. 2013).

Hypoxia tolerance limits differ dramatically among aquatic organisms (Davis 1975). For example, fish cannot generally withstand dissolved oxygen concentrations lower than 2 mg L⁻¹, while several species of zooplankton can tolerate concentrations lower than 0.5 mg L⁻¹ (Doudoroff and Shumway 1970, Davis 1975, Eby and Crowder 2002, Ekau et al. 2010, Klumb et al. 2014). By tolerating severe hypoxia, it is possible for zooplankton to inhabit regions of the water column that are lethal to their fish predators (Vanderploeg et al. 2009). Consequently, by engaging in DVM some zooplankton not only make use of a low-light refuge from fish predation, but also use a hypoxia refuge from fish predation (Hanazato et al. 1989, Vanderploeg et al. 2009, Larsson and Lampert 2011, Keister and Tuttle 2013).

Aquatic invertebrates have developed a number of physiological adaptations to manage hypoxia stress including regulating ventilatory activity at low oxygen concentrations, increasing production of the oxygen-binding molecule hemoglobin, and relying temporarily on anaerobic metabolism (Grieshaber et al. 1994, Childress and Seibel 1998, McMahon 2001). Not all species of zooplankton are capable of utilizing these adaptations to the same degree (Davis 1975). Thus, different species of zooplankton have different hypoxia tolerance limits.

Studies characterizing zooplankton hypoxia tolerance have historically used in situ studies and laboratory experiments. In situ studies involve collecting depth-discrete zooplankton samples along with measurements of the local temperature, light, and dissolved oxygen profiles (Longhurst 1967, Pothoven et al. 2012). Such studies are useful in characterizing the depth distribution of a population as well as lake-specific patterns of diel vertical migration. In situ studies have described *B. longimanus* survival at dissolved oxygen concentrations as low as 2.4 mg L⁻¹ (Grigorovich et al. 1998, Vanderploeg et al. 2009). *L. kindtii*, on the other hand, has been reported to survive at dissolved oxygen concentrations below 1 mg L⁻¹ (Moshiri et al. 1969, Vogt et al. 2013). Unfortunately, these studies were purely observational and did not identify the absolute hypoxia thresholds, or duration of exposure, of either species.

Laboratory hypoxia experiments involve monitoring zooplankton survival at known dissolved oxygen concentrations (Herbert 1954, Goto et al. 2012). Hypoxic water can be easily prepared in a laboratory setting via nitrogen sparging, a technique that has been used for decades in hypoxia studies of fish and aquatic invertebrates (Shepard 1955,

Tinson and Laybourn-Parry 1962, Brown 2008). Nitrogen sparging is not only easier but also more cost-effective than other hypoxia preparation techniques such as microbial respiration or the use of sodium sulfite reduction reactions (Bevan and Kramer 1988).

To date, only one laboratory hypoxia experiment on *B. longimanus* and *L. kindtii* has been published (Herbet 1954). This study involved placing 20 conspecifics in 3-L bottles of nitrogen-sparged tap water for four hours. The survival thresholds (LC₅₀) generated by this experiment were 1.61 mg L⁻¹ for *B. longimanus* and 1.17 mg L⁻¹ for *L. kindtii*.

Several shortcomings of this study (Herbert 1954) warrant consideration. First, the experiments used *B. longimanus* from Lago Maggiore (Italy) and thus were likely testing the small-bodied form of *Bythotrephes (longimanus)* rather than the larger-bodied conspecific form of *Bythotrephes (cederstroemi)* that is commonly found in the United States (Therriault et al. 2002). Second, since the experiments were conducted with 20 animals in one bottle, there is a distinct possibility that the results were influenced by interaction between multiple animals, which for *B. longimanus* may have been cannibalism (Brown et al. 2012). Third, the study (Herbert 1954) did not test *B. longimanus* and *L. kindtii* at conditions that they would encounter in the hypolimnion of a stratified temperate lake. For example, the experiments used tap water instead of lake water, which may have influenced the health and respiratory activity of the tested animals, and were run at 18°C, which is warmer than a typical hypolimnion of a stratified temperate lake. Since the respiratory rates of both species increase with increasing temperature, the hypoxia tolerance limits reported may be different than limits in a 5-

10°C hypolimnion (Moshiri et al. 1969, Yurista 1999). Finally, the study lacked inter-seasonal and inter-lake replication.

The objective of this study was to fully describe the oxygen tolerance limits of *B. longimanus* and *L. kindtii* using laboratory hypoxia experiments. I hypothesized that *L. kindtii* is more tolerant of fish-based hypoxia ($<2 \text{ mg L}^{-1}$) than *B. longimanus*, allowing *L. kindtii* to avoid fish predation by inhabiting hypolimnetic regions of eutrophic lakes.

METHODS

Study Sites

Due to the difficulty of culturing *B. longimanus* (Kim and Yan 2010) and *L. kindtii* (Pichlová et al. 2004) in a laboratory setting, all animals in this study were collected from the field. *B. longimanus* were collected from Island Lake Reservoir and Mille Lacs Lake, while *L. kindtii* were collected from Island Lake Reservoir and Caribou Lake. Island Lake Reservoir, hereafter referred to as Island Lake, is a large (surface area = 29.7 km²), deep (max depth = 28.7 m), moderately productive temperate reservoir located 30 km north of Duluth, Minnesota (Branstrator et al 2006). *B. longimanus* was first discovered in Island Lake in 1990 and has since established itself as an enduring member of the zooplankton community (Brown et al 2012). *L. kindtii* is native to Island Lake, but is found at relatively unknown locations, densities, and times of year (D.K. Branstrator, personal communication). Mille Lacs Lake is a large (surface area = 518.9 km²), shallow (max depth = 12.8 m) lake in central Minnesota that has supported *B. longimanus* since at least 2009, when it was detected and reported by the Minnesota Department of Natural Resources (www.dnr.state.mn.us/lakefind). Caribou Lake is a small (surface area = 2.2 km²), shallow (max depth = 6.4 m) lake located 10 km northwest of Duluth, Minnesota that supports *L. kindtii* but not *B. longimanus* (www.dnr.state.mn.us/lakefind). All three study lakes are generally well-oxygenated (> 5 mg L⁻¹) throughout the water column, even in the late summer months (Brown et al. 2012, MPCA Surface Water Data).

Field and Laboratory

B. longimanus and *L. kindtii* were collected for hypoxia survival trials during the open water months of 2014 and 2015 (Table 1). The goal of the trials was to determine the dissolved oxygen LC₅₀ of each species. For the purposes of this study, LC₅₀ is defined as the concentration of dissolved oxygen at which 50% of the tested individuals died after 12 hours of exposure.

Live animals were collected the evening before a trial took place by towing a Puget Sound-style net (0.5-meter-opening, 1.5-meter-length, 200- μ m-pore aperture mesh, solid cod end) through the water column from near the sediments to the surface. Island Lake zooplankton were collected from the Rice Lake Road bridge, while Mille Lacs Lake and Caribou Lake zooplankton were collected from a 16 foot boat. Mille Lacs Lake sampling was conducted at Isle Bay (latitude 46°09'07" N, longitude 93°29'00" W).

After collection, the zooplankton were gently dispensed from the cod end into 10 liter carboys for transportation to the lab. In addition, 50 liters of lake water was filtered through a 65 μ m-pore aperture mesh and stored in 10 and 20 liter plastic carboys to be used in the trials. Zooplankton and lake water were stored overnight in a darkened 10°C incubator.

The following morning, zooplankton were poured into white plastic sorting trays and individuals were isolated into wells of 12-well 5 mL tissue culture plates filled with filtered lake water. *B. longimanus* was transferred by forceps applied to the caudal spine while *L. kindtii* was transferred using a wide-aperture glass pipette.

The carboys of filtered lake water were then individually sparged with pure nitrogen gas. Sparging took place inside the incubator, with the nitrogen tubing running into the ports on the side of the incubator. To reduce gas exchange at the water's surface, the carboys were covered with a lid that had one hole for the nitrogen tubing and another hole for a YSI 5010 dissolved oxygen probe. The YSI 5010 is a membrane-type oxygen probe that is accurate to ± 0.1 mg/L or $\pm 2\%$ of the reading, whichever is greater (YSI Instruction Manual). The probe membrane was changed and the probe was calibrated to Winkler wet chemistry tests once every 2-3 weeks throughout the duration of the hypoxia survival trials.

When the water reached its target dissolved oxygen concentration (hereafter sometimes referred to as D.O. concentration), the carboy was removed from the incubator and placed on the laboratory countertop. Three siphons of aquarium tubing were used simultaneously to transfer water from the carboy to twenty numbered 300 mL glass biological oxygen demand (hereafter BOD) bottles, which were allowed to overflow in order to remove any trapped oxygen bubbles. When each bottle was filled, its glass stopper was replaced. The order of bottle filling was noted.

Twenty replicates at each of five different dissolved oxygen concentrations constituted a typical trial. In each trial, one of the five treatments consisted of non-sparged control. Preliminary testing during early summer 2014 was used to identify which dissolved oxygen concentrations were relevant to the LC_{50} thresholds of *B. longimanus* and *L. kindtii* so that enhanced resolution (more test concentrations) could be

used near those thresholds. Testing indicated that *B. longimanus* threshold concentrations were near 1.50-2.50 mg L⁻¹, while those of *L. kindtii* were near 0.25-1.00 mg L⁻¹.

When all 100 bottles were filled for a trial, pre-trial dissolved oxygen concentrations (YSI 5010) were recorded in 30% of the BOD bottles. Then, a single live animal was added to each and returned to the dark incubator for the duration of the 12 hour trial. Typically, trials were conducted at 10°C. However, during 2-3 July 2015, 50 *B. longimanus* were tested at each of 10, 15, 20, and 25°C to identify the effect of trial temperature on dissolved oxygen LC₅₀. Previous trials, conducted on *B. longimanus* and *L. kindtii* in June-July 2014, demonstrated that 5°C was too cold for both species, reducing their level of swimming activity to the point where they laid on the bottom of the test bottle.

At the end of a 12 hour trial, dissolved oxygen concentration was measured immediately in 50% of the BOD bottles, staggered evenly across the sequence in which the bottles were initially filled. Bottles that were not measured for post-trial D.O. were assigned a concentration based on the average concentration in the bottles that had been filled immediately before and after at the trial start. For example, the bottle that was filled second was assigned an average D.O. concentration of the bottles that were filled first and third. This approach saved a considerable amount of time and allowed for the trial duration to be as close to 12 hours as possible. All BOD bottles were then poured out onto 65µm-pore aperture mesh and each of the tested animals was submerged in filtered lake water before being declared dead or alive based on the presence of a heartbeat

observed under a dissecting microscope (Leica MZ125). Post-trial, all *B. longimanus* and *L. kindtii* were preserved in 100% ethanol.

Analysis

The developmental stage of *B. longimanus* was scored based on the number of lateral barbs on the caudal spine. Both *B. longimanus* and *L. kindtii* were measured for total length according to Branstrator (2005). Shrinkage in ethanol was corrected for as 19.05% for *B. longimanus* and 16.67% for *L. kindtii* (M. Pawlowski, unpublished data). Shrinkage-corrected lengths were converted to dry weights based on Branstrator (2005) as:

$$L. \textit{kindtii} \text{ dry weight } (\mu\text{g}) = 1.018 \cdot \text{mm}^{2.042} \quad (\text{Eq. 1}),$$

and

$$B. \textit{longimanus} \text{ dry weight } (\mu\text{g}) = 45.2 \cdot \text{mm} - 287.2 \quad (\text{Eq. 2}),$$

where mm equals total unpreserved length in millimeters.

The LC₅₀ values and their associated 95% confidence intervals were calculated for each trial by logistic regression using the statistical software R (Appendix A). The adequacy of the model was assessed by a Chi-Square Goodness of Fit test. All of the tested animals were treated independently for the logistic regression, rather than grouping them by treatment. The logistic regressions and LC₅₀ estimates were based on post-test D.O. concentrations, which on average (mean) were only 4.8% higher than the pre-test

D.O. concentrations ($n=321$, $SD=14.5\%$). Two LC_{50} values were considered significantly different from one another ($p < 0.05$) if their 95% confidence intervals did not overlap.

The results of all *B. longimanus* and *L. kindtii* trials at 10°C were pooled and analyzed to produce an average LC_{50} for each species at this temperature. Additionally, a Mann Whitney-rank sum test was used to determine whether the LC_{50} estimates of the *B. longimanus* trials were significantly different than those of the *L. kindtii* trials. For *B. longimanus*, a developmental stage-based LC_{50} threshold was determined.

Pooled results from all trials at 10°C were also used to test for size-based hypoxia LC_{50} thresholds. To do this, *L. kindtii* dry weights were separated into quartiles and *B. longimanus* dry weights were separated into eight percentile-based weight classes. Regressions were performed using R to identify the extent to which the mean weights of each *B. longimanus* and *L. kindtii* weight class were able to predict the dissolved oxygen LC_{50} . The results of both species were first analyzed via linear regression using the equation:

$$LC_{50} = a \cdot W + b \quad (\text{Eq. 3}),$$

where LC_{50} is the dissolved oxygen LC_{50} estimate in mg L^{-1} , W is mean organism dry weight in μg , and a and b are determined empirically.

Additionally, results were fit to a power law model. To accomplish this, mean dry weights and LC_{50} estimates were transformed with a natural log operator and then used in linear regression. The power law model is commonly used to explain the scaling of physiological processes (Peters 1983):

$$LC_{50} = a \cdot W^b \quad (\text{Eq. 4}),$$

where LC_{50} is the dissolved oxygen LC_{50} estimate in mg L^{-1} , W is mean organism dry weight in μg , and a and b are determined empirically.

The results of these regressions were compared to determine which model, linear or power law, best predicted dissolved oxygen LC_{50} .

RESULTS

B. longimanus (n=690) and *L. kindtii* (n=380) were subjected to hypoxia trials during the open water months of 2014 and 2015. Seven of eight *B. longimanus* trials were conducted on animals from Island Lake Reservoir and one was conducted on animals from Mille Lacs Lake (Table 1). Three of four *L. kindtii* trials were conducted on animals from Island Lake Reservoir and one was conducted on animals from Caribou Lake (Table 1).

The LC₅₀ estimates of the *B. longimanus* trials ranged from 1.36 mg L⁻¹ to 2.80 mg L⁻¹ dissolved oxygen (D.O.), while the LC₅₀ estimates of the *L. kindtii* trials ranged from 0.46 mg L⁻¹ to 0.82 mg L⁻¹ D.O. (Tables 2, 3). The *B. longimanus* trial LC₅₀ estimates (n=8) were significantly different from the *L. kindtii* trial LC₅₀ estimates (n=4) (Mann-Whitney, $U = 3.96$, $df = 1$, $p < 0.01$). A sample result of a hypoxia trial is depicted in Figure 1.

When the results of all *B. longimanus* trials conducted at 10°C from both lakes were pooled, the LC₅₀ estimate was 1.65 mg L⁻¹ D.O. (95% confidence interval: 1.59, 1.72) (Table 4, Fig. 2). When the results of all *L. kindtii* trials done at 10°C from both lakes were pooled, the LC₅₀ estimate was 0.58 mg L⁻¹ D.O. (0.51, 0.65) (Table 4, Fig. 2). These pooled 10°C hypoxia tolerance limits (12 hour LC₅₀) of *B. longimanus* and *L. kindtii* were significantly different from one another at a level of $p = 0.05$.

Size- and stage-based hypoxia tolerance

Tested *B. longimanus* covered a length range of 6.5-13.5 mm and an estimated dry weight range of 5.8-322.2 μg (Figs. 3, 4). The mean length of the tested *B. longimanus* was 9.3 mm, while the estimated mean dry weight was 131.5 μg . Tested *L. kindtii* covered a length range of 2.1-11.2 mm and an estimated dry weight range of 4.7-141.3 μg (Figs. 5, 6). The mean length of the tested *L. kindtii* was 5.4 mm, while the estimated mean dry weight was 34.9 μg which roughly corresponds to the size at which *L. kindtii* reach sexual maturity (Branstrator 2005). The *L. kindtii* from 20 August 2014 were neither measured nor saved, and thus, were not included in these size-based analyses.

All tested *B. longimanus* were scored for developmental stage based on the number of barbs on their caudal spine. Pooled LC_{50} estimates were 1.40 mg L^{-1} (1.28, 1.51) for stage 1 *B. longimanus* (n=203), 1.68 mg L^{-1} (1.59, 1.76) for stage 2 *B. longimanus* (n=322), and 1.95 mg L^{-1} (1.82, 2.14) for stage 3 *B. longimanus* (n=165) (Table 5, Fig. 7). The LC_{50} estimates of all three *B. longimanus* developmental stages were significantly different from one another at a level of $p = 0.05$.

Since *B. longimanus* developmental stage is largely descriptive of the life cycle and may not be physiologically relevant regarding respiration, the pooled *B. longimanus* results were also analyzed in eight dry weight classes. Weight class 1, the smallest *B. longimanus* (weight range: 5.8-53.8 μg), had a LC_{50} of 1.37 mg L^{-1} D.O. (1.19, 1.48), while weight class 8, the largest *B. longimanus* (weight range: 196.4-322.2 μg), had a LC_{50} of 2.11 mg L^{-1} D.O. (1.91, 2.61) (Table 6). The weight class analysis produced

similar results to the developmental stage analysis in that hypoxia tolerance decreased with increasing body size (Table 6). The single most hypoxia-tolerant *B. longimanus* survived a dissolved oxygen concentration of 1.13 mg L⁻¹ D.O. This individual, at 7.5 mm and 53.8 µg, was in the smallest weight class of tested *B. longimanus*.

Pooled *L. kindtii* dry weights were analyzed in quartiles. Quartile 1 (weight range: 0-19.0 µg) *L. kindtii* had a LC₅₀ of 0.54 mg L⁻¹ D.O. (0.47, 0.68), quartile 2 (19.1-32.7 µg) *L. kindtii* had a LC₅₀ of 0.50 mg L⁻¹ D.O. (0.19, 1.88), quartile 3 (32.8-45.1 µg) *L. kindtii* had a LC₅₀ of 0.52 mg L⁻¹ D.O. (0.39, 0.63), and quartile 4 (45.2-141.3 µg) *L. kindtii* had a LC₅₀ of 0.62 mg L⁻¹ D.O. (0.55, 0.74) (Table 7). These LC₅₀ estimates were not different from one another at a level of $p = 0.05$. The wide confidence interval around the LC₅₀ of quartile 2 was due to one *L. kindtii* that died in an oxygen saturation treatment (control) on 14 July 2015. It is assumed that this *L. kindtii* died due to injury during collection or transportation and not due to the dissolved oxygen exposure. It was the only animal in the study among both species to die in a control. The single most hypoxia-tolerant *L. kindtii* was 5.5 mm and 32.7 µg, and survived a D.O. concentration of 0.26 mg L⁻¹.

Linear regression was used to analyze the mean weights of *B. longimanus* and *L. kindtii* weight classes as predictors of dissolved oxygen LC₅₀. For the linear model (Eq. 3), *B. longimanus* mean weight was a significant predictor of LC₅₀ ($t = 7.24$, $p < 0.01$, adjusted $R^2 = 0.88$) (Table 8, Fig. 8). However, *L. kindtii* mean weight was not a significant predictor of LC₅₀ ($t = 1.63$, $p = 0.24$) (Table 8, Fig. 8).

Mean dry weights and LC₅₀ estimates were also transformed with a natural log operator and subjected to linear regression in order to produce estimates of power law parameters (Eq. 4). Again, *B. longimanus* mean weight was a significant predictor of LC₅₀ ($t = 4.40$, $p < 0.01$, adjusted $R^2 = 0.72$) and *L. kindtii* mean weight was not a significant predictor of LC₅₀ ($t = 0.95$, $p = 0.44$) (Table 9). The *B. longimanus* power law regression produced estimates of 0.56 for coefficient a , and 0.23 for coefficient b (Table 9). The adjusted R^2 value for the power law model was 0.72, which is less than that of the linear regression model (adjusted $R^2 = 0.88$) (Table 10). Thus, *B. longimanus* dissolved oxygen LC₅₀ is better explained as increasing linearly with dry weight, than in accordance with the power law.

Inter-lake hypoxia tolerance

Mille Lacs Lake *B. longimanus* yielded a LC₅₀ estimate that was significantly lower than the pooled results of all Island Lake hypoxia survival trials (Table 11). It is worth noting, though, that the Mille Lacs Lake trial was dominated by small *B. longimanus* (mean weight = 57 μg), which were significantly more hypoxia tolerant than large *B. longimanus* (Tables 2, 6). Caribou Lake *L. kindtii* yielded an LC₅₀ estimate of 0.46 mg L^{-1} (0.34, 0.57) that was not significantly different than the pooled LC₅₀ of all Island Lake hypoxia survival trials, 0.65 mg L^{-1} (0.55, 0.76) (Table 12).

A simulated trial was used to explore whether the inter-lake *B. longimanus* results were due to a true lake specific difference in hypoxia tolerance of the populations or, alternatively, the effect of testing a large proportion of small-bodied animals from Mille

Lacs Lake. The simulated trial was aimed at answering the question: What would have been the results of a trial with Island Lake *B. longimanus* that occurred in the same relative developmental stage proportions as the Mille Lacs Lake *B. longimanus*? First, pooled LC₅₀ estimates were calculated for each developmental stage of Island Lake *B. longimanus* (Table 13). A mock population was then established that reflected the relative proportions of stages 1, 2, and 3 *B. longimanus* tested from Mille Lacs Lake (Table 14). Then, the LC₅₀ and 95% confidence interval estimates of Island Lake *B. longimanus* were assigned a weighted average, to reflect the differing proportion of each developmental stage in the Mille Lacs Lake trial (Table 14). The calculation resulted in a LC₅₀ estimate of 1.59 mg L⁻¹ (1.39, 1.75), which is well within the confidence bounds of the Mille Lacs Lake *B. longimanus* trial, 1.32 mg L⁻¹ (1.24, 1.46) (Table 15). The data do not support an inter-lake difference in hypoxia tolerance for either species, but rather suggest the occurrence of different population size structures including a high proportion of small, hypoxia tolerant *B. longimanus* in Mille Lacs Lake at the time of collection.

Inter-season hypoxia tolerance

B. longimanus displayed a greater magnitude of temporal variation in LC₅₀ estimates (1.36-2.80 mg L⁻¹) than *L. kindtii* (0.46-0.82 mg L⁻¹) (Tables 2, 3). However, this may have been related to a size effect, since the mean weight of *B. longimanus* across the study also spanned a greater magnitude (57-175 µg) than that of *L. kindtii* (31.9-39.0 µg) (Tables 2, 3). Trials with high relative proportions of stage 1 *B. longimanus* tended to

have low LC₅₀ values (e.g. 20 July 2015), while the opposite was typical of trials with high relative proportions of stage 3 *B. longimanus* (e.g. 17 September 2015) (Table 2).

Hypoxia tolerance over a range of temperatures

The LC₅₀ of *B. longimanus* was significantly higher at 20°C and 25°C than at 10°C (Table 16) indicating that *B. longimanus* became less tolerant of hypoxia as trial temperatures increased. However, the statistical significance of this relationship is not clear, due in part to treatment concentrations that did not properly surround the LC₅₀ of the tested organisms (Table 17). For example, since proportion survival of the 20°C trial dropped from 1 at 7.43 mg L⁻¹ to 0.5 at 1.98 mg L⁻¹, the upper estimate of the 95% confidence interval is very wide (Tables 16, 17).

DISCUSSION

With a pooled LC_{50} of $0.58 \text{ mg L}^{-1} \text{ D.O.}$ (0.51, 0.65), *L. kindtii* is significantly more tolerant of fish-based hypoxia than *B. longimanus* which had a pooled LC_{50} of $1.65 \text{ mg L}^{-1} \text{ D.O.}$ (1.59, 1.71). These tolerance limits agree with previous in situ studies, which depict *B. longimanus* survival down to 2.4 mg L^{-1} and *L. kindtii* survival below 1 mg L^{-1} (Grigorovich et al. 1998, Vanderploeg et al. 2009, Vogt et al. 2013). These lab-based physiological results are consistent with landscape-scale ecological observations that *B. longimanus* successfully invades primarily mesotrophic and oligotrophic lakes while *L. kindtii* thrives across a wider range of lakes, including eutrophic ones (MacIsaac et al. 2000, Palmer et al. 2001).

B. longimanus displayed a considerable amount of inter-trial variation in hypoxia tolerance (Table 2). This variation likely resulted from a number of factors including seasonal changes in animal physiology, seasonal changes in population size distribution (e.g. proportion of stage 1 to stage 3 individuals), and the extent to which trial treatment levels properly surrounded the LC_{50} . It is difficult to differentiate between these effects with the present data set. However, some degree of seasonal variation in hypoxia tolerance is expected since other physiological traits, such as thermal tolerance, are well known to change seasonally (Garton et al. 1990).

This study compliments the results of a field study by Vanderploeg et al. (2009) conducted in the central basin of Lake Erie during the summers of 2005 and 2007. That study involved collecting depth-discrete zooplankton samples in August before peak hypoxia (hypolimnion $\text{D.O.} \geq 2.0 \text{ mg L}^{-1}$) and again in September during peak hypoxia

(hypolimnion D.O. < 2.0 mg L⁻¹). Before peak hypoxia, *B. longimanus* exhibited typical diel vertical migration (DVM), retreating to the dark hypolimnion during the day, and rising to the epilimnion at night. However, during peak hypoxia, the vertical distribution of *B. longimanus* was compressed and *B. longimanus* remained in the oxygenated epilimnion at all times of day despite a high risk of predation. In the same study, *L. kindtii* remained in the epilimnion during all sample dates and times. The only herbivorous zooplankton exhibiting avoidance of D.O. concentrations ≤ 2.0 mg L⁻¹ was *Daphnia mendotae*, a common prey item of both *B. longimanus* (Schultz and Yurista 1999) and *L. kindtii* (Lunte and Luecke 1990). The remaining species (e.g. *Bosmina longirostris*, *Daphnia longiremis*, etc.) were more hypoxia tolerant than *B. longimanus*, and were able to inhabit regions of the water column with D.O. concentrations ≤ 1.0 mg L⁻¹.

Vanderploeg et al. (2009) concluded that *B. longimanus* was forced to engage in DVM during periods of normoxia due to its conspicuous appearance (e.g. pigmented eyespot and brood chamber), while *L. kindtii* was permitted to remain in the epilimnion due to its body transparency. This is in agreement with De Bernardi and Giussani's (1975) observations from Lago Maggiore where planktivorous fish select *B. longimanus* over *L. kindtii*. Thus, *B. longimanus* appears to be more vulnerable to fish predation and this compounds the ecological implications of *B. longimanus* hypoxia intolerance.

Diel vertical migration in *L. kindtii*, while not noted in the aforementioned study, has been documented in eutrophic lakes where *L. kindtii* occurs without *B. longimanus* (Costa and Cummins 1969, Vijverberg 1991, Vogt et al. 2013). Under those conditions, it

is likely that *L. kindtii* engages in DVM to avoid higher levels of fish predation. My results, which indicate that *L. kindtii* is well-equipped to tolerate hypoxia ($> 0.6 \text{ mg L}^{-1}$) for up to 12 hours, are consistent with its migration to a hypoxic refuge as a survival strategy.

Hypoxia tolerance limits of other cladoceran zooplankton

The ecological implications of this study are further understood in the context of the hypoxia tolerance limits of other cladoceran zooplankton. *Daphnia pulex*, *Daphnia pulicaria*, *Daphnia magna*, *Daphnia longispina*, *Simocephalus vetulus*, and *Bosmina longirostris* have all been documented to survive D.O. concentrations below 1.0 mg L^{-1} (Herbert 1954, Kobayashi 1982, Weider and Lampert 1985, Hanazato et al. 1989, LaBerge and Hann 1990, Vanderploeg et al. 2009, Larsson and Lampert 2011). *Daphnia pulex*, in particular, has been well-studied, and seems to use a combination of hemoglobin production and filtering rate regulation to survive D.O. concentrations as low as 0.1 mg L^{-1} (Fox 1948, Kring and O'Brien 1976, Weider and Lampert 1985, LaBerge and Hann 1990). Thus, *B. longimanus* hypoxia intolerance may not only influence its ability to find refuge from fish predation, but also its ability to find prey in hypoxic eutrophic lakes. *L. kindtii*, on the other hand, is capable of withstanding levels of hypoxia that are comparable to the tolerance limits of many of its cladoceran prey species.

Daphnia mendotae is one species of cladoceran that is able to coexist effectively with *B. longimanus* in North American lakes. Interestingly, *D. mendotae* is less tolerant of hypoxia than all other *Daphnia* species, exhibiting 30% survival after six hours of

exposure to 2.0 mg L⁻¹ D.O. (Schultz and Yurista 1999, Vanderploeg et al. 2009, Pichlová and Vanderploeg 2011, Goto et al. 2012). *D. mendotae* does not have the ability to regulate filtering rates (and therefore oxygen uptake) at low D.O. concentrations, and instead exhibits filtering rates that decrease linearly with decreasing D.O. concentration (Heisey and Porter 1977). This physiological limitation does not allow *D. mendotae* to meet its oxygen requirements at concentrations below 2.0 mg L⁻¹. However, it is able to overlap habitat with *B. longimanus* due to its remarkably quick escape response, and large postabdominal claw, which is thought to provide significant predator avoidance (Pichlová-Ptáčnicková and Vanderploeg. 2011).

Size-based hypoxia tolerance

The smaller *B. longimanus* tended to be more hypoxia tolerant than their larger conspecifics. This is in agreement with the size-specific respiration model developed by Yurista (1999) and first principles on how physiological processes scale with body size (Peters 1983). Although mass-specific oxygen demand declines with increasing size, total oxygen demand increases with increasing size, consistent with the observed pattern in LC₅₀ (Peters 1983, Yurista 1999). A similar size-driven trend is likely occurring in *L. kindtii*, but it cannot be confirmed with the present data set.

Several physiological processes, such as respiration, scale in accordance to the power law equation: $R = a \cdot W^b$, where R is the biological variable of interest (e.g. rate of respiration), W is the weight of the organism, and a and b are determined empirically (Peters 1983). For many organisms, the parameter b is estimated at approximately 0.75

(Peters 1983). Yurista (1999) measured size and temperature-dependent respiration in *B. longimanus* and reported a b parameter estimate of 0.68. In comparison, when *B. longimanus* weights from this study were used to predict LC_{50} , the b estimate was 0.23. This means that *B. longimanus* hypoxia tolerance (LC_{50}) did not scale proportionately with respiration rate. Further, the results of this study revealed that *B. longimanus* LC_{50} scaled with weight better as a linear function (adjusted $R^2 = 0.88$) than a power function (adjusted $R^2 = 0.72$).

The size-based hypoxia tolerance pattern of *B. longimanus* has interesting implications for habitat-based selection related to refuge availability within a lake. The pattern would suggest that if *B. longimanus* is using the hypolimnion as refuge from fish predators, smaller-bodied *B. longimanus* should be more heavily selected for in lakes experiencing more severe hypoxia. Bilkovic and Lehman (1997) demonstrated that in Lake Erie, *B. longimanus* tended to be smaller-bodied than conspecifics from Lakes Heron and Michigan. They explained this as top-down control by visual planktivory, since the largest *B. longimanus* are the often most visible to fish (Zaret and Kerfoot 1975, Jarnigan et al. 2000). The pattern may be explained more effectively, however, through an interaction between top-down control and size-specific hypoxia tolerance, since the largest *B. longimanus* were likely the least tolerant of hypoxia stress during the season of collection (Scavia et al. 2014), forcing them into more significant spatial overlap with fish.

Hypoxia tolerance over a range of temperatures

B. longimanus appeared to be more tolerant of hypoxia when experiments were conducted at cooler temperatures (10-15°C) than at warmer temperatures (15-20°C) (Table 16). However, the statistical significance of this relationship is not clear due to trial treatment levels that did not properly surround the LC₅₀ (Table 17). Temperature-dependent survival and respiration has been measured in *B. longimanus* and *L. kindtii* (Moshiri et al. 1969, Garton et al. 1990, Yurista 1999). For both species, respiratory activity reached a minimum at around 5°C and increased at warmer temperatures. The 10°C trials presented here represent conditions that are elevated only slightly over optimal temperatures for reducing respiration rates, and therefore promoting hypoxia tolerance. In lakes where sharp declines in dissolved oxygen intersect warm water, as for example in the metalimnion of productive stratified lakes, temperature could play a major role in further reducing refuge for *B. longimanus*.

Mechanism of *L. kindtii* hypoxia tolerance

This study has shown that *L. kindtii* is significantly more tolerant of hypoxia than *B. longimanus*, even among individuals of similar body size (Fig. 8). The underlying mechanisms, however, are unclear. *Daphnia* makes use of the oxygen binding molecule hemoglobin as a facultative response to hypoxia exposure (Fox 1948). While adaptive in the presence of hypoxia, *Daphnia* also turns progressively more red as hemoglobin accumulates, rendering this tradeoff risky in the presence of visually searching predators. It is unlikely that *L. kindtii* uses hemoglobin to enhance hypoxia tolerance. First, red-

colored *L. kindtii* have never been detected. Second, it is incongruent with extreme body transparency as their major form of fish defense (Branstrator 2005). Interestingly, deep-red colored *B. longimanus* have been noted on occasion in collections from Island Lake Reservoir, both by myself and others, offering an intriguing avenue for future research on hypoxia tolerance.

Other than hemoglobin production, there are three mechanisms that may be responsible for the observed differences in hypoxia tolerance between *B. longimanus* and *L. kindtii*. First, it is possible that *L. kindtii* has a greater surface area to mass ratio than *B. longimanus*, permitting greater gas exchange per unit mass in *L. kindtii*. Cladoceran zooplankton obtain oxygen via diffusion across the body surface (Pennak 1989, Pirow et al. 1999a, Pirow et al. 1999b). The most striking morphological differences between the species include the presence of a stiff, chitinous caudal spine on *B. longimanus* and the presence of long, branched second antennae and a long, plump abdomen on *L. kindtii*. While it is unlikely that oxygen diffusion occurs through the chitinous spine on *B. longimanus*, the long branched antennae and long abdomen of *L. kindtii* may together provide enough additional surface area to allow *L. kindtii* to gather oxygen more efficiently than *B. longimanus*.

Second, it is possible that *B. longimanus* and *L. kindtii* have evolved different levels of oxygen demand per unit mass in relation to swimming style and overall activity. *B. longimanus* uses a frantic and constant swimming method during which the second antennae beat constantly. By comparison, *L. kindtii* propels itself with periodic long strokes of the second antennae often followed by an extended glide that may be more

energetically-favorable. These differences deserve further attention as they may have metabolic implications that directly relate to oxygen demand.

Third, it is possible that *L. kindtii* is able to engage in anaerobic metabolism to a greater extent than *B. longimanus*. Anaerobic metabolism has been documented in several aquatic invertebrate taxa but never directly studied in *B. longimanus* or *L. kindtii* (Grieshaber et al. 1994). A future study could quantify the relative amounts of lactate and succinate, common anaerobic respiration byproducts (Grieshaber et al. 1994), in *L. kindtii* and *B. longimanus* after they are exposed to hypoxia. Such studies have successfully identified biochemical differences between hypoxia-tolerant invertebrates (e.g. the midge *Chaoborus crystallinus*) and hypoxia intolerant invertebrates (e.g. the mosquito *Culex pipiens*) (Scholz and Zerbst-Boroffka 1998).

***B. longimanus* habitat modeling**

The results of this study have implications for understanding current and future range distributions of *B. longimanus*. First, the hypoxia tolerance limits of *B. longimanus* could be used to better inform habitat niche modeling efforts that predict lake susceptibility to invasion. Several studies have predicted ecosystem suitability to invasion based on lake variables such as surface area, maximum depth, and primary productivity (MacIsaac 2000, Potapov et al. 2011). My study supports dissolved oxygen content as an additional variable that should be considered when modeling *B. longimanus* habitat suitability. The presence of a well-oxygenated, low-light refuge from fish predation is

likely of more significance in determining habitat suitability for *B. longimanus* than it is for *L. kindtii*.

Many temperate lakes are becoming more eutrophic (Dodds et al. 2008). Cultural eutrophication of fresh waters causes an estimated \$2.2 billion of annual damages in the United States and will likely continue to impact North American lakes throughout the 21st Century (Dodds et al. 2008). Despite its problems for water quality overall, eutrophication has the interesting potential to reduce habitat quality for the invasive *B. longimanus* (Jankowski et al. 2006).

My results also have implications for *B. longimanus* habitat quality models in the face of climate change. It is predicted that continued warming of temperate lakes will prolong summer stratification, and exacerbate the severity and duration of hypolimnetic oxygen-depletion (De Stasio et al. 1996, Jankowski et al. 2006, Adrian et al. 2009). Minnesota lakes, in particular, are projected to be heavily impacted by climate change (Stefan and Fang 1994). The long-term model developed by Stefan and Fang (1994) predicts that climate change over the next several decades will decrease hypolimnetic oxygen concentrations by 2-8 mg L⁻¹ and extend periods of hypolimnetic anoxia by up to 80 days per year. Taken together, climate change and cultural eutrophication may provide additional barriers to *B. longimanus* success in North American lakes.

Finally, this study is relevant to the international and domestic issue of ballast water as a vector of species invasion. Ballast water from cargo ships has the potential to transport countless pathogens, plants, and animals in addition to those such as *B. longimanus* that have already established themselves in North America (Carlton and

Geller 1993). Nitrogen sparging and yeast-based bioreactors are being explored as anoxia ballast water treatment methods (Tamburri et al. 2002, De Lafontaine and Despatie 2014). Ballast water deoxygenation systems should be designed to not only affect planktonic adult *B. longimanus*, but also the resting egg stage. Brown (2008) documented that *B. longimanus* resting eggs are highly tolerant of hypoxia, surviving after 4 months at dissolved oxygen concentrations as low as 1.4 mg L⁻¹. My results, by contrast, indicate that maintaining a dissolved oxygen concentration of 1.0 mg L⁻¹ for 12 hours would be lethal to the planktonic life stage of *B. longimanus*, providing a benchmark for water treatment systems targeting this life stage.

Future directions

This study was limited by the availability of *B. longimanus* and *L. kindtii* in Island Lake. Greater availability of both species, as might come from reliable laboratory cultures, would allow for greater replication in identifying inter-temperature responses to hypoxia. To accurately represent natural conditions in a stratified lake, future studies could test *B. longimanus* and *L. kindtii* in response to repeated diel hypoxia exposure (12 hours hypoxia: 12 hours normoxia). It is possible that the hypoxia tolerance limits of an individual animal changes through acclimatization after repeated exposure. Additionally, very few studies have identified the long-term (> 1 week) effects of hypoxia exposure on zooplankton (Homer and Waller 1983). Long-term exposure may influence life-history traits such as birth size, age at maturation, and clutch size (Homer and Waller 1983).

Such traits carry great importance in determining *B. longimanus* establishment and success in newly-invaded lakes (Young et al. 2011, Kim and Yan 2013).

Conclusions

This study provides greater explanation for why *B. longimanus* is limited to mesotrophic and oligotrophic lakes while *L. kindtii* is able to persist under a wider range of lake conditions. Hypoxia intolerance and a conspicuous appearance (e.g., large eyespot, large caudal spine) likely make it difficult for *B. longimanus* to find a low-light refuge from fish predation in eutrophic lakes (Fig. 9). *L. kindtii*, on the other hand, is well equipped to tolerate hypoxia ($> 0.6 \text{ mg L}^{-1}$) for long periods of time (up to 12 hours). The combined effects of climate change and cultural eutrophication throughout the 21st Century will likely increase the occurrence of temperate lake hypoxia, and provide a barrier to *B. longimanus* invasion of North American lakes. Future attempts to manage the spread of *B. longimanus* (e.g. boat washing stations) should be concentrated on well-oxygenated oligotrophic lakes, such as those in northern Minnesota.

TABLES

Table 1. Dates and locations of zooplankton collections for 10°C hypoxia survival trials ordered by date for *B. longimanus* and then *L. kindtii*.

Species	Date of Collection	Lake
<i>B. longimanus</i>	5-Sep-2014	Island
<i>B. longimanus</i>	16-Sep-2014	Island
<i>B. longimanus</i>	7-Oct-2014	Island
<i>B. longimanus</i>	2-Jun-2015	Island
<i>B. longimanus</i>	18-Jun-2015	Island
<i>B. longimanus</i>	1-Jul-2015	Island
<i>B. longimanus</i>	19-Jul-2015	Mille Lacs
<i>B. longimanus</i>	19-Aug-2015	Island
<i>L. kindtii</i>	19-Aug-2014	Island
<i>L. kindtii</i>	7-Jul-2015	Caribou
<i>L. kindtii</i>	13-Jul-2015	Island
<i>L. kindtii</i>	12-Sep-2015	Island

Table 2. Summary of *B. longimanus* hypoxia survival trials conducted at 10°C including source lake, date of experiment, number of individuals per trial (n), proportions of animals in each developmental stage comprising a trial, and the mean dry weight of all animals in a trial, χ^2 (df = 1) goodness of fit statistic, and χ^2 goodness of fit p-value. A 95% confidence interval could not be calculated for the 17 September 2014 trial due to the small number of tested individuals (n = 40).

Species	Lake	Date	n=	LC ₅₀ (mg L ⁻¹)	95% Confidence Interval	Proportion Stage 1	Proportion Stage 2	Proportion Stage 3	Mean Weight ± 1 SD (µg)	χ^2 (1)	p-value
<i>Bythotrephes</i>	Island	6-Sep-14	80	1.81	(1.41, 1.97)	0.35	0.26	0.39	136 ± 56.4	91.9	< .001
<i>Bythotrephes</i>	Island	17-Sep-14	40	2.80	-	0.05	0.08	0.88	175 ± 36.5	37.6	< .001
<i>Bythotrephes</i>	Island	8-Oct-14	100	2.08	(1.95, 2.21)	0.12	0.76	0.12	153 ± 49.6	64.4	< .001
<i>Bythotrephes</i>	Island	3-Jun-15	100	1.86	(1.69, 1.99)	0.31	0.51	0.18	141 ± 47.4	61.2	< .001
<i>Bythotrephes</i>	Island	19-Jun-15	100	1.52	(1.33, 1.67)	0.13	0.64	0.23	144 ± 40.6	52.1	< .001
<i>Bythotrephes</i>	Island	2-Jul-15	50	1.59	(1.29, 1.80)	0.15	0.85	0.25	174 ± 42.5	37.0	< .001
<i>Bythotrephes</i>	Mille Lacs	20-Jul-15	120	1.36	(1.24, 1.45)	0.80	0.18	0.03	57 ± 33.1	69.0	< .001
<i>Bythotrephes</i>	Island	20-Aug-15	100	1.47	(1.38, 1.56)	0.19	0.53	0.28	135 ± 52.4	69.8	< .001

Table 3. Summary of *L. kindtii* hypoxia survival trials conducted at 10°C including source lake, date of experiment, number of individuals in a trial (n), mean dry weight of all animals in a trial, χ^2 (df = 1) goodness of fit statistic, and χ^2 goodness of fit p-value. *L. kindtii* from 20 August 2014 were not saved and were not included in any length or weight-based analyses.

Species	Lake	Date	n=	LC ₅₀ (mg L ⁻¹)	95% Confidence Interval	Mean Weight ± 1 SD (µg)	χ^2 (1)	p-value
<i>L. kindtii</i>	Island	20-Aug-14	80	0.62	(0.67, 0.73)	-	11.4	< .001
<i>L. kindtii</i>	Caribou	8-Jul-15	100	0.46	(0.34, 0.57)	39.0 ± 15.0	71.9	< .001
<i>L. kindtii</i>	Island	14-Jul-15	100	0.82	(0.24, 2.82)	33.9 ± 14.4	23.8	< .001
<i>L. kindtii</i>	Island	13-Sep-15	100	0.56	(0.49, 0.62)	31.9 ± 31.8	68.7	< .001

Table 4. Pooled results of all *B. longimanus* and *L. kindtii* hypoxia trials conducted at 10°C during the summers of 2014 and 2015. The results from both study lakes were included for both species. B₀ and B₁ represent the parameter estimates of the logistic regression. χ^2 indicates the logistic regression model goodness of fit statistic (df = 1), followed by the goodness of fit p-value.

Species	n=	LC ₅₀ (mg L ⁻¹)	95% Confidence Interval	B ₀	B ₁	χ^2 (1)	p-value
<i>B. longimanus</i>	690	1.65	(1.59, 1.72)	-2.49	4.12	386.3	< .001
<i>L. kindtii</i>	380	0.58	(0.51, 0.65)	-2.40	4.11	193.6	< .001

Table 5. Pooled results of *B. longimanus* developmental stage analysis. All *B. longimanus* tested at 10°C from both lakes (Island and Mille Lacs) were used for this analysis. B₀ and B₁ represent the parameter estimates of the logistic regression. χ^2 indicates the logistic regression model goodness of fit statistic (df = 1), followed by the goodness of fit p-value.

Developmental Stage	n=	LC₅₀ (mg L⁻¹)	95% Confidence Interval	B₀	B₁	χ^2 (1)	p-value
1	203	1.40	(1.28, 1.51)	-4.97	3.54	99.8	< .001
2	322	1.68	(1.59, 1.76)	-7.20	4.29	209.9	< .001
3	165	1.95	(1.82, 2.14)	-6.36	3.26	108.1	< .001

Table 6. Pooled results of *B. longimanus* weight class analysis. *B. longimanus* dry weights were separated into eight even-width percentile ranges, and an LC₅₀ estimate was calculated for each. The proportion of each developmental stage in each weight class is noted. All *B. longimanus* tested at 10°C from both lakes (Island and Mille Lacs) were used for this analysis. B₀ and B₁ represent the parameter estimates of the logistic regression. χ^2 indicates the logistic regression model goodness of fit statistic (df = 1), followed by the goodness of fit p-value. Superscript numbers on the 95% confidence intervals indicate significant difference at a level of p = 0.05. Lack of common superscripts indicates significant difference.

Weight Class	Percentile	Weight Range (µg)	n=	Mean Weight ± 1 SD (µg)	LC ₅₀ (mg L ⁻¹)	95% Confidence Interval	Proportion Stage 1	Proportion Stage 2	Proportion Stage 3	B ₀	B ₁	χ^2 (1)	p-value
1	0-12.5	5.8-53.8	89	36.1 ± 12.6	1.37	(1.19, 1.48) ^a	0.99	0.01	0.00	-7.36	5.38	44.8	< 0.01
2	12.6-25.0	53.9-90.9	87	73.7 ± 11.4	1.44	(1.16, 1.66) ^{a,b}	0.80	0.18	0.01	-4.32	2.99	42.2	< 0.01
3	25.1-37.5	91.0-118.0	84	104.7 ± 8.0	1.45	(1.25, 1.63) ^{a,b}	0.33	0.63	0.04	-5.80	4.00	50.3	< 0.01
4	37.6-50.0	118.1-133.4	86	125.6 ± 5.0	1.67	(1.49, 1.82) ^{b,c}	0.10	0.84	0.06	-6.81	4.09	46.8	< 0.01
5	50.1-62.5	133.5-154.2	85	143.7 ± 5.6	1.65	(1.45, 1.82) ^{a,b,c}	0.02	0.84	0.14	-6.67	4.03	50.6	< 0.01
6	62.6-75.0	154.3-173.4	87	164.4 ± 5.5	1.69	(1.49, 1.86) ^{b,c}	0.02	0.67	0.31	-7.17	4.24	58.6	< 0.01
7	75.1-87.5	173.5-196.3	85	184.0 ± 6.5	1.95	(1.81, 2.10) ^{c,d}	0.04	0.49	0.47	-11.43	5.86	72.8	< 0.01
8	87.6-100	196.4-322.2	87	222.5 ± 24.5	2.11	(1.91, 2.61) ^d	0.00	0.11	0.89	-6.20	2.93	54.0	< 0.01

Table 7. Pooled results of *L. kindtii* weight class analysis including four quartiles, the number of individuals per quartile, the mean weight and standard deviation of each quartile, and the LC₅₀ estimate. None of the weight classes were different from one another at a level of $p = 0.05$. All *L. kindtii* tested at 10°C from both lakes (Island and Caribou) were used for this analysis. B₀ and B₁ represent the parameter estimates of the logistic regression. χ^2 indicates the logistic regression model goodness of fit statistic (df = 1), followed by the goodness of fit p-value.

Weight Range	n=	Mean Weight ± 1 SD (µg)	LC₅₀ (mg L⁻¹)	95% Confidence Interval	B₀	B₁	χ^2 (1)	P-value
0-19.0 µg	79	12.6 ± 4.2	0.54	(0.47, 0.68)	-4.41	8.03	54.8	< .001
19.1 µg – 32.7 µg	74	25.7 ± 3.6	0.50	(0.19, 1.88)	-0.24	0.48	17.5	< .001
32.8 µg – 45.1 µg	75	39.1 ± 3.5	0.52	(0.39, 0.63)	-3.97	7.69	51.4	< .001
45.2 µg – 141.3 µg	72	64.7 ± 21.1	0.62	(0.55, 0.74)	-9.34	15.17	75.7	< .001

Table 8. Weight class linear regression results. The mean weight of each *B. longimanus* and *L. kindtii* weight class was used to predict dissolved oxygen LC₅₀. The results from both study lakes were included for both species. *a* represents the slope coefficient of the linear regression.

Species	n=	<i>a</i> estimate	<i>a</i> estimate p-value	Adjusted R ²
<i>B. longimanus</i>	690	4.0 x 10 ⁻⁴	3.5 x 10 ⁻⁴	0.88
<i>L. kindtii</i>	300	1.8 x 10 ⁻³	0.24	0.36

Table 9. Weight class power law regression results. The mean weight of each *B. longimanus* and *L. kindtii* weight class was used to predict dissolved oxygen LC₅₀. The results from both study lakes were included for both species. *b* represents the slope coefficient of the linear regression.

Species	n=	<i>b</i> estimate	<i>b</i> estimate p-value	Adjusted R ²
<i>B. longimanus</i>	690	0.23	4.5 x 10 ⁻³	0.72
<i>L. kindtii</i>	300	0.08	0.44	0

Table 10. Comparison of linear and power law models used for predicting *B. longimanus* (n=690) dissolved oxygen LC₅₀ based on mean dry weight of eight percentile-based weight classes. *W* in each equation represents mean dry weight.

Model	Equation	Adjusted R²
Linear	$LC_{50} = 0.004 \times W + 1.1331$	0.88
Power Law	$LC_{50} = 0.56 \times W^{0.23}$	0.72

Table 11. Inter-lake results of *B. longimanus* hypoxia survival trials including lake of collection, dates of trial, total number of individuals (n) tested, and dissolved oxygen LC₅₀ estimate. χ^2 indicates the logistic regression model goodness of fit statistic (df = 1), followed by the goodness of fit p-value.

Species	Lake	Date(s)	n=	LC ₅₀ (mg L ⁻¹)	95% Confidence Interval	χ^2 (1)	p-value
<i>B. longimanus</i>	Island	See Table 2	570	1.74	(1.67, 1.81)	352.8	< 0.001
<i>B. longimanus</i>	Mille Lacs	20 July 2015	120	1.36	(1.24, 1.45)	69.0	< 0.001

Table 12. Inter-lake results of *L. kindtii* hypoxia survival trials including lake of collection, dates of trial, total number of individuals (n) tested, LC₅₀ estimate, and dissolved oxygen LC₅₀ estimate. χ^2 indicates the logistic regression model goodness of fit statistic (df = 1), followed by the goodness of fit p-value.

Species	Lake	Date(s)	n=	LC ₅₀ (mg L ⁻¹)	95% Confidence Interval	χ^2 (1)	p- value
<i>L. kindtii</i>	Island	20 August 2014, 14 July 2015, 13 September 2015	280	0.65	(0.55, 0.76)	137.2	< 0.001
<i>L. kindtii</i>	Caribou	6 July 2015	100	0.46	(0.34, 0.57)	71.9	< 0.001

Table 13. Pooled results of *B. longimanus* developmental stage analysis for Island Lake hypoxia survival trials only. B_0 and B_1 represent the parameter estimates of the logistic regression. χ^2 indicates the logistic regression model goodness of fit statistic (df = 1), followed by the goodness of fit p-value.

Developmental Stage	n=	LC₅₀ (mg L⁻¹)	95% Confidence Interval	B₀	B₁	χ^2 (1)	p-value
1	107	1.55	(1.33, 1.74)	-4.65	3.00	58.6	< 0.001
2	301	1.69	(1.61, 1.77)	-7.34	4.33	195.7	< 0.001
3	162	1.97	(1.84, 2.17)	-6.47	3.28	108.9	< 0.001

Table 14. Calculation of simulated *B. longimanus* LC₅₀ based on the proportion of each developmental stage found in the Mille Lacs Lake trial. The value in Island Lake trials column was multiplied by the proportion of Mille Lacs Lake trial column to produce a stage estimate. The sum of the stage estimate values for each developmental stage produced an LC₅₀ and associated confidence interval, 1.59 mg L⁻¹ (1.39, 1.75).

Parameter	Value in Island Lake Trials (mg L ⁻¹)	Proportion of Mille Lacs Lake Trial	Stage Estimate (mg L ⁻¹)	Pooled Estimate (mg L ⁻¹)
LC ₅₀ – Stage 1	1.55	0.8	1.24	
LC ₅₀ – Stage 2	1.69	0.175	0.30	1.59
LC ₅₀ – Stage 3	1.97	0.025	0.05	
LC ₅₀ - Low – Stage 1	1.33	0.8	1.06	
LC ₅₀ - Low – Stage 2	1.61	0.175	0.28	1.39
LC ₅₀ - Low – Stage 3	1.84	0.025	0.05	
LC ₅₀ - High – Stage 1	1.74	0.8	1.39	
LC ₅₀ - High – Stage 2	1.77	0.175	0.31	1.75
LC ₅₀ - High – Stage 3	2.17	0.025	0.05	

Table 15. Results of the simulated *B. longimanus* hypoxia survival trial comparing the Island Lake and Mille Lacs Lake populations of *B. longimanus*.

Species	Lake	Date(s)	n=	LC₅₀ (mg L⁻¹)	95% Confidence Interval
<i>B. longimanus</i>	Mille Lacs	20 July 2015	120	1.32	(1.24, 1.46)
<i>B. longimanus</i>	Simulated	-	100	1.59	(1.39, 1.75)

Table 16. *B. longimanus* temperature test on 2-3 July 2015. Superscript numbers on the 95% confidence intervals indicate significant difference at a level of $p = 0.05$. Lack of common superscripts indicates significant difference. χ^2 indicates the logistic regression model goodness of fit statistic ($df = 1$), followed by the goodness of fit p-value.

Temperature (°C)	n=	LC ₅₀ (mg L ⁻¹)	95% Confidence Interval	χ^2 (1)	p-value
10	50	1.59	(1.29, 1.80) ¹	37.0	< 0.001
15	50	1.51	(1.25, 1.86) ^{1, 2}	34.2	< 0.001
20	50	2.00	(1.81, 6.54) ²	38.7	< 0.001
25	50	1.92	(1.64, 2.13) ^{1, 2}	48.0	< 0.001

Table 17. *B. longimanus* temperature test treatments on 2-3 July 2015. Five treatment D.O. concentrations with ten replicates each were tested at each temperature.

Temp. (°C)	Median D.O. Level ± 1 SD (mg L ⁻¹)	n=	Proportion Survival
10	8.22 ± 0.15	10	1
10	2.21 ± 0.05	10	1
10	2.00 ± 0.05	10	0.8
10	1.56 ± 0.07	10	0.5
10	0.87 ± 0.05	10	0
15	7.57 ± 0.14	10	1
15	2.55 ± 0.03	10	1
15	2.18 ± 0.02	10	0.9
15	1.30 ± 0.02	10	0.3
15	1.14 ± 0.07	10	0.2
20	7.43 ± 0.08	10	1
20	1.98 ± 0.04	10	0.5
20	1.71 ± 0.05	10	0.2
20	1.18 ± 0.04	10	0
20	0.60 ± 0.04	10	0
25	7.08 ± 0.05	10	1
25	2.30 ± 0.04	10	0.9
25	1.90 ± 0.06	10	0.5
25	1.17 ± 0.08	10	0
25	1.02 ± 0.05	10	0

FIGURES

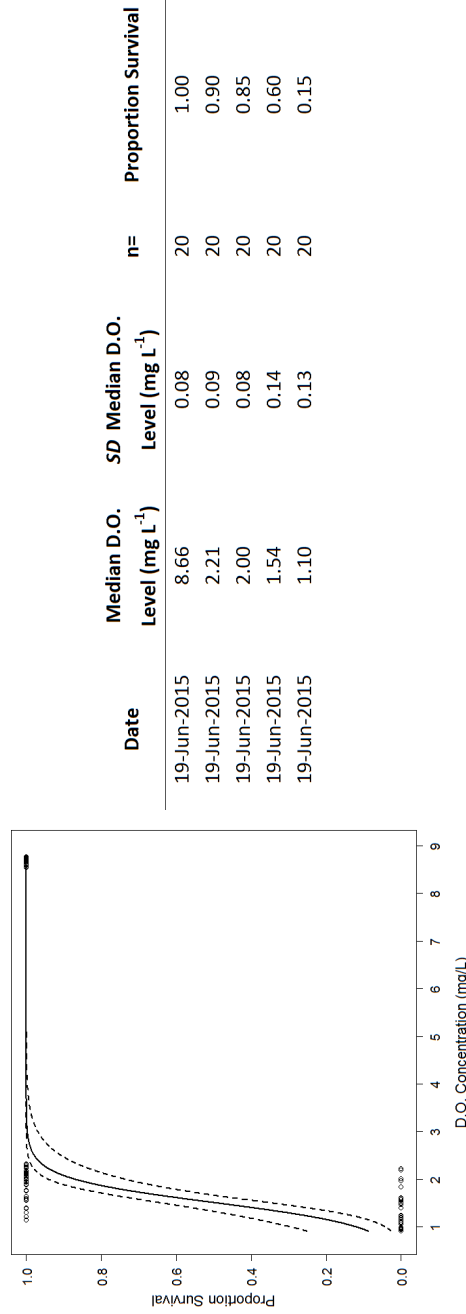


Figure 1. Sample result of a 10°C *B. longimanus* hypoxia trial from 19 June 2015. A typical trial consisted of five D.O. treatment levels, one of which served as a control and did not involve any nitrogen sparging. Ideally, the D.O. treatment levels would produce a range of survival proportions, both above and below 0.5. For this particular trial, logistic regression produced an LC₅₀ estimate of 1.52 mg L⁻¹ (1.33, 1.67).

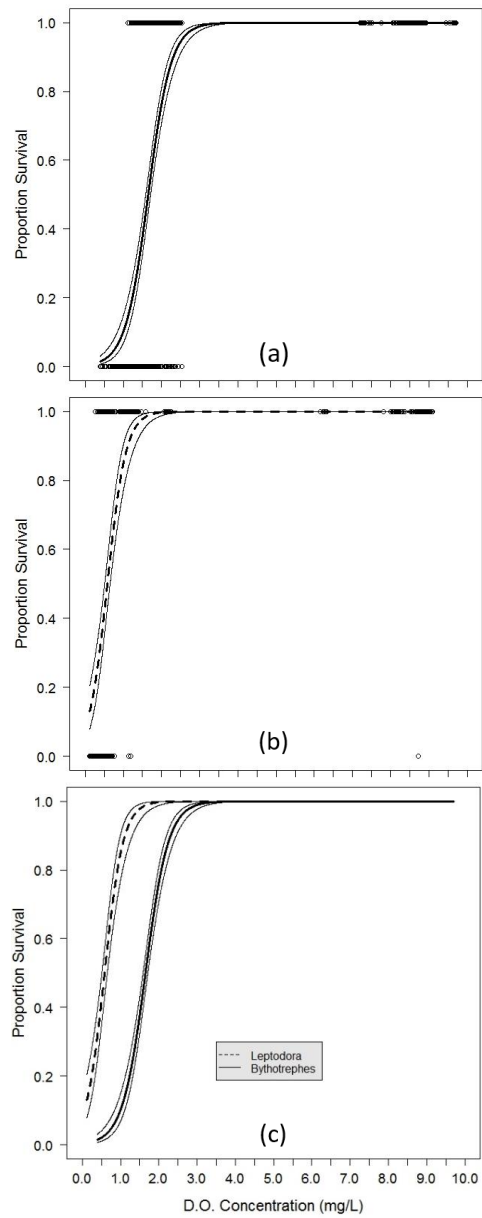


Figure 2. Hypoxia tolerance curves and raw data of *B. longimanus* (n=690) (panel a) and *L. kindtii* (n=380) (panel b), and the curves only for both species (panel c) based on the pooled results of all experiments conducted at 10°C during 2014 and 2015. The thin solid lines represent 95% confidence limits.

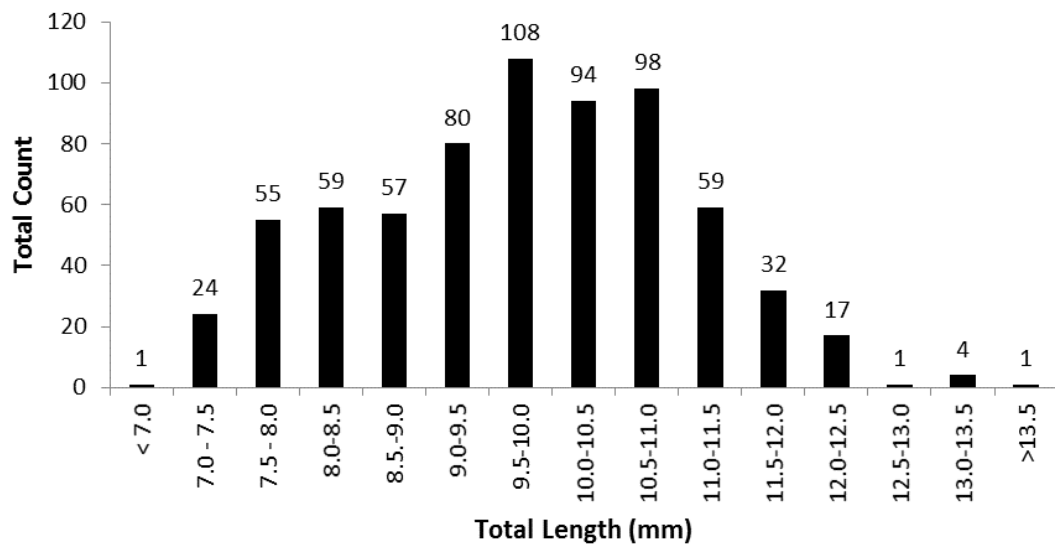


Figure 3. Length distribution of all *B. longimanus* (n=690) subjected to hypoxia trials during 2014 and 2015. All *B. longimanus* tested at 10°C from both lakes (Island and Mille Lacs) are shown.

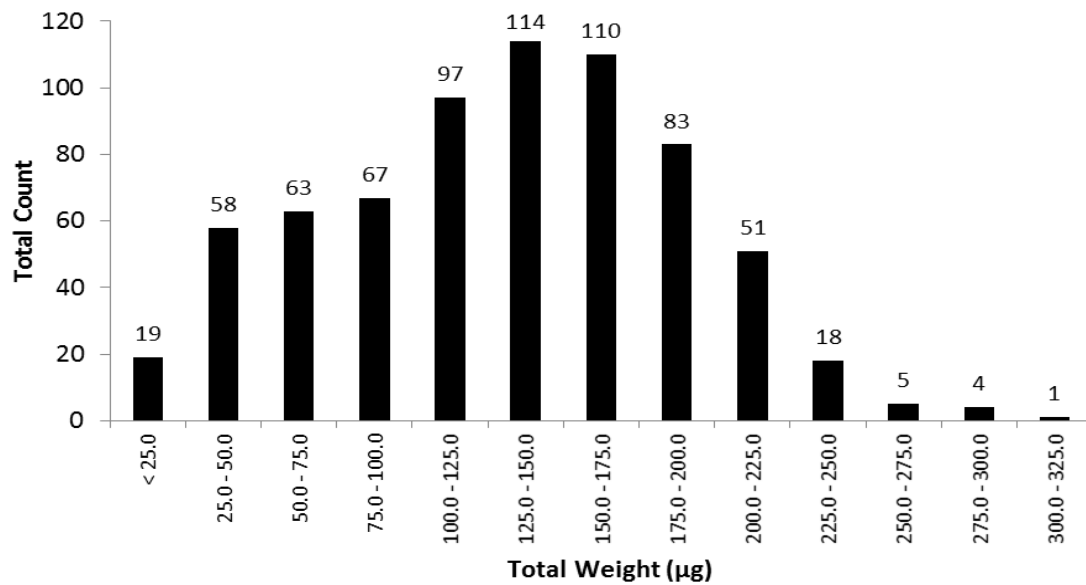


Figure 4. Weight distribution of all *B. longimanus* (n=690) subjected to 10°C hypoxia trials during 2014 and 2015. All *B. longimanus* tested at 10°C from both lakes (Island and Mille Lacs) are shown.

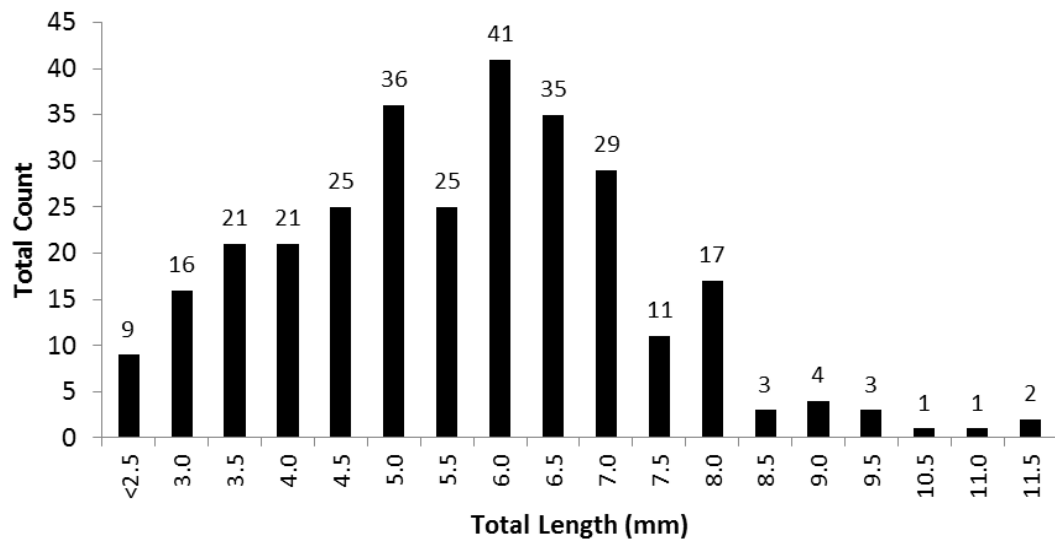


Figure 5. Length distribution of most *L. kindtii* (n=300) subjected to 10°C hypoxia trials during 2014 and 2015. The total length of *L. kindtii* spans from the center of the compound eye to the base of the caudal stylets. *L. kindtii* from 20 August 2014 were not saved and were not included in this analysis.

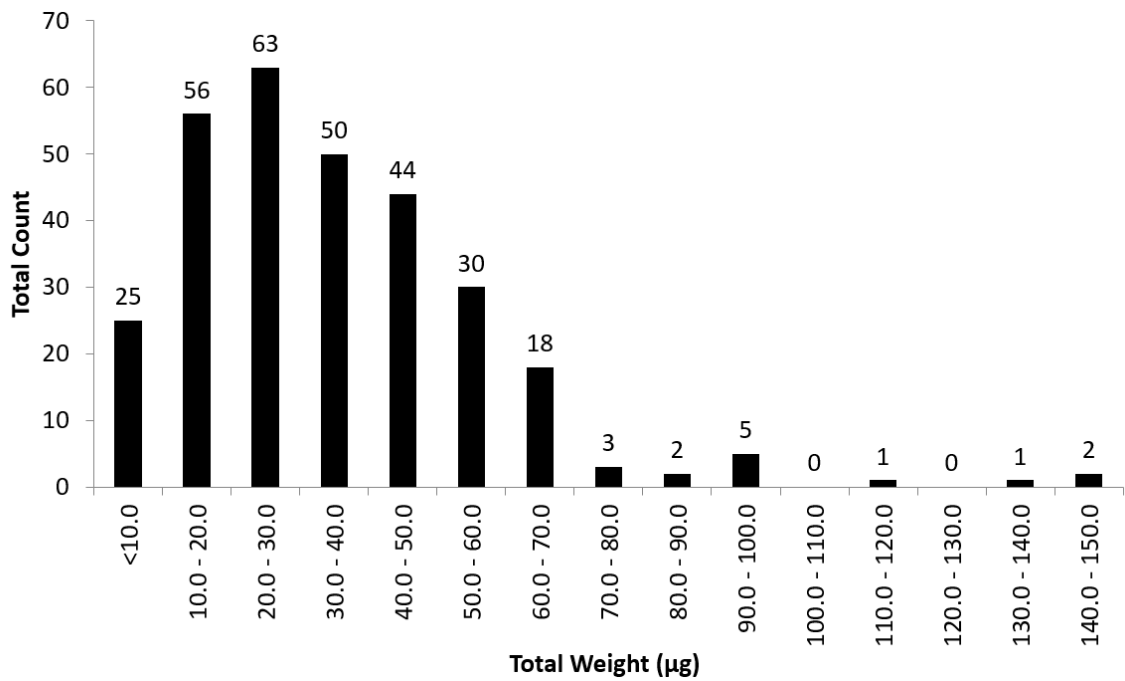


Figure 6. Weight distribution of most *L. kindtii* (n=300) subjected to hypoxia trials during 2014 and 2015. *L. kindtii* from 20 August 2014 were not saved and were not included in this analysis.

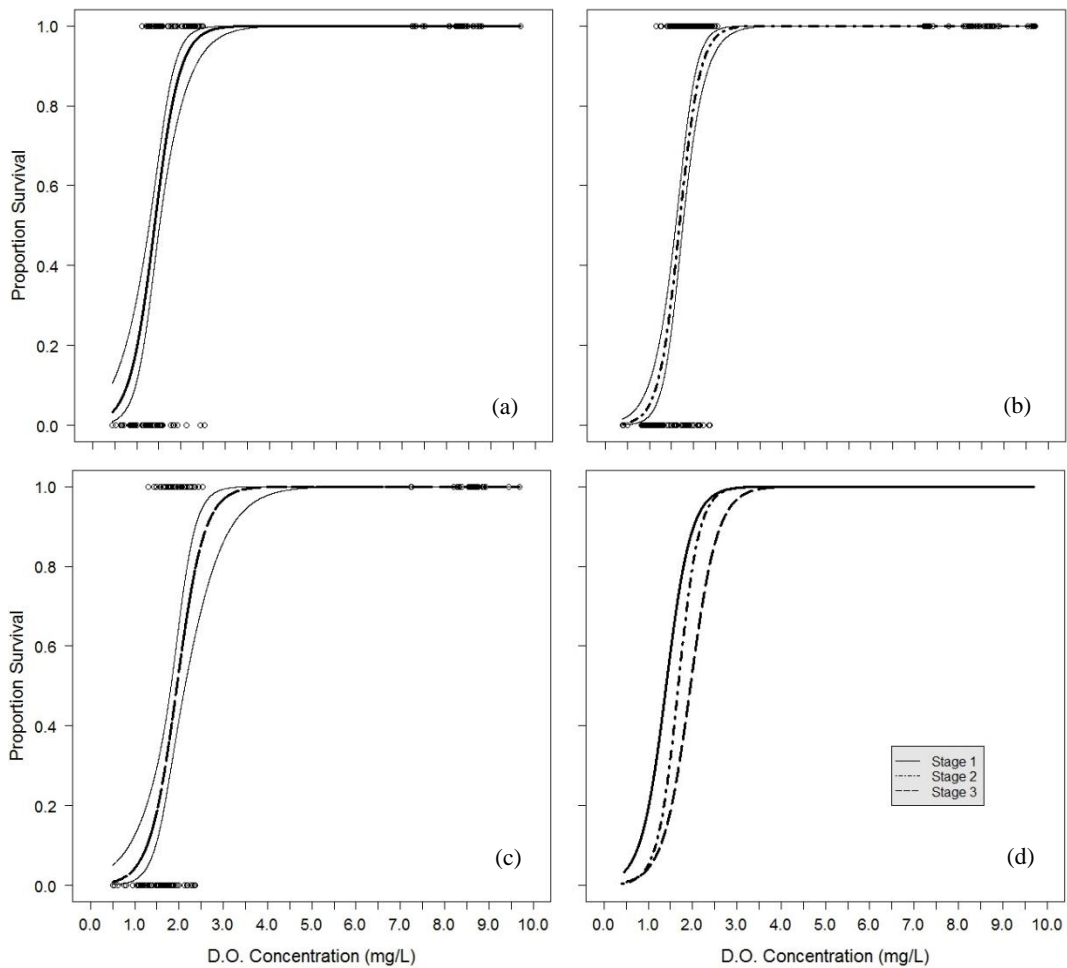


Figure 7. Hypoxia tolerance curves and raw data for *B. longimanus* stage 1 (panel a), stage 2 (panel b), and stage 3 (panel c), and the curves only for all stages (panel d) based on the pooled results of all 10°C experiments conducted during 2014 and 2015. The 95% confidence limits in panel d were excluded in order to see the regression lines. The LC50 estimates of all three stages were significantly different at a level of $p = 0.05$.

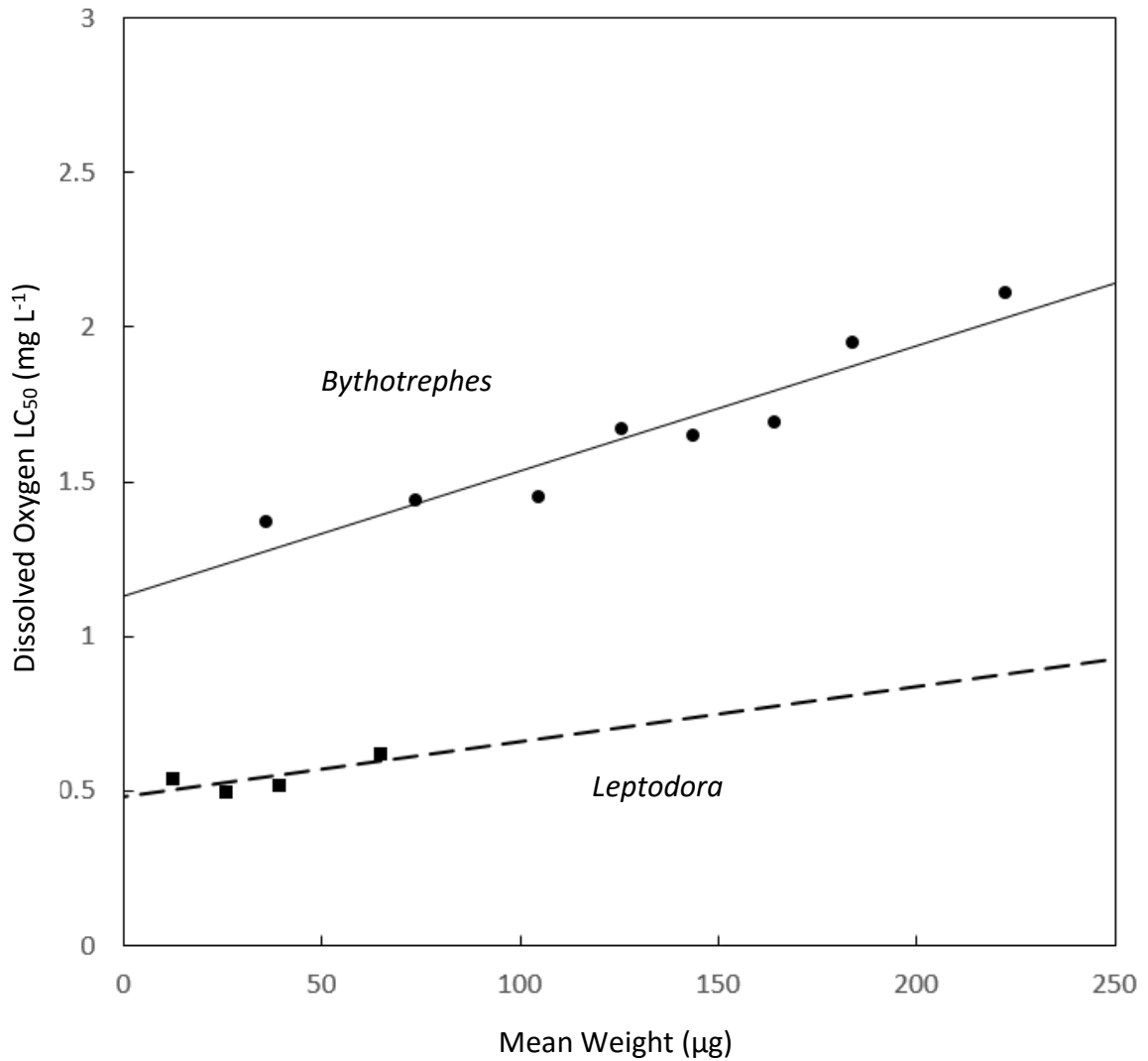


Figure 8. Linear regression using mean weight as a predictor of dissolved oxygen LC₅₀. *B. longimanus* (n=690) dry weights were apportioned into eight weight classes at 12.5 percentile intervals, while *L. kindtii* (n=300) dry weights were split into quartiles. Each circle represents the mean weight of a *B. longimanus* weight class, while each square represents the mean weight of a *L. kindtii* quartile. The slope of the *B. longimanus* line was significantly different from zero (ANOVA, $F_{1,6} = 52.5$, $p < 0.001$), while the slope of the *L. kindtii* line was not (ANOVA, $F_{1,2} = 2.7$, $p = 0.24$).

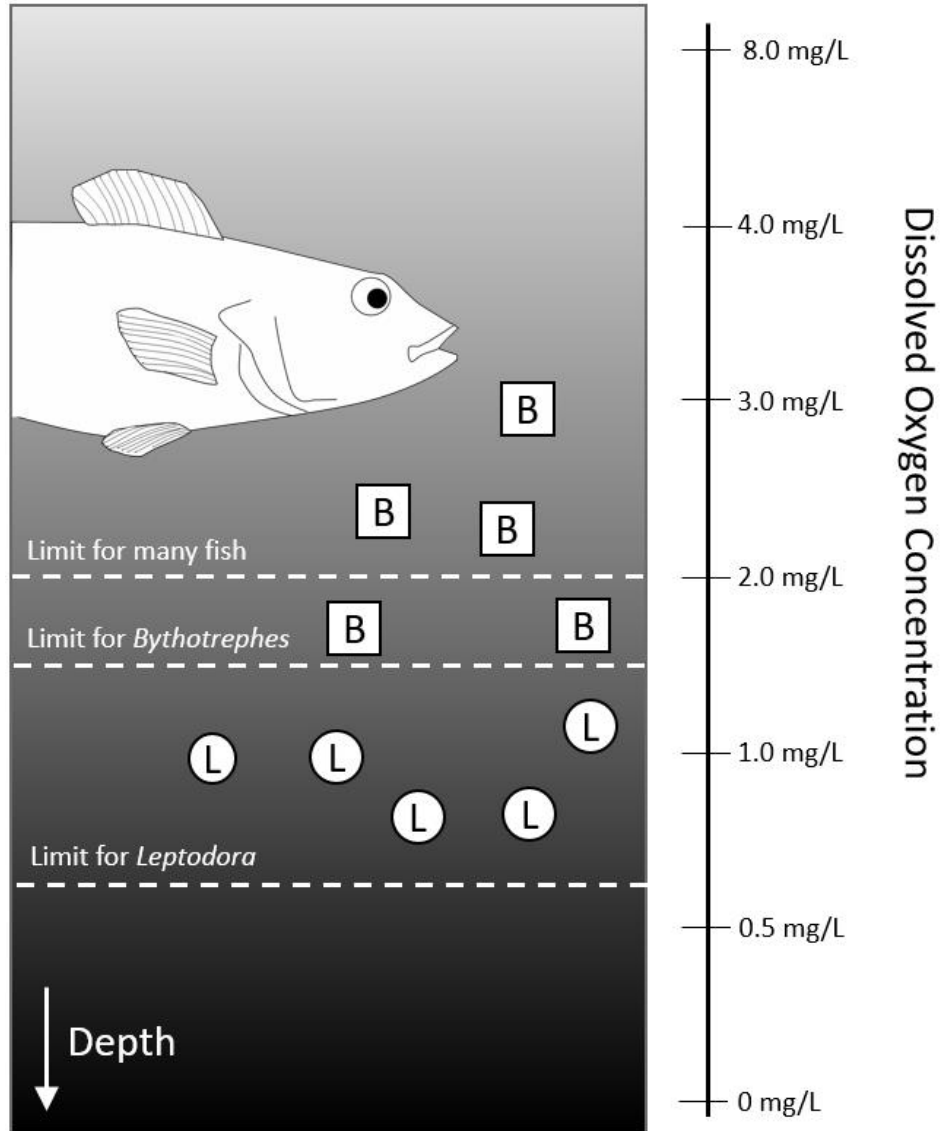


Figure 9. Implications of hypoxia tolerance for deep water refuge of *B. longimanus* (square symbol) and *L. kindtii* (circle symbol) in a eutrophic lake. Many fish cannot withstand hypoxia below 2 mg/L (Vanderploeg et al. 2009), while the hypoxia limits (12 hour LC₅₀ at 10°C) for *B. longimanus* and *L. kindtii* are 1.65 mg/L and 0.58 mg/L, respectively.

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APPENDIX A: LOGISTIC REGRESSION R CODE

```
1 dat <- read.table(file = "PooledBythotrephe.csv", header = T, sep =
2 head(dat)
3
4 fit <- glm(Survival ~ D.O., family = binomial, data = dat)
5 summary(fit)
6 xhat <- seq(min(dat$D.O.), max(dat$D.O.), 0.001)
7 preDat <- data.frame(D.O. = xhat)
8 mike <- predict(fit, newdata = preDat, type = "link", se.fit = TRUE)
9 uPred <- mike$fit + 1.96 * mike$se.fit
10 lPred <- mike$fit - 1.96 * mike$se.fit
11 uCI <- plogis(uPred)
12 lCI <- plogis(lPred)
13
14 plot(xhat,
15       plogis(mike$fit), ylim = c(0, 1), type = "l", lwd = 2,
16       ylab = "survival", xlab = "D.O.", xaxt = "n", las = 1)
17
18 axis(1, at = seq(0.38, 8.33, 0.1))
19 lines(xhat, uCI, lty = 2, lwd = 2)
20 lines(xhat, lCI, lty = 2, lwd = 2)
21
22 xhat[which.min(abs(uCI - 0.5))]
23 xhat[which.min(abs(lCI - 0.5))]
24 -fit$coef[1] / fit$coef[2]
25 |
```