Indirect versus Direct Effects of Freshwater Browning on Larval Fish Foraging

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ABSTRACT

1) The larval stage in fish development is characterized as a 'critical period' with unusually high mortality rates. Additional stress may be imposed on fish larvae as fresh waters 'brown' in color due to increased inputs of dissolved organic matter from the terrestrial watershed. Both energy transfer and foraging efficacy are predicted to decrease as light availability declines with browning.
2) Here, we investigated the balance between indirect versus direct effects of browning on zooplankton-larval fish interactions. Experimental tanks were established in the laboratory where water color was altered with increasing concentrations of SuperHume (water color measured as absorbance at 440 nm = 1.6 – 10.8 m^{-1}). Phytoplankton and zooplankton densities were monitored across treatments for one month to assess possible changes in community structure leading up to fish spawning (i.e., late Spring in central Virginia, USA). We then introduced larval largemouth bass to the tanks to assess indirect effects on larval fish feeding rate and growth. Additionally, direct effects on the foraging efficiency and selectivity of largemouth bass and bluegill were determined with separate short-term feeding experiments using the same experimental setup.
3) Browning did not directly alter the ability of larval fish to see and capture prey across our brown water treatments. However, significant indirect effects on larval fish foraging, growth, and survival were observed as phytoplankton and zooplankton densities decreased with increasing water color.
4) Our data suggest lake browning will reduce energy transfer to larval fish, during a critical stage of development, due to a reduction in prey availability but not visual foraging.

1. INTRODUCTION

In recent decades, many freshwater and coastal ecosystems in the Northern Hemisphere have become browner in color due to an increased export of chromophoric dissolved organic matter (cDOM) from the terrestrial watershed (Monteith et al. 2007; Erlandsson et al. 2008; Haaland et al. 2010; Solomon et al. 2015). The mechanisms underlying this 'browning' are currently debated and include changes in hydrology and land use, increased global temperatures, reduced atmospheric acid deposition, and increased inputs of dissolved iron (Freeman et al. 2001; Monteith et al. 2007; Erlandsson et al. 2008; Kritzberg and Ekström 2012). Whatever the mechanism, this phenomenon has far reaching ecological consequences for the structure and function of aquatic ecosystems, including energy flow.

One concern is the effect of browning on the underwater light environment. As waters become browner in color, the quantity of light in the water column is reduced, resulting in a shallower euphotic zone (Bukaveckas and Robbins-Forbes 2000; Einem and Graneli 2010). In addition, shorter wavelength ultraviolet and visible radiation are more readily absorbed by cDOM compared to longer wavelengths (Morris et al. 1995; Wetzel 2001). Consequently, the light environment shifts to the red portion of the visible spectrum. These alterations in the quantity and quality of light have the potential to affect zooplankton-fish interactions--indirectly through reduced energy transfer up the food chain and directly through reduced foraging efficiency.

While recent studies have shown that the initial browning of low productive systems can stimulate primary production (Ask et al. 2012; Seekell et al. 2015; Williamson et al. 2015), excessive browning of fresh waters can reduce rates of photosynthesis due to competition for

photons (Kirk 1994). cDOM has been reported to sequester 10 times more photons within the visible spectrum (400 - 700 nm) compared to photosynthetic pigments (Thrane et al. 2014). As a consequence, areal primary production often decreases with increased water color (Jones 1992; Carpenter et al. 1998; Ask et al. 2009; Karlsson et al. 2009; Thrane et al. 2014), and pelagic primary production typically exceeds benthic primary production (Vasconcelos et al. 2018; Vasconcelos et al. 2019). Phytoplankton community composition can also shift to predominantly cyanobacteria, which are generally less nutritious or inedible to zooplankton consumers (Ekvall et al. 2013; Robidoux et al. 2015). Overall, aquatic ecosystems often become net heterotrophic with increased browning as bacterial production exceeds primary production (Cole et al. 1994; Ask et al. 2012).

Reductions in the quantity and quality of basal resources then 'cascade up' the grazer food chain to influence zooplankton and fish. For example, Robidoux et al. (2015) observed decreases in crustacean zooplankton biomass and density with increased water color while Craig et al. (2017) found that bluegill in lakes of increasing color were smaller in size and had lower fecundity compared to those living in lakes with less color. Taipale et al. (2018) noted that zooplankton and fish had poorer nutritional quality in browner systems because the phytoplankton on which they fed had lower concentrations of essential fatty acids, proteins, lipids, and carbohydrates. Combined, these results suggest that freshwater browning indirectly alters energy flow to higher trophic levels, reducing ecosystem productivity.

Freshwater browning may also directly affect fish foraging behavior by altering the light environment to which fish are adapted. Many fish are visually orienting predators, depending on the quantity (i.e. intensity) and quality (i.e. spectra) of underwater light to locate and

capture prey (Guthrie and Muntz 1993; Leech and Johnsen 2009). However, results from studies investigating direct effects of browning on fish foraging are varied. Some have noted reductions in foraging efficiency as water color darkens (Estlander et al. 2010; Ranaker et al. 2012; Stasko et al. 2012; Jönsson et al. 2013; Weidel et al. 2017) while others have found no effect of browning (Jönsson et al. 2103; Nurminen et al. 2014) or enhanced effects (Jönsson et al. 2103). This suggests that the effects of browning on fish foraging may be species-specific. It is also important to note that most studies have focused on juvenile or adult life history stages.

The larval stage represents an especially critical phase in fish development that ultimately affects population growth and fish biomass (Hjort 1914; Fuiman and Werner 2002; Karlsson et al. 2009; Karlsson et al. 2015). Because of their dependence on shorter wavelengths of light for foraging, early life history stages may be particularly affected by freshwater browning. Over evolutionary time, the visual system of fishes spectrally tunes to the intensity and wavelengths of light present in their environment (Douglas and Djamgoz 1990). This spectral tuning often varies with age and a fish's behavior. Larval fish spend most of their time foraging in top few meters of the eplimnion, relying on short-wavelength ultraviolet and blue radiation, while older fish predominantly forage at deeper depths on larger prey, relying on longer wavelength green, yellow, and red light (Leech and Johnsen 2009).

Here, we use laboratory experiments to assess the indirect versus direct effects of browning on larval fish foraging at the time of hatching (i.e., late spring/early summer in central Virginia, USA). We hypothesized that increased browning will reduce phytoplankton biomass, leading to a reduction in zooplankton abundance, and consequently larval fish foraging efficiency, growth, and survival during an early, critical stage in development. We then used

the same laboratory set up to examine the direct effects of browning on fish feeding rate and prey selectivity when given equal zooplankton prey, hypothesizing that fish larvae will consume less prey as water color increases due to a reduction in light availability for foraging.

2. METHODS

2.1 Indirect Effects of Browning

Laboratory experiments were conducted in twelve 20 L glass aquaria assembled on three shelves with four tanks per shelf. The entire shelving unit, including individual shelves, was covered in black plastic with additional black plastic placed in between each tank. Light was provided by 1.2 m long grow lamps containing two Lumichrome ® Full Spectrum Plus fluorescent, 32W bulbs suspended approximately 10 cm above the water surface of each tank on each shelf. While lamps cannot exactly reproduce the solar spectrum, these specific bulbs were selected based on their broad coverage of the ultraviolet and visible specta. The relative light intensity (i.e., photon count) in each tank was measured with an Ocean Optics Red Tide USB650 UV spectrophotometer from 250 to 800 nm (Figure 1). Lights were placed on a 14-hr light, 10-hr dark cycle.

Experimental tanks were arranged in a randomized block design, with at least one tank from each treatment on each shelf. Nine tanks were filled with 17 L of artificial lake water made from the COMBO medium recipe (Kilham et al. 1998). SuperHume, a commercially available source of humic acid, was then added to the tanks at varying concentrations to adjust the brown color of the water (Lennon et al. 2013). Three tanks received 7 μg/L of SuperHume to serve as a light brown treatment (water color measured as absorbance at 440 nm (a_{440}) = 1.6 m^{-1}), three tanks received 33 μg/L to serve as a moderate brown treatment (a_{440} = 5.7 m^{-1}), and

150 three tanks received 66 μg/L to serve as a dark brown treatment (a_{440} = 10.8 m^{-1}). The pH of
151 each tank was then adjusted to 7 with approximately 2 μL of 2N HCl. The remaining three tanks
152 were filled with 17 L of lake water from Sandy River Reservoir, Farmville, VA to compare with
153 the artificial SuperHume treatments (a_{440} = 1.5 m^{-1}). Lake water was passed through a GF/F
154 filter to remove bacterio-, phyto-, and zooplankton, as best as possible. All tanks were allowed
155 to stabilize at room temperature, 22 - 23 °C.

156 At the beginning of the experiment, each tank received a dense mixture of
157 phytoplankton containing equal parts of *Ankistrodesmus* sp., *Chlorella* sp., *Scenedesmus* sp.,
158 and *Selenastrum* sp., which resulted in an initial chlorophyll-*a* concentration of approximately
159 10 μg/L in all tanks. Algae were purchased from Carolina Biological and cultured in COMBO
160 medium prior to the experiment. A mixed assemblage of zooplankton was then added to each
161 tank. Zooplankton were collected from Sandy River Reservoir the day before the experiment by
162 towing a 64 μm mesh bongo net from 0 - 6 m several times. Prior to introduction, zooplankton
163 were concentrated into a single 4 L container, mixed, and then 50 mL aliquots were introduced
164 into each tank to provide a starting density of approximately 26 zooplankton per liter in each
165 tank. This initial relatively low density permitted observations of population growth over time.
166 Proportionally, the zooplankton assemblage consisted of 80% copepods (mostly cyclopoids),
167 15% cladocerans (*Daphnia* sp. and *Bosmina* sp.), and 5% rotifers (*Keratella* sp.). We removed all
168 *Chaoborus* before introducing the zooplankton to the tanks to minimize zooplankton mortality
169 due to predation.

170 Over the course of the next month, phytoplankton biomass and zooplankton abundance
171 were measured at 0, 3, 7, 14, 21 and 28 days. Tanks were gently mixed with a broad, plastic

172 spoon before each sampling. For phytoplankton, two replicate 50 mL water samples were
173 removed from each tank and filtered through Whatman GF/F filters to measure chlorophyll-*a*
174 concentration as a proxy for phytoplankton biomass. Filters were placed in 90% acetone
175 overnight in the freezer and then chlorophyll-*a* concentration was measured on a Shimadzu
176 Trilogy Fluorometer using the non-acidification module. Chlorophyll-*a* concentrations are
177 reported as the average of these two replicates. One hundred milliliters of DI water were added
178 to each tank to replace the 100 mL removed for chlorophyll-*a* analysis, keeping the volume at
179 ~17 L.

180 Because bacteria can serve as an alternate food source for zooplankton, either directly
181 or through the microbial loop (Sanders and Porter 1990; Wylie and Currie 1991), we measured
182 bacterial abundance at Days 0 and 28. Five milliliters of water were collected from each tank
183 with a serological pipet, placed in a sterile culture tube, and preserved with glutaraldehyde.
184 Samples were refrigerated until analysis. Bacterial abundance was determined by counting
185 DAPI stained cells under an epifluorescent microscope at a magnification of 100x, based on the
186 methods of Porter and Feig (1980). A minimum of 400 cells were counted per sample to
187 determine cell density per milliliter.

188 For zooplankton, 3 L of water from each tank was passed through a 64 µm mesh cup
189 and then poured back into the tank. Zooplankton collected on the mesh were rinsed into a
190 sample cup and preserved with 70% ethanol. Zooplankton were identified and enumerated
191 under a dissecting microscope at 40x in a Ward counting wheel. Zooplankton density
192 (individuals per liter) was determined by counting the total number of rotifers, copepods, and

cladocerans in each sample collection and then dividing by 3, or the total volume of water sampled.

After 28 days, two larval largemouth bass (*Micropterus salmonoides*), 10-12 mm in length, were introduced to each tank. Preliminary experiments determined the fish behaved better in pairs compared to single introductions. Because we were unable to successfully obtain fish larvae from Sandy River Reservoir prior to the experiment, we used larvae from a local pond with similar optical characteristics. Fish larvae were collected using light traps (Aquatic Instruments, Inc., Hope, ID) anchored in the littoral zone of the pond overnight. Fish were immediately transported back to the lab and housed for 24 hours without food. Fish collection and care followed approved institutional animal care and use protocols.

Once introduced to the experimental tanks, the fish larvae were allowed to feed for 24 hours. After which, zooplankton were sampled as described above. This provided an estimate of daily zooplankton consumption in each treatment, assuming that total zooplankton consumed was represented by the difference in zooplankton density at the beginning and end of fish feeding. We also assumed that the two fish in each tank fed equally, such that the difference in zooplankton density was divided by 2 to estimate daily consumption rates per fish larvae.

Fish were then allowed to feed in the tanks for another 5 days. After which, fish larvae were euthanized in MS-222, and final measurements were taken of their body length under a dissecting microscope to estimate growth. Because we could not clearly distinguish the two fish in each tank, growth was estimated by first adding together the length of the two fish in each tank at the beginning and end of the experiment. We then took the difference of these two

numbers and divided by 2 to estimate growth per larvae. A final collection was also made of zooplankton density in the tanks. However, zooplankton abundance was too low in all the tanks to make accurate counts.

Throughout the experiment, a YSI 600 XLM sonde was used to measure water temperature, conductivity, pH, and dissolved oxygen concentration in each tank. To monitor potential changes in water color over time, a Shimadzu UV/Vis spectrophotometer was used to measure absorbance at 440 nm of water filtered through a GF/F filter. Dissolved organic carbon (DOC) concentration was also monitored during the experiment using GF/F filtrate. Samples were run on a Shimadzu TOC-L analyzer.

2.2 Direct Effects of Browning

The same experimental setup was used to investigate direct effects of browning on fish foraging efficiency. However, we did not use a lake water control. Light to dark brown water color treatments consisted of four replicate tanks assembled in a randomized, block design on the three shelves. After adding SuperHume to the tanks, we noted that water color ranged from a_{440} = 1.6 to 13.1 m^{-1}. In each tank, the pH was adjusted to approximately 7, and the water was allowed to equilibrate to room temperature (22 - 23°C). A YSI 600 XLM sonde was used to determine the water temperature, conductivity, dissolved oxygen concentration, and pH of each tank prior to the beginning of each feeding experiment.

Both zooplankton and larval *Micropterus salmonoides* were collected from Sandy River Reservoir. Fish larvae were starved for 24 hours prior to the experiment. For each experimental tank, two larval fish (~13 mm in length) were introduced and allowed to acclimate for a minimum of 1 hour. After which, a known concentration of zooplankton prey (i.e. ~ 20

zooplankton/L) was added, and the fish were allowed to feed for 30 minutes. We staggered the introduction of the zooplankton every 10-15 minutes to allow time for disassembling each tank at the end of the timed feeding trial. The zooplankton community consisted of approximately 40% cladocearans (mostly *Daphnia* sp.), 27% adult calanoid copepods, 24% *Chaoborus*, 7% copepodids, and 2% adult cyclopoid copepods.

Fish were removed from the tank and placed in MS-222 for euthanization. The remaining zooplankton were collected by filtering the water in each tank through a 64 μm mesh. Zooplankton were rinsed off the mesh and into a sample cup with 70% ethanol. Zooplankton were identified and enumerated under a dissecting microscope at 10 - 40x in a Ward counting wheel. Zooplankton density per liter was calculated as the total number of counted zooplankton divided by 17, or the total volume of water in the tank. The total zooplankton consumed per minute by each fish larvae was then calculated as the difference in zooplankton density at the beginning versus the end of an experiment divided by 30, or the number of minutes the fish fed, and then divided by 2, or per larvae. We assumed each fish in each tank fed equally during the experiment. We chose this method rather than examining gut contents to be consistent with previous experiments. Preliminary studies filling and emptying tanks with zooplankton resulted in ~99% recovery, providing confidence in our methodology (unpublished data).

To further explore the direct effects of browning on larval fish foraging, we performed three additional experiments using the same experimental setup but with bluegill *Lepomis machrochirus* (~13-15 mm in length) collected from a local pond. The goal of these experiments was to test the limits of larval fish foraging under increasing water color by reducing the

foraging time and number of prey. For each experiment, fish larvae were collected with light traps 24 - 48 hours before the experiment. Two larvae were placed in each tank and allowed to acclimate for 24 hours prior to the introduction of zooplankton prey. A YSI sonde was used to confirm that temperature, pH, conductivity, dissolved oxygen concentrations were similar across all tanks. For the first experiment, fish larvae fed for 24-hours with a relatively high density of prey (i.e., 130 zooplankton per liter) to estimate daily feeding rates under ideal conditions. We then conducted two experiments with reduced time and prey concentration to test the limits of larval fish foraging: 1) a 10-minute feeding trial with 4 zooplankton per liter and 2) a 5-minute feeding trial with 2 zooplankton per liter. Zooplankton prey remaining in each tank were collected at the end of each experiment to assess fish feeding rates and prey selectivity. For all three experiments, zooplankton were collected from the same pond as the fish larvae and consisted of approximately 80% cladocearans (mostly *Daphnia* sp.), 15% cyclopoid copepods, 3% *Chaoborus*, and 2% rotifers (*Keratella* sp. and *Asplanchna* sp.).

2.3 Statistical Analyses

For the first experiment investigating indirect effects, repeated measures one-way ANOVAs were used to assess potential differences in water temperature, conductivity, pH, dissolved oxygen, and chlorophyll-*a* concentration over the course of the experiment in each treatment. A two-way ANOVA was used to compare bacterial abundance across treatments between Day 0 and Day 28. Total zooplankton density was assessed only on Day 28 with a one-way ANOVA as densities were similar on all other dates.

For both the indirect and direct effects experiments, linear regressions were performed to determine differences in fish feeding rates with increasing water color. Zooplankton

consumption served as the dependent variable and absorbance at 440 nm the independent variable. We also used a linear regression to calculate differences in fish growth with increasing water color during the 6-day feeding trial in the indirect effects experiment. Pearson correlation coefficients were calculated to estimate the effect of zooplankton densities on larval fish consumption rates and growth. E*Indices were calculated to determine potential differences in prey selectivity with browning (Lechowicz 1982). All statistical tests were performed using the R Statistical Environment (R Core Team 2018).

3. RESULTS

3.1 Indirect Effects of Browning

Over the course of the 28-day experiment, there were no significant differences in water temperature, pH, or dissolved oxygen concentration among the 12 tanks with time or treatment (Table 1). Conductivity did not significantly vary with time but was significantly lower in the lake water treatment compared to the SuperHume treatments (Table 1). Absorbance at 440 nm was similar in the light brown and lakewater treatments but significantly different from the moderate and dark brown treatments (Table 1). Water color decreased during the first week in all treatments and then remained relatively constant. On average, absorbance coefficients at 440 nm were 1.3 m^{-1} in the light brown and lake water treatments, 5.0 m^{-1} in the moderate brown treatment, and 9.7 m^{-1} in the dark brown treatment. DOC concentration significantly differed among the four treatments, with the light brown treatment having the lowest DOC concentration (2.6 mg/L) and the lake water treatment having the highest DOC concentration (5.1 mg/L) (Table 1). However, DOC concentration did not significantly vary over time. Interestingly, only marginal increases in DOC concentration were observed with

303 SuperHume additions despite an approximate 10 times increase in water color. Higher DOC
304 concentrations in the lake water treatment, compared to the SuperHume treatments, were due
305 to high inputs of non-chromophoric, algal-derived organic carbon in the eutrophic reservoir.
306 Bacterial abundance was significantly higher on Day 28 compared to Day 0 across all
307 treatments (F=20.34, DF=1, P= 0.0002). The lake water treatment had an initial bacterial
308 abundance of 2.86 x 10^6 cells per mL $\pm$ 1.4 x 10^5 S.E. compared to approximately 7.1 x 10^5 cells
309 per mL $\pm$ 1.5 x 10^5 S.E. in the light brown treatment and approximately 1.3 x 10^6 cells per mL $\pm$ 9
310 x 10^5 S.E. in the moderate and dark brown treatments. This suggests that not all bacteria were
311 removed from the lake water treatment during the initial set-up, and bacteria were introduced
312 with the addition of SuperHume. By the end of the experiment, bacterial abundance did not
313 significantly differ across the light, moderate, and dark brown treatments but was significantly
314 higher in the lake water treatment (i.e., ~ 3.19 x 10^6 cells per mL in the lake water treatment
315 compared to ~ 2 x 10^6 cells per mL in the SuperHume treatments (F=24.61, DF=3, P>0.0001)).
316 Interestingly, bacterial abundance differed by only 10 $\pm$ 9% between Day 0 and Day 28 in the
317 lake water treatment while in the low, moderate, and dark brown treatments, bacterial
318 abundance was 64 $\pm$ 5%, 35 $\pm$ 5%, and 23 $\pm$ 3% higher on Day 28, respectively.
319 Phytoplankton biomass, as estimated by chlorophyll-*a* concentration, significantly
320 differed with treatment but not time (Figure 2). The highest chlorophyll-*a* concentrations
321 throughout the experiment were observed in the light brown treatment (i.e., 46.1 µg/L by Day
322 28, Figure 2) while there was no significant difference in the moderate brown, dark brown and
323 lake water treatments (i.e., declining to 2 -7 µg/L by Day 28, Figure 2). Based on slopes from
324 regression analyses, algal growth during the first week was fastest in the light brown treatment

(5.61 µg/L per day ± 0.25 S.E.) followed by the lake water (4.15 µg/L per day ± 0.22 S.E), moderate brown (2.36 µg/L per day ± 0.28 S.E), and dark brown treatments (1.51 µg/L per day ± 0.2 S.E).

After 28 days, total zooplankton density was significantly greater in the lake water treatment (mean = 190 individuals per L ± 25 S.E.) followed by the light brown (mean = 120 individuals per L ± 35 S.E.), moderate brown (mean = 72 individuals per L ± 7 S.E.), and dark brown treatments (mean = 47 individuals per L ± 10 S.E.) (Figure 3A). Overall, the cladoceran *Bosmina* sp. was the most abundant zooplankton in all treatments by Day 28 (Figure 3B). Despite being the dominant zooplanktors at the beginning of the experiment, copepods represented only 12% of the zooplankton community in the lake water treatment and 5% of the zooplankton community in the SuperHume treatments by Day 28 (Figure 3D & 3E). *Daphnia* sp. abundance was low in all treatments by Day 28, except for the light brown treatment (Figure 3C). While counting the zooplankton under the microscope, flocculant SuperHume was observed, but not quantified, in the guts of cladocerans (i.e., *Daphnia* and *Bosmina*) but not calanoid or cyclopoid copepods (Figure 4). Rotifers rapidly decreased in abundance and were not observed in any of the treatments after Day 7.

All fish survived the 6 day incubation in the lake water and light brown treatments. However, there was a 33% mortality rate in both the moderate and dark brown treatments, with one dead fish found in two replicate tanks of each treatment after 5-6 days. As predicted, fish foraging rate decreased with increasing water color (Figure 5A). In the first 24 hours, fish consumed twice as many prey in the lake water treatment (i.e. ~610 zooplankton per larva) compared to the light brown treatment (~323 zooplankton per larva) and four times as many

prey than in the dark brown treatment (~143 zooplankton per larva). Daily zooplankton consumption correlated highly with zooplankton densities at the time fish were introduced to the experimental tanks (Pearson Correlation Coefficient =0.88, P=0.0002). Proportionally, fish larvae primarily consumed *Bosmina* in all treatments as they were the most numerous species in the tanks. However, *Daphnia* had a higher electivity value (E*= 0.24 $\pm$ 0.0004), followed by *Bosmina* (E*= -0.12 $\pm$ 0.02) and then copepods (E*= -0.64 $\pm$ 0.02). No statistical differences in electivity were observed across treatments.

Fish growth over the 6-day feeding experiment was greatest in the lake water and light brown treatments followed by the moderate and dark brown treatments (Figure 5B). On average, fish grew ~1.2 – 1.5 mm in the lake water and light brown treatments, ~0.66 mm in the moderate brown treatment, and ~0.33 mm in the dark brown treatment. However, based on linear regression, water color explained only 33% of the variation in larval fish growth (y= -0.13x + 1.51, P=0.05), and growth was not significantly correlated with total zooplankton consumption (Pearson Correlation Coefficient =0.45, P=0.15). After 6 days, the surviving fish larvae in the moderate and dark brown treatments displayed empty and concave stomachs while those in the lake water and light brown treatments were full with zooplankton prey (Figure 5C). Note that previous studies have reported that it takes 4 – 6 hours for larvae to fully evacuate their guts (Werner 1969).

3.2 Direct Effects of Browning

In the experiment with largemouth bass, we observed no significant effect of increased browning on fish foraging efficiency or prey selectivity over the 30-minute feeding trial (Figure 6). Fish consumed on average 2.5 zooplankton per minute $\pm$ 0.3 S.E. across all treatments,

consuming mostly *Daphnia* sp. (percent consumed = 61% ± 0.03 S.E.) and *Chaoborus* sp. (percent consumed = 57% ± 0.07 S.E.) followed by copepods (percent consumed = 0.07% ± 0.02 S.E.). E*Index values did not significantly differ across treatments, with *Daphnia* sp. (E* Index= 0.19 ± 0.02 S.E.) and *Chaoborus* sp. (E* Index = 0.15 ± 0.03 S.E.) prey preferred over copepods (E* Index= -0.7 ± 0.07 S.E.).

Similar patterns were observed in the experiments with bluegill. Despite reducing zooplankton prey concentration and foraging time, no significant differences in foraging rate or prey selectivity were detected with increasing water color (Table 2). Similar to largemouth bass, larval bluegill primarily consumed *Daphnia* sp. and *Chaoborus* sp. prey compared to copepods in all treatments (Table 2). In the 24-hour experiment, larval bluegill in all treatments consumed ~80% of the zooplankton in their respective tanks, with low consumption rates for rotifers (Table 2).

4. DISCUSSION

The transition from endogenous to exogenous feeding is a 'critical period' in larval fish development characterized by unusually high mortality rates that influence longer-term population growth (Hjort 1914; Fuiman and Werner 2002). Here, we demonstrate that browning will likely add stress to this critical stage by decreasing zooplankton prey abundance. Browning did not, however, directly alter larval fish feeding efficiency or prey selectivity, suggesting plasticity in foraging behavior under varying light conditions. Understanding the balance between direct versus indirect effects of browning on the early life history of fishes will improve conservation and management strategies in response to continued organic matter loading to aquatic ecosystems with future changes in climate and land use.

4.1 Indirect Effects of Browning

The transfer of energy through the traditional grazer food chain depends on basal resources to support higher trophic levels. Therefore, one of the most profound effects of freshwater browning is the reduction in primary production, due to increased light attenuation (Wetzel 2001; Thrane et al. 2014; Solomon et al. 2015), that subsequently reduces energy flow to zooplankton and fish (Jones et al. 2012; Solomon et al. 2015; Creed et al. 2018). Our study supports these general observations and raises concerns for larval fish. With increasing brown color, we observed a reduction in the quantity of light, decreased phytoplankton biomass, and decreased zooplankton densities. In turn, the foraging rate, growth, and survival of larval largemouth bass significantly declined.

Starvation is more serious for larval fish than juvenile or adult stages because of their high metabolic demands coupled with low energy reserves in their tissues (Fuiman 2002). Within 4 - 5 days at 25 - 30 ^{o}C, larvae will likely starve to death given low to no food rations (Fuiman 2002). In the present study, after 6 days at 22 - 23°C, fish larvae in the moderate and dark brown treatments exhibited a 33% mortality rate while the surviving larvae in these treatments displayed concave-shaped, empty stomachs. Although we did not quantify gut fullness, these observations suggest a positive relationship between browning and starvation risk.

Fish larvae are primarily zooplanktivorous, and therefore, zooplankton abundance and community composition (e.g. small- vs. large-bodied) are of critical importance to larval fish survival and growth. Generally, increased primary production in the late Spring and early

Summer leads to increased zooplankton production. Shortly thereafter, many fish species begin to spawn, matching larval fish hatching with an abundance of zooplankton prey (Mills et al. 1989; Mehner and Thiel 1999; Hansson et al. 2007). However, as fresh waters continue to brown and energy flow to zooplankton is reduced, planktivorous fishes of all stages may be forced to compete for fewer zooplankton prey or switch to alternate resources (Batt et al. 2015). Competition may be further intensified as fish density in the epilimnion increases due to a shallowing of the thermocline with increased browning (Solomon et al. 2015; Koizumi et al. 2018). Fish larvae may be especially negatively affected as they are not as apt at locating and capturing prey as larger juveniles and adults (Fuiman and Werner 2002).

At the time fish larvae were introduced to our experimental tanks, zooplankton densities in the moderate and dark brown treatments were 2 to 4 times less than in the light brown and lake water treatments (i.e. 46 - 72 individuals per liter compared to 120 - 190 individuals per liter). Daily consumption rates for larval fish are often greater than a larvae's own biomass (Post 1990). We did not directly measure zooplankton or larval fish biomass in our study. However, based on published length-weight regressions (reviewed in Watkins et al. 2011) and assuming average body length, we roughly estimate that zooplankton biomass was ~93 μg dry weight in the dark brown treatment, ~175 μg dry weight in the moderate brown treatment, ~373 μg dry weight in the light brown treatment, and ~473 μg dry weight in the lake water treatment at the time the fish larvae began to forage. Approximately 70-80% of the zooplankton were consumed in their respective tanks, suggesting that zooplankton prey availability was potentially low in all of our treatments.

We placed two larvae in each tank to encourage routine behavior, which resulted in a fish density of ~118 larvae m^{-3}. This is relatively high but not uncommon in nature, particularly during the early months of fish hatching (Santucci et al. 2003). Previous studies have shown that when zooplankton consumption outweighs zooplankton reproduction, fish larvae may rapidly deplete their food source, affecting future growth and survival (Mehner and Thiel 1999; Santucci et al. 2003; Hansson et al. 2007). This may occur more often in brown systems if zooplankton abundances are low at the time of fish hatching. Furthermore, as surface water temperatures rise as a result of global climate change, larval fish feeding rates are predicted to increase as a consequence of higher metabolic rates (Van Dorst 2017; Van Dorst et al. 2017). Brown waters are likely to increase in temperature more rapidly due to the increased absorption of solar radiation (Solomon et al. 2015), further exacerbating competition for limited resources in these systems.

Unlike prey abundance, we did not see significant differences in zooplankton community composition with increased water color. Others studies have also noted minimal changes in zooplankton community composition with freshwater browning, and there does not seem to be a clear pattern in positive versus negative effects on specific zooplankton groups (Nicolle et al. 2012; Ekvall and Hansson 2012; Kelly et al. 2014; Robidoux et al. 2015; Lebret et al. 2018; Leech et al. 2018). In the present study, the zooplankton community shifted from copepod to cladoceran dominated across all treatments, with smaller-bodied *Bosmina* the dominant zooplankton prior to larval fish introduction. Abundances of large-bodied *Daphnia* were similar in all treatments during the first two weeks of the experiment, but for unknown reasons, continued to increase only in the light brown treatment (i.e., ~ 30 individuals per L at

Day 28). It is possible that the light brown treatment provided the ideal diet for *Daphnia*, with sufficient carbon and nutrients from both algal- and terrestrially-derived resources (Lennon et al. 2013; Gall et al. 2017; Tang et al. 2018).

Conversely, copepod abundances declined in all treatments over the 28 days, especially in treatments with SuperHume. Cladoceran zooplankton may have outcompeted copepods because of their faster life cycle combined with their consumption of flocculant organic matter and/or bacteria as phytoplankton biomass declined with browning (Tang et al. 2018). Alternatively, SuperHume has been suggested to be toxic to zooplankton (Robidoux et al. 2015). After our experiment, we reared gravid calanoid females and copepod nauplii in beakers with COMBO medium, algae, and increasing concentrations of SuperHume to investigate possible inhibitory effects. The results were variable, but we did see some females drop their egg sacs, which remained unhatched, in the moderate and dark brown treatments (unpublished data). We therefore cannot rule out potential toxic effects of SuperHume that affected our results. However, the *in situ* zooplankton community of Sandy River Reservoir was also observed to shift from copepod to cladoceran-dominated over time. For example, cladocerans were the dominant zooplankton at the time the direct effect experiment was conducted with larval largemouth bass.

Overall, the zooplankton community that developed in our experiment provided a standard diet for fish larvae. Typically, larger crustacean zooplankton promote foraging success, growth, and survival (Crowder et al. 1987; Mills et al. 1989). However, for younger larvae that are gape-limited, smaller prey, like *Bosmina* spp., can be more beneficial (Mehner and Thiel

1999). Nevertheless, zooplankton prey abundance in the moderate and dark brown treatments was too low to support healthy larval development.

Similar to previous studies (Batt et al. 2015; Karlsson et al. 2015; Solomon et al. 2015), reduced zooplankton abundance coincided with reduced phytoplankton biomass as browning increased. Interestingly, despite having similar water color, the light brown and lake water treatments displayed opposite trends in phytoplankton biomass after Day 14. In the lake water treatment, increased zooplankton abundance likely resulted in greater consumption of phytoplankton biomass. However, we are uncertain why the same pattern did not occur in the light brown treatment.

Phytoplankton species composition and nutritional quality were not assessed as part of our study; however, we recognize that these factors can also influence energy and nutrient availability for zooplankton, and consequently larval fish (Taipale et al. 2018; Creed et al. 2018). Because of logistics, we chose to use an artificial assemblage of green algal species that are generally favorable foods. In nature, phytoplankton communities can shift towards cyanobacteria with increased water color, which could further reduce zooplankton densities (Ekvall et al. 2013; Robidoux et al. 2015). It is also possible that the algae used in our experiment may not have been adapted to the low light conditions of brown water systems given that they were purchased from Carolina Biological. However, our results are comparable to field studies examining natural communities along a water color gradient (Ask et al. 2009; Karlsson et al. 2009; Thrane et al. 2014).

Reductions in prey consumption, and possibly less nutritious prey, ultimately led to reductions in fish growth. Fish larvae in the lake water and light brown treatments, on average,

grew twice as fast as the larvae in the moderate brown treatment and four times faster than the larvae in the dark brown treatment. Growth rates are important for several reasons. Larval fish become better at avoiding predators and detecting prey as swimming strength and visual acuity increases with body size (Fuiman 2002). In addition, larger larvae consume bigger, more energy-rich prey. Growth in juvenile and adult fish is often slower in brown compared to blue lakes, resulting in a smaller length-at-age (Estlander et al. 2010; Horppila et al. 2010; Benoit et al. 2016; van Dorst et al. 2018), and our data suggest similar patterns for larval fish. These reductions in individual growth lead to further reductions in population growth and fish biomass as lakes darken in color (Karlsson et al. 2009, 2015; Finstad et al. 2014), which can alter food web structure and lower fisheries yields.

4.2 Direct Effects of Browning

When given an equal abundance of zooplankton prey, neither the foraging efficiency nor prey selectivity of larval largemouth bass or bluegill were affected by increased browning. Our results are similar to Stasko et al. (2012), which found no significant effect of browning on juvenile roach feeding rates but are contrary to Jönsson et al. (2013), which reported decreases in reactive distance and capture success of piscivorous Northern pike (*Esox lucius*) feeding on roach (*Rutilus rutilus*) with increased water color. Weidel et al. (2017) reported significant effects of browning on juvenile largemouth bass and bluegill foraging, but water color explained only ~25-28% of the variation in foraging rates. Combined, results from our study and others (Estlander et al. 2012; Ranaker et al. 2012; Nurminen et al. 2014) suggest that effects of browning on fish foraging rates may be age- or species-specific.

It is also possible that larval fish used in our experiments were pre-adapted to the low-light environment of brown water systems. In general, the man-made reservoirs and ponds of central Virginia, USA have high light attenuation due to increased cDOM inputs and/or increased phytoplankton growth. Compared to local lakes and ponds in the region, our dark brown treatment increased water color approximately 10 times. However, we may have seen direct effects of browning on fish feeding had we continued to add organic matter to the tanks. Moreover, fish inhabiting clear, blue lakes may show a greater response to freshwater browning than those in our study (Stasko et al. 2015), and we encourage further experimentation with fish larvae from these systems.

Because foraging success plays a critical role in survival and reproduction, there is likely strong selection pressure to adapt to changing environmental conditions. As light availability for visual foraging declines with browning, fish may rely on other sensory mechanisms, such as mechanoreception or olfactory cues, to detect zooplankton prey. Non-visual feeding in fish larvae is not well studied, but previous research ablating the function of superficial neuromasts with neomycin or streptomycin has shown reduced feeding rates in marine fish larvae (Jones and Janssen 1992; Cobcroft and Pankhurst, 2003; Sampson et al. 2013). Larval zebrafish have been shown to learn to use mechanoreception to feed in the dark (Carillo and McHenry 2016). There is also recent evidence that juvenile bluegill may actually feed more in the open pelagic water column at night and horizontally migrate towards the littoral zone during the day to avoid piscivorous predators (Shoup et al. 2014). Future research should investigate the potential for alternative feeding strategies as waters brown in color.

4.3 Small Enclosures versus Natural Systems

We recognize that our experiment was conducted in relatively small experimental containers and that caution must be applied when scaling up results to natural systems. Nevertheless, our observations of phytoplankton, zooplankton, and fish responses to browning are supported by recent field-based studies (Karlsson et al. 2009, 2015; Finstad et al. 2014 and others mentioned above). Working with fragile fish larvae is challenging, and smaller containers minimize some of the logistical constraints. Yet, the small, confined tank could have influenced feeding rates. Given the size of larvae used in our experiments (10 - 13 mm), their visual acuity, or reaction distance, is approximately one body length (Werner 1969). However, we do not know how long it took larvae to search the tank. Larger containers may have revealed greater differences in feeding rates across treatments, similar to Weidel et al. (2017). We also did not quantify the spatial distribution of zooplankton in the tanks, which could have influenced feeding rates. Experimental tanks were covered during the feeding trials to avoid distracting the fish. Zooplankton are known to swim downward in the presence of light and fish, and it is possible that the zooplankton could have clumped at the bottom of the tanks, making them easier to find and capture. However, this behavioral pattern in the zooplankton was not obviously apparent to us while breaking down the tanks.

Interestingly, Seekell et al. (2018) recently reported that the relationship between fish biomass and water color was more negative in deep lakes compared to shallow lakes within the boreal region of Sweden. The authors state that the negative effects of browning associated with light extinction and decreased primary production are minimized in shallow lakes because light often reaches the lake bottom. The authors further noted that most fish were observed in

the littoral zone of lakes. Moving into shallow waters may provide fish adequate light to forage in brown waters, as supported by our results in shallow experimental tanks (i.e., ~0.2 m depth).

4.4 Implications and Applications

Given increasing trends in temperature and precipitation with global climate change, the browning of inland and coastal waters is predicted to continue, with far reaching consequences for aquatic ecosystems (de Wit et al. 2016; Weyhenmeyer et al. 2016). Here, we demonstrate that freshwater browning reduces energy flow to higher trophic levels, negatively affecting larval fish growth and survival during a 'critical period'. Because of their ecological and economic value, fish population dynamics are closely monitored and predicted by natural resource managers and conservationists. While current population growth models focus on the importance of temperature and nutrients (e.g., Deslauriers et al. 2017), our study and others, highlight the need to incorporate water color (i.e. cDOM) to more accurately predict recruitment strength, sustainable yields, and food web stability in systems affected by browning.

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TABLE 1. Mean ± S.E. of major physical and chemical parameters across the water color treatments. Significance is denoted in the last column, with NS = not significant, T = significant treatment effect, and D = significant time effect (i.e. day sampled). Lowercase letters indicate which treatments were significantly different from one another.

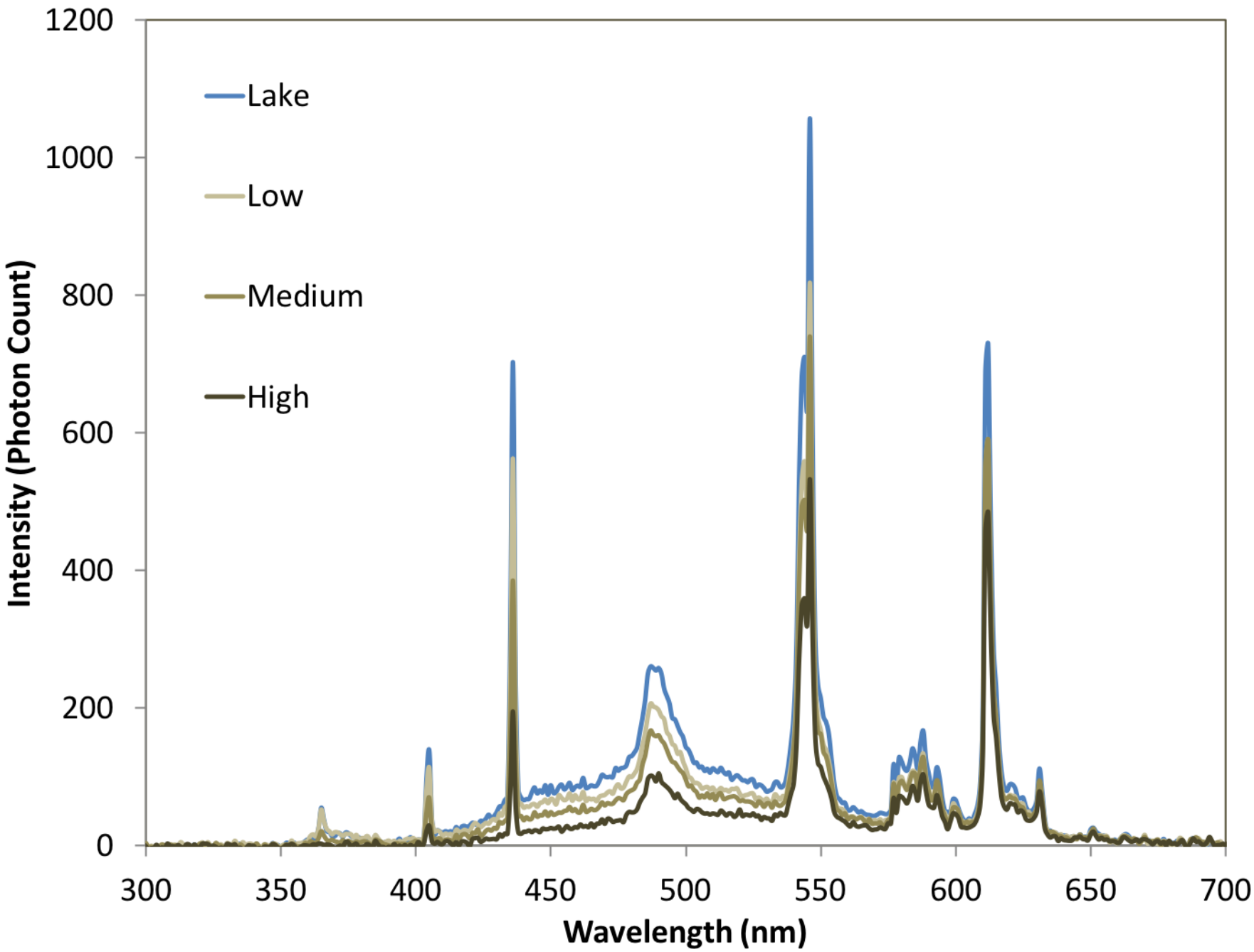
	Lake Water	**Light Brown**	**Moderate Brown**	**Dark Brown**	**Significance**
Temperature (^{o}C)	22.1 ± 0.18	22.09 ± 0.16	22.04 ± 0.16	22.03 ± 0.15	NS
Dissolved Oxygen (mg/L)	9.05 ± 0.09	9.12 ± 0.09	8.89 ± 0.04	8.83 ± 0.03	NS
Conductivity (mS/cm)	0.08 ± 0 a	0.25 ± 0.01 b	0.25 ± 0 b	0.25 ± 0.01 b	< 0.0001 (T)
pH	7.36 ± 0.04	7.25 ± 0.04	7.15 ± 0.22	7.14 ± 0.02	NS
a440	1.77 ± 0.65 a	0.99 ± 0.08 a	4.84 ± 0.12 b	9.83 ± 0.17 c	< 0.0001 (T) 0.008 (D)
Chl-*a* (ug/L)	16.34 ± 2.69 a	29.65 ± 3.83 b	12.4 ± 2.12 a	8.66 ± 1.57 a	< 0.0001 (T)
DOC (mg/L)	5.22 ± 0.05 a	2.58 ± 0.07 b	3.40 ± 0.06 c	4.66 ± 0.07 d	< 0.0001 (T)

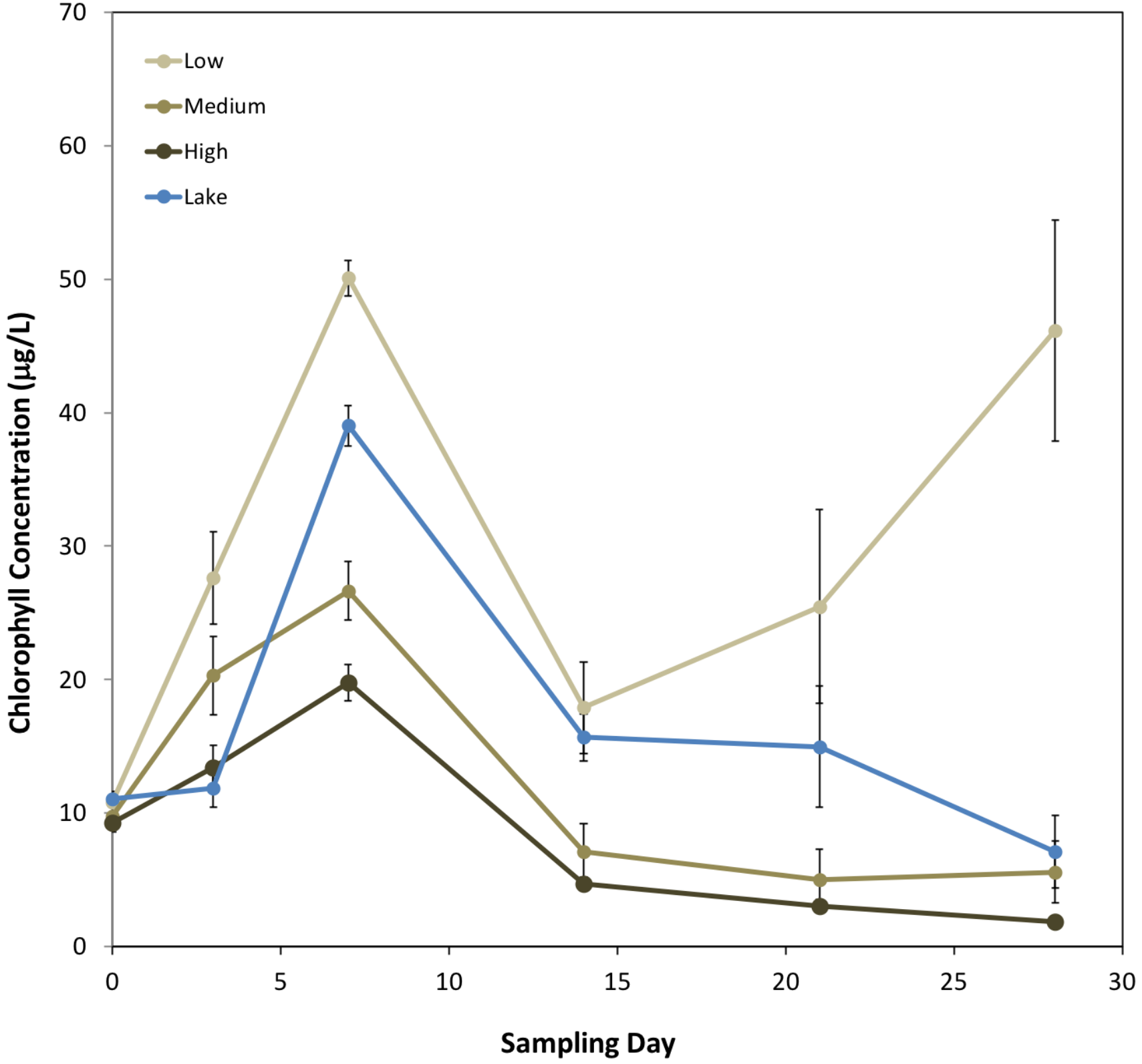
954 **TABLE 2**. Results from the three foraging experiments with larval bluegill (*Lepomis*
955 *machrochirus*), including consumption rates and E* index values for prey selectivity. The E*
956 Index ranges from 1 to -1, where 1 represents a strong selection for a prey type, -1 equals
957 strong avoidance of a prey type, and 0 equals random feeding. The approximate total number
958 of each zooplankton type in the tanks at the start of a feeding trial is provided in parentheses in
959 the first column.
960

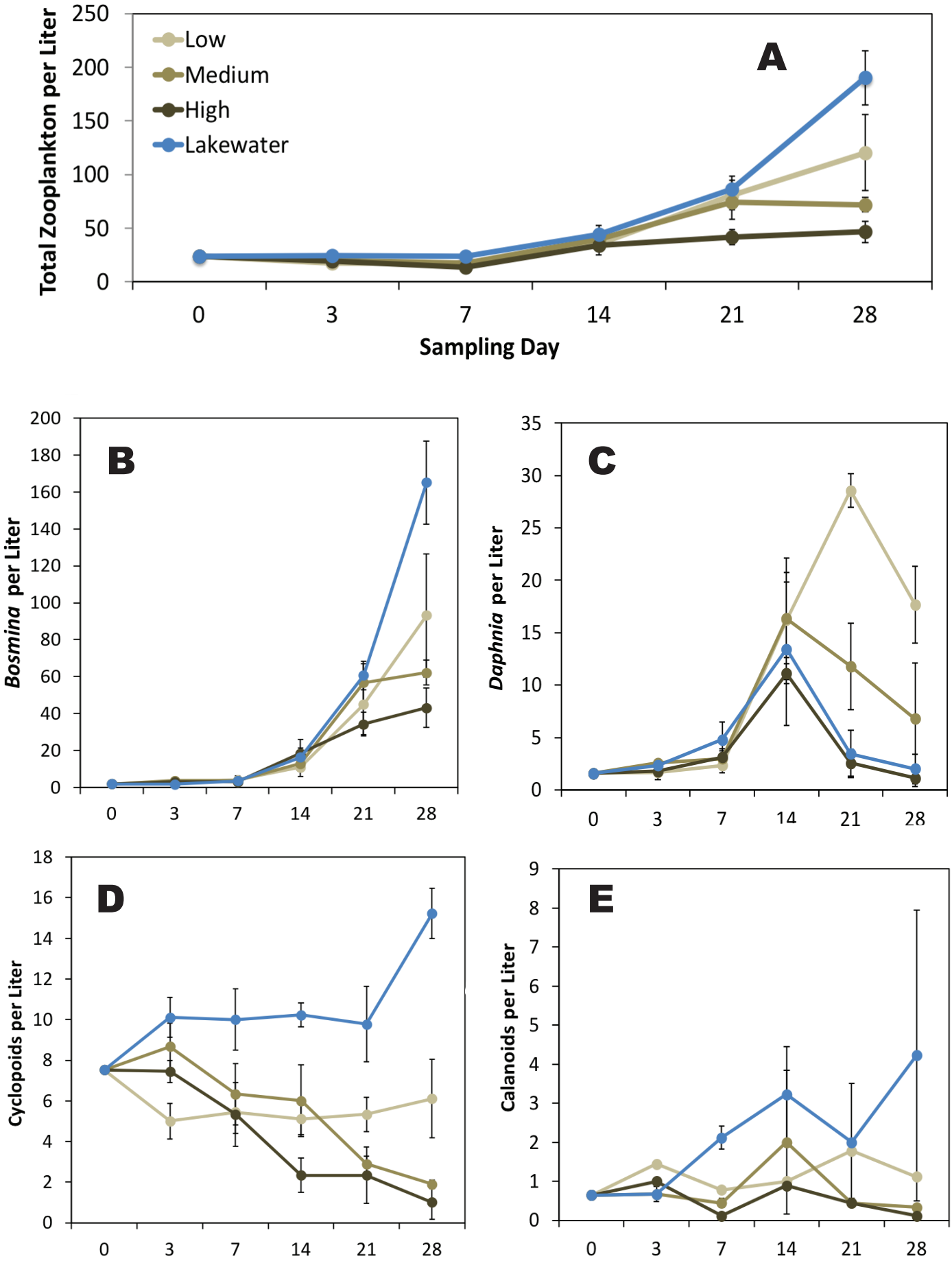
Zooplankton Type	Consumption Rates (day^{-1} $larva^{-1}$ or min^{-1} $larva^{-1}$)			E* Index		
	Light	**Moderate**	**Dark**	**Light**	**Moderate**	**Dark**
24 hours, ~130 zooplankton L^{-1}						
Large Cladocerans (210)	71	85	89	-0.05	0.02	0.04
Small Cladocerans (1817)	769	728	726	0.07	0.02	0.01
Chaoborus (33)	17	17	17	0.15	0.13	0.12
Copepods (47)	21	21	19	0.10	0.06	0.005
Rotifers (77)	10	15	18	-0.48	-0.35	-0.25
10 minutes, ~4 zooplankton L^{-1}						
Large Cladocerans (39)	2	2	1.7	0.006	0.18	0.11
Chaoborus (5)	0.25	0.25	2.5	0.01	0.17	0.17
Copepods (23)	1	0.85	1.7	-0.02	0.07	0.15
5 minutes, ~2 zooplankton L^{-1}						
Large Cladocerans (22)	1.4	1.6	1.5	0.15	0.03	0.10
Small Cladoceran (7)	0.25	0.45	0.38	-0.13	-0.02	-0.02
Copepods (11)	0.45	0.73	0.5	-0.07	-0.01	-0.10

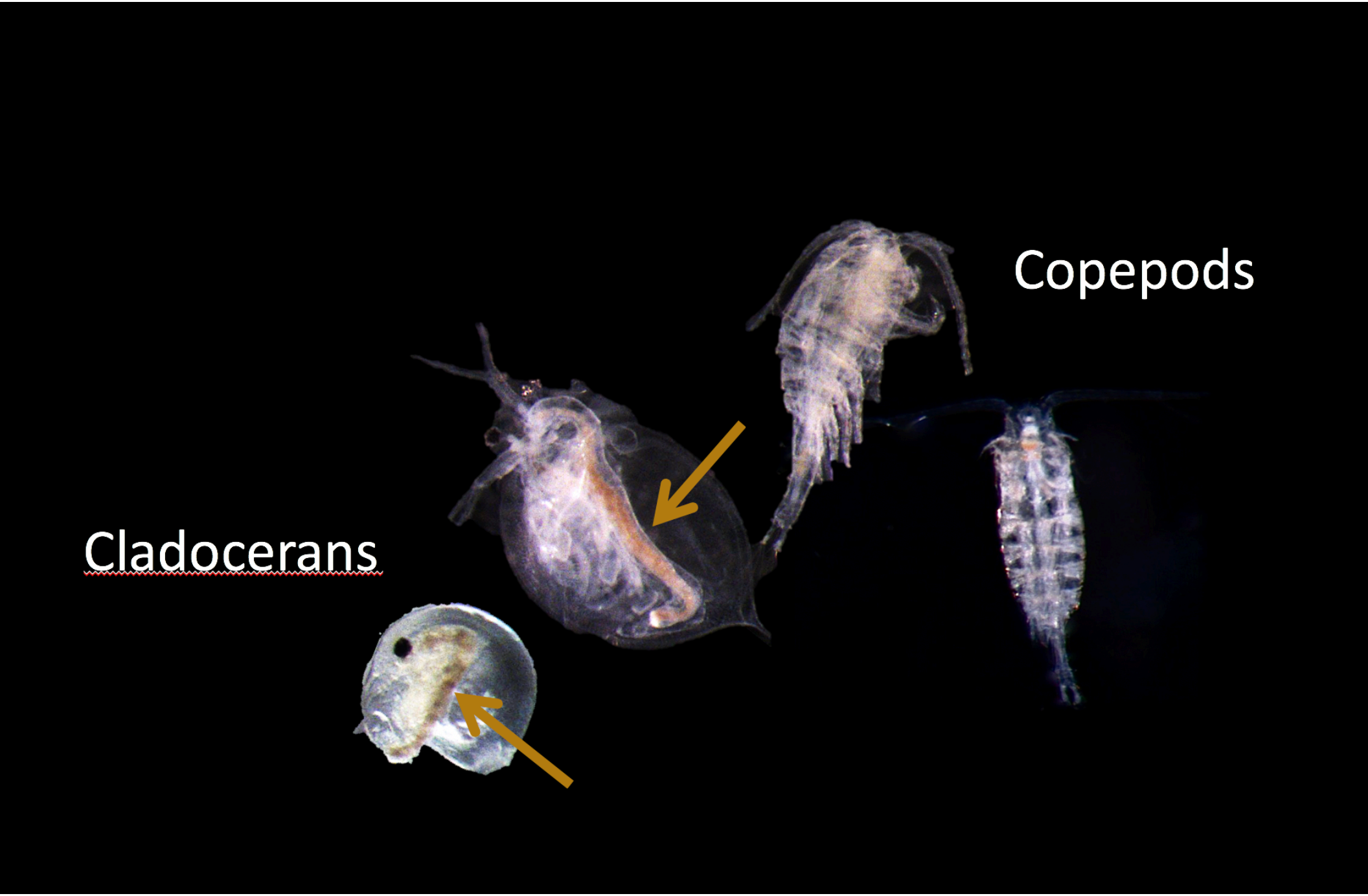
961
962

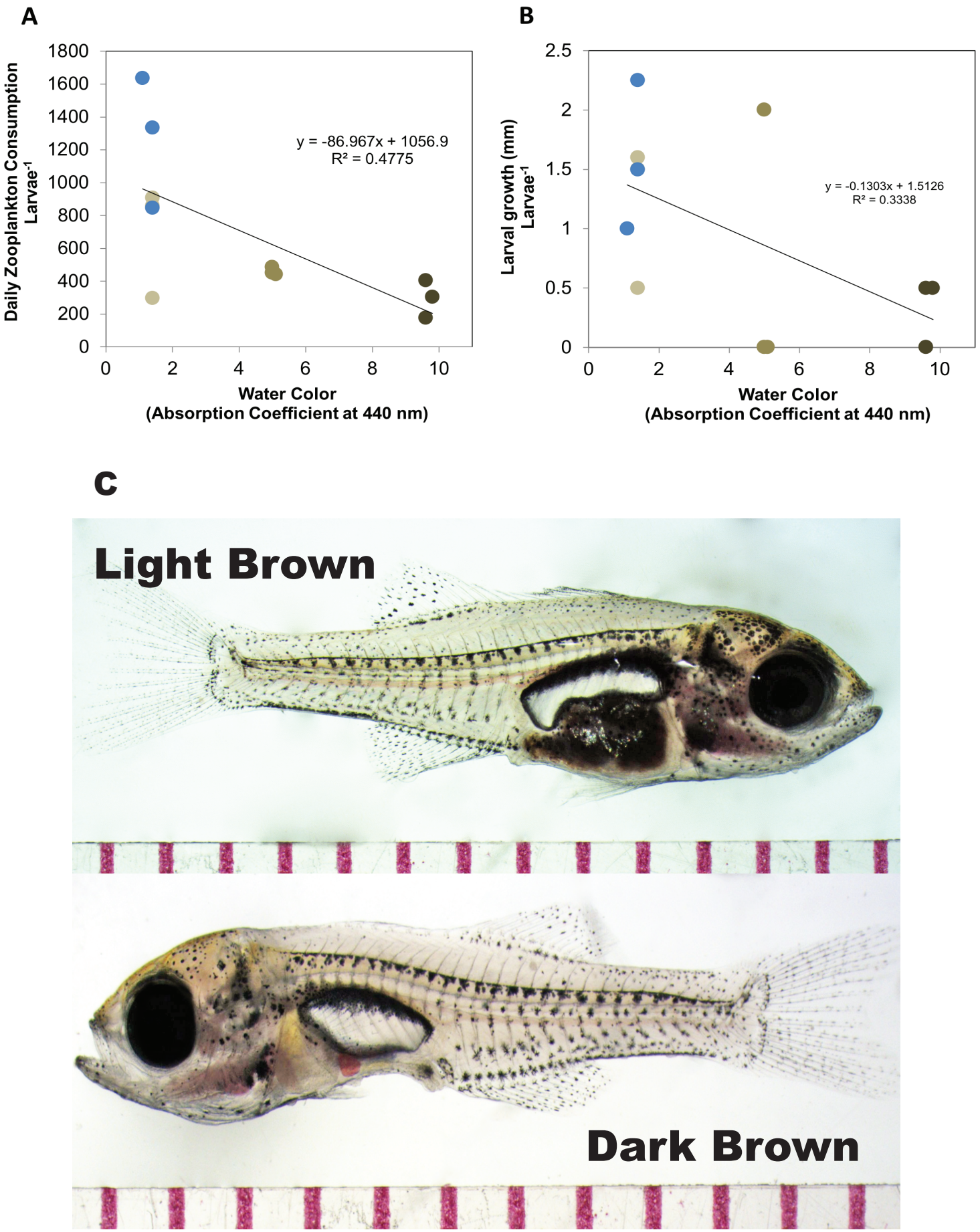
963 **FIGURE LEGENDS**
964
965 **Figure 1**. Spectral characteristics of the four treatments measured in photon counts per
966 wavelength. Data were collected by an Ocean Optics Red Tide USB650 UV spectrophotometer
967 from 250 to 800 nm with the sensor placed at the bottom of the tank pointing straight up
968 toward the overhead grow lamps.
969
970 **Figure 2**. Mean phytoplankton biomass, as estimated by chlorophyll-*a* concentration, in the
971 four treatments during the 28-day experiment. Error bars represent the standard error of three
972 replicate tanks per treatment. Phytoplankton biomass increased in all treatments during the
973 first 7 days but was comparatively lower in treatments with increased water color throughout
974 the experiment.
975
976 **Figure 3**. Zooplankton densities (individuals per Liter) in the four treatments during the 28-day
977 experiment. Error bars represent the standard error of three replicate tanks per treatment. The
978 top panel displays changes in total zooplankton densities (A) while the bottom panels display
979 individual zooplankton genera or groups, including the cladocerans *Bosmina* (B) and *Daphnia*
980 (C) as well as cyclopoid (D) and calanoid (E) copepods. In general, the zooplankton community
981 shifted from copepod to cladoceran dominated and abundance was significantly lower in
982 treatments with increased water color.
983
984 **Figure 4**. Flocculant organic matter was observed in the guts of cladocerans, including *Bosmina*
985 and *Daphnia*, but not copepods in the moderate and dark brown treatments. Brown arrows
986 highlight cladoceran gut contents. Image taken at 40x magnification.
987
988 **Figure 5**. Both daily prey consumption (A) and total growth over 6 days (B) were negatively
989 correlated with water color, as measured by absorbance at 440 nm. After 6-days, surviving
990 larval fish in the moderate and dark brown treatments displayed concave empty stomachs
991 while those in the light brown and lake water treatments had full stomachs. Photos show
992 example larvae from the light brown and dark brown treatments (C). Red tick marks below fish
993 are in millimeters.
994
995 **Figure 6**. Zooplankton consumption rates for larval largemouth bass with increasing water
996 color, as measured by absorbance at 440 nm. When given equal prey concentrations in all
997 treatments (i.e., 20 individuals per liter), there was no significant difference in the number of
998 prey consumed during the 30-minute feeding trial. This suggests no direct effect of browning
999 on visual foraging.

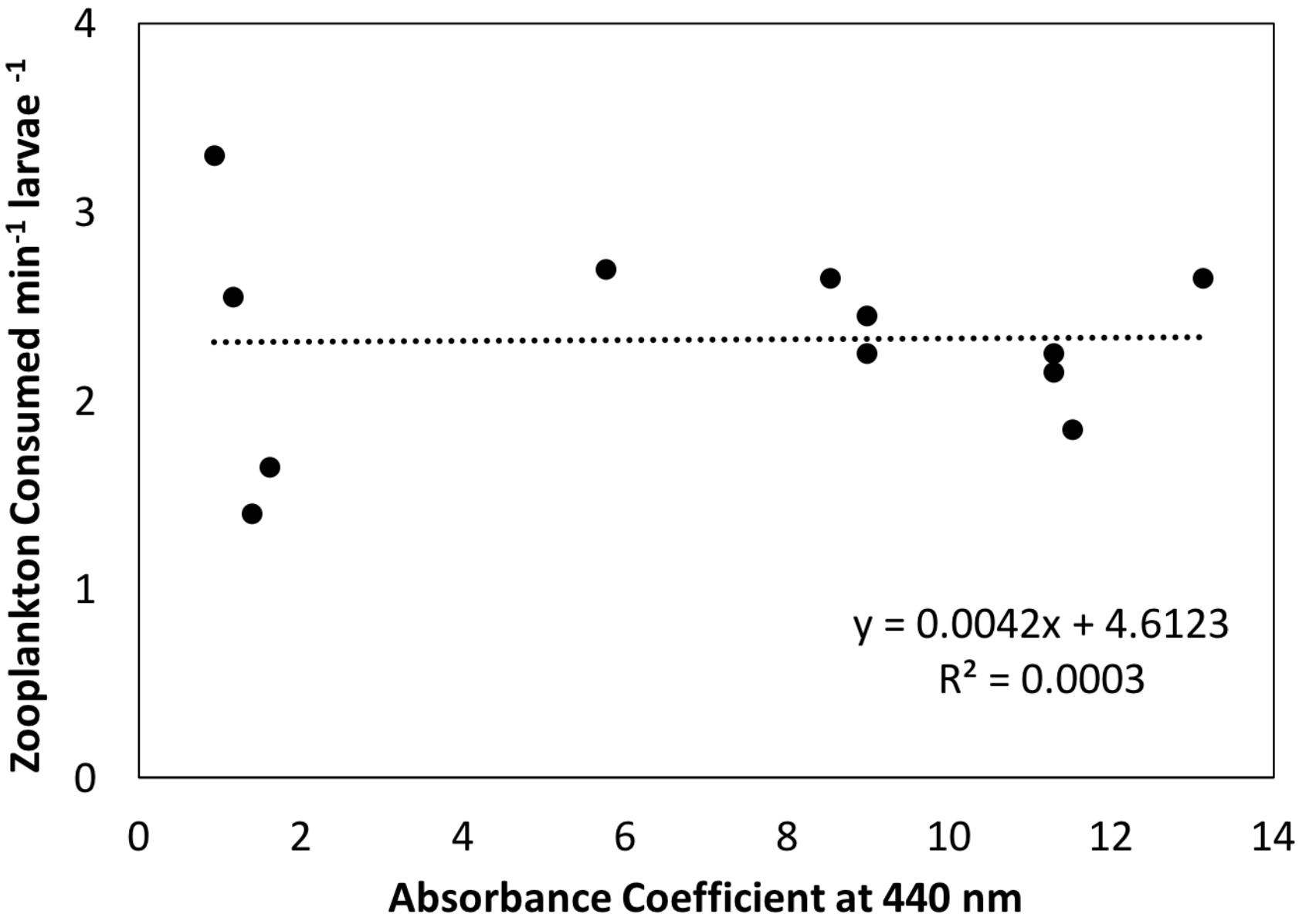

Lake
Low
Medium
High
Intensity (Photon Count)
Wavelength (nm)
0
200
400
600
800
1000
1200
300
350
400
450
500
550
600
650
700


Low
Medium
High
Lake
Chlorophyll Concentration (µg/L)
Sampling Day
0
10
20
30
40
50
60
70
0
5
10
15
20
25
30


A
Total Zooplankton per Liter
Low
Medium
High
Lakewater
Sampling Day
B
Bosmina per Liter
C
Daphnia per Liter
D
Cyclopoids per Liter
E
Calanoids per Liter


Copepods
Cladocerans


A
Daily Zooplankton Consumption Larvae-1
1800
1600
1400
1200
1000
800
600
400
200
0
y = -86.967x + 1056.9
R² = 0.4775
0
2
4
6
8
10
Water Color
(Absorption Coefficient at 440 nm)
B
Larval growth (mm) Larvae-1
2.5
2
1.5
1
0.5
0
y = -0.1303x + 1.5126
R² = 0.3338
0
2
4
6
8
10
Water Color
(Absorption Coefficient at 440 nm)
C
Light Brown
Dark Brown


Zooplankton Consumed min-1 larvae -1
4
3
2
1
0
0
2
4
6
8
10
12
14
Absorbance Coefficient at 440 nm
y = 0.0042x + 4.6123
R² = 0.0003