Specific Gravity of Ova and Larvae of Pelagophilic Broadcast-spawning Cyprinids

By

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TABLE OF CONTENTS

ABSTRACT

Many cyprinids native to streams and rivers of the US Great Plains belong to a reproductive guild that spawns by broadcasting non-adhesive semi-buoyant ova into the current. After fertilization these ova increase in size and buoyancy and remain suspended in the water column as long as sufficient flow is available. After hatching, early larval stages are incapable of sustaining buoyancy. They rely on a swim-up movement and flow to keep in suspension until the swim bladder is fully developed, which takes about four to six days post-hatching depending on temperature. It is believed to be important that ova and larvae remain in suspension to prevent settling to the river bottom where they are susceptible to being buried by shifting sediments. Specific gravity can be used as a means of estimating the minimal flow required for ova and larvae to remain in suspension. Previous studies have measured the specific gravity of ova from a small number of these cyprinids, yet no study has measured the specific gravity of larvae. The purpose of this study was to measure specific gravity of fertilized ova and larvae from five broadcast-spawning cyprinids using a calibrated, density-gradient column ranging from 0 to 30 ppt salinity concentration (ova) or 30 to 125 ppt salinity concentration (larvae). Mean specific gravity of fertilized ova ranged from 1.0050 to 1.0080, and were similar to measurements of specific gravity from previous studies. Mean specific gravity of larvae one day post-hatch ranged from 1.0440 to 1.0459 and were significantly denser than ova. Given that larvae are significantly denser than ova and likely require higher flow to stay in suspension, I conclude that the

hypothesized effects of river discharge on reproductive success of pelagophilic broadcast-spawning cyprinids most likely is attributable to survival of early larval stages.

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Streams and rivers of the US Great Plains often are characterized as harsh, due to highly variable and unpredictable runoff patterns (Poff and Ward 1989). During summer months, these streams and rivers exhibit large variations in runoff due to sporadic precipitation events. When precipitation events do not occur over extended periods of time, high temperatures and low runoff from the surrounding prairie can cause intermittent flow conditions (Dodds et al. 2004). High flows from large sporadic precipitation events are important to native fish communities within these systems. They provide fish with an opportunity for dispersal by connecting more variable upstream reaches with more perennial downstream reaches and creating deep pools for fish to use as refugia during intermittent conditions (Labbe and Fausch 2000; Dodds et al. 2004). In order for native fish to sustain populations within these systems and successfully produce young, reproductive strategies must be adapted to variable runoff patterns.

Most cyprinids native to streams and rivers of the US Great Plains are pelagophilic obligate-riverine species (Balon 1975). These cyprinids belong to a reproductive guild that spawns by broadcasting non-adhesive semi-buoyant ova into the water column (Moore 1944; Bottrell et al. 1964; Lehtinen and Layzer 1988; Johnston and Page 1992; Platania and Altenbach 1998; Durham 2007; Durham and Wilde 2008). After fertilization,

these ova absorb water into the perivitelline space, increase in size and buoyancy, and remain suspended in the water column as long as sufficient flow is available (Moore 1944; Bottrell et al. 1964; Balon 1975; Platania and Altenbach 1998). Developing ova hatch within one or two days depending on water temperature. Sufficiently cold temperatures can cause development of ova to slow. After hatching, early developing larvae are incapable of sustaining horizontal swimming. Larvae rely on instinctive upward swimming and sufficient flow to keep in suspension until the swim bladder is fully developed, about four to six days post-hatch, again depending on temperature (Moore 1944).

Studies on the reproductive ecology of broadcast-spawning cyprinids using gonadal-somatic indices and other measures of reproductive activity have shown that populations spawn in synchrony in association with high flow events (Lehtinen and Layzer 1988; Bestgen et al. 1989; Taylor and Miller 1990; Bonner 2000; Durham 2007; Durham and Wilde 2008; Urbanczyk 2012). Some studies also have shown members of this reproductive guild spawn individually throughout the spawning season with no apparent association to flow (Bonner 2000; Durham 2007; Durham and Wilde 2008; Urbanczyk 2012). Bonner (2000) studied the reproductive ecology of two pelagophilic broadcast-spawning cyprinids from the Canadian River and found evidence that spawning occurred even during periods of intermittency. Thus, spawning can occur during high flow, low flow, or intermittent flow conditions. Durham and Wilde (2008) concluded that alterations in flow conditions, such as reduced flood pulses, and the

effects they have on initiating spawning may be of less concern to species persistence than the effects they have on the survival of the early developmental stages.

Durham and Wilde (2006) examined otoliths from four species of age-0 pelagophilic broadcast-spawning cyprinids from the Canadian River and used the daily growth increments to back-calculate hatch dates. They found no evidence that young fish were successfully produced during periods of intermittency. Durham and Wilde (2009) later found similar results for two members of this guild in the Brazos River. Therefore, although spawning can occur during intermittent conditions, Durham and Wilde (2006; 2009) concluded for six broadcast-spawning cyprinids, flow is required to successful produce young.

Studies on early development stages and the dispersal of ova and larvae are common for many fish species in marine systems. Studies have shown that ova and larvae develop at specific vertical positions within the stratified water column. This provides suitable habitat for ova and larvae development, including areas of greater dissolved oxygen concentration, reduced predation, reduced water movement, and greater food availability (Vallin and Nissling 2000; Ådlandsvik et al. 2001; Boyra et al. 2003; Dominguez-Petit et al. 2013).

Specific gravity is equal to the density of an object relative to the density of water or other reference substances. Coombs (1981) developed a method for measuring specific gravity of ova and larvae in the laboratory using a salt-gradient density-column. Using the method developed by Coombs (1981) and with an understanding of the environmental conditions, several studies have modeled the

position of ova and larvae within the water column (Sundby 1990; Ådlandsvik et al. 2001; Boyra et al. 2003; Ospina-Álvarez et al. 2012; Dominguez-Petit et al. 2013). Studies have also observed changes in specific gravity during the early developmental stages, which resulted in ova or larvae passively moving to different positions within the water column (Dominguez-Petit et al. 2013; Jung et al. 2012).

In flowing rivers, specific gravity can be used as a means of estimating the minimal flow required for ova and larvae to remain in suspension. Although it is believed ova and larvae require flow to stay in suspension and prevent abrasion and suffocation by shifting sediment, previous studies have only measured specific gravity of ova from pelagophilic broadcast-spawning cyprinids and none have measured the specific gravity of larvae.

The first objective of this study was to spawn and measure specific gravity of ova of several broadcast-spawning cyprinid species. Species included in this study were Plains Minnow *Hybognathus placitus,* Shoal Chub *Macrhybopsis hyostoma,* Smalleye Shiner *Notropis buccula*, Arkansas River Shiner *Notropis girardi*, and Sharpnose Shiner *Notropis oxyrhynchus*.

The second objective of this study was to measure specific gravity of larvae of these same species. I hypothesized that larvae have a similar specific gravity as ova and require similar flow to stay suspended within the water column as suggested by previous studies (Platania and Altenbach 1998; Dudley and Platania 2007; Medley et al. 2007)

CHATER II

METHODS

Fish were kept at the Prairie Stream Fish Conservation and Propagation Laboratory at the Institute of Environmental and Human Health facility (TIEHH).

Spawning

To prepare fish for spawning, they were anesthetized with buffered tricaine methanesulfonate (MS-222) (100 mg/l MS-222 buffered with 233 mg sodium bicarbonate). Sex was determined by the presence or absence of expressed milt. To induce ovulation and increase sperm production, each individual was injected with a concentration of 1.10-mg/ml of acetone dried carp pituitary. During prior spawning attempts, males produced the most milt when given 24 hours after injection. Therefore, males were injected approximately 24 hours in advance of spawning. During initial spawning attempts, females did not begin to ovulate until six to eight hours after injection. Therefore, females were injected six hours in advance of expected time of spawning. Beginning six hours after females were injected, they were checked hourly for ovulation by gently pressing the abdominal region. At the first sign of ovulation, males were stripped of milt, which was kept on ice until used. Females were stripped of ova by gently applying pressure to the abdomen in a posterior direction and ova were expelled into a petri dish. Milt was then mixed with the ova using a feather, water was added to activate fertilization, and fertilized ova were placed in 2.5 l plastic bags filled with tank water for transportation. Ova were transported to Texas Tech University, placed in 37.9 l tanks, and acclimated to room temperature, approximately 20°C.

Throughout the entire process, adults, ova, and larvae were kept in water with salinity of 2 ppt.

Measuring Specific Gravity of Ova

Specific gravity of ova was measured using the methods of Coombs (1981) as modified by Cowley et al. (2005). A salt gradient was formed in a density-column (Techne [Cambridge] Ltd) using one liter of water with 45 ppt salinity and one liter reverse osmosis (RO) water (Figure 1) (Cowley et al. 2005). The two solutions were in separate Erlenmeyer flasks and connected at the base by a thin glass tube. From the RO flask, water flowed through a glass tube to the bottom of the column. As the column received water from the RO flask, gravity forced water with 45 ppt salinity to enter the RO flask. A magnetic stirrer mixed the salt water with the RO water, which increased the salinity of the water in the RO flask and increased the salinity of the water entering the column. The movement of water into the column was slow enough not to disturb the layers already established. The salinity of the water in the RO flask continued to increase until the gradient-column was full and a salinity gradient was established. This yielded a graded column with 0 ppt salinity at the top to 30 ppt at the bottom. The gradient was calibrated using standardized beads, each with a known density at 23°C (1.0009 1.0020, 1.0031, 1.0042, 1.0053, 1.0064, 1.0075, 1.0086, 1.0097, 1.0108, 1.0119, 1.0130 g/cm^3) (Techne [Cambridge] Ltd.). Density is temperature-dependent and specific gravity is temperature-independent. To account for using water at slightly different temperatures, the densities of the standardized beads were converted to

specific gravity. This was done by dividing the density of the beads by density of water at which they were calibrated (23°C water = 0.997538 g/cm^3).

Cowley et al. (2005) found after 12 hours that the specific gravity of ova stabilized and that inserting ova into the column 24 hours after fertilization provided consistent estimates. Following the methods of Cowley et al. (2005), ova were inserted into the density-gradient column using a long pipette 24 hours after fertilization. Ova then were inserted just below the salinity concentration expected to equal the specific gravity. This allowed ova to be exposed to the surrounding environment for the shortest period of time, which limited water loss due to osmosis. The expected specific gravity of ova was based on results of previous experiments (Cowley et al. 2005; Alleman 2008), as well as results of pilot trials. Ova rose within the column to the height (density) at which they were neutrally buoyant. A few seconds were allowed to ensure ova had stopped rising, and then a height was recorded. Twenty ova were measured per column, five ova at a time. After several ova had been placed in the column, the integrity of the gradient was compromised and a new column was created. Standardized bead heights were recorded after every two insertions to take into consideration the displacement by the ova. Prior to each insertion, diameters of the ova were measured using a dissecting microscope and calibrated ocular micrometer.

Specific Gravity of Larvae Measurements

During pilot trials, larvae were found to be much denser than of ova. Therefore, a denser and steeper salt gradient was required to measure the specific gravity of larvae. Water with 30 ppt salinity replaced the RO water in the first flask and water with 250

ppt salinity replaced 45 ppt salinity in the second flask. The end result was a graded column with water that ranged from approximately 30 ppt (at the top) to 125 ppt salinity (at the bottom). The gradient was calibrated using three standardized beads with known specific gravities (1.025, 1.050, and 1.075) (American Density Material, Inc.).

To measure the specific gravity of larvae, larvae were anesthetized with buffered MS-222 (100 mg/l MS-222 buffered with 233 mg sodium bicarbonate) and were inserted into the column using a long pipette, below the height at which salinity concentration was expected to equal specific gravity of the larvae. This allowed larvae to be exposed to the surrounding environment for the shortest period of time, limiting water loss due to osmosis. In the column, larvae rose in a head-up orientation to the height (density) at which they were neutrally buoyant. During initial trials, larvae were observed to rotate into a head-down orientation. In subsequent trials, this was taken as an indication larvae had reached neutral buoyancy. Prior to inserting the larvae into the column, lengths of larvae were measured using a dissecting microscope and calibrated ocular micrometer. Specific gravity and length of larvae were measured Day 1 post-hatch (within 24 hours post-hatch), Day 3 post-hatch (within 72 hours post-hatch), and Day 5 post-hatch (within 120 hours post-hatch).

The objectives of this study were to measure the specific gravity of both ova and larvae for five broadcast-spawning cyprinids. Because larvae were so much denser than ova, new standardized beads were ordered to calibrate the denser column. These beads were not received until early August, which is near the end of the spawning season for

these species (Bestgen et al. 1989; Taylor and Miller 1990; Durham 2007; Durham and Wilde 2008; Williams 2011). After receiving the beads, I was unable to spawn *H. placitus*, thus specific gravity of *H. placitus* larvae was not measured. In addition, not all species produced sufficient number of ova to estimate specific gravity for all larval stages.

Statistical Analysis

Linear regression of heights and specific gravities of the standardized beads was used to measure the gradient within the column. These regressions were then used to estimate specific gravity of ova and larvae (Figures 2, 3, 4 and 5). Differences in specific gravity and size measurements were assessed using analysis of variance followed by pairwise tests using Fisher's Least Significant Differences model. If zero was not within the 95% confidence limits, species or age comparisons were considered to be statistically significant (α=0.05). Linear regression was used to model the dependence of specific gravity on size (volume of ova or length of larvae). All statistical analyses were performed using SAS statistical software.

CHAPTER III

RESULTS

Specific gravity of ova

Mean specific gravity of ova ranged from 1.0050 to 1.0080 (Table 1) for five species of broadcast-spawning cyprinids. Specific gravity of M*. hyostoma* ova was significantly less ($P < 0.05$) than that of the other four species (Figure 6, Table 2). *Macrhybopsis hyostoma* ova also were significantly larger ($P < 0.05$) in diameter than were ova of the other four species (Figure 7, Table 3). There was a negative correlation, $r = -0.6 \pm 0.1$ (r ± SE), between specific gravity and mean ovum volume across all species (Figure 8).

Specific gravity of larvae

Mean specific gravity of larvae ranged from 1.0440 to 1.0459 (Table 1) for four species of broadcast-spawning cyprinids. For each species, the specific gravity of larvae was significantly ($P < 0.05$) greater than that of ova (Figure 9). Specific gravity of Day 1 post-hatch *M. hyostoma* and *N. girardi* larvae was significantly ($P < 0.05$) less than that of both *N. oxyrhynchus* and *N. buccula* (Figure 10, Table 4). Lengths of Day 1 post-hatch larvae were significantly ($P < 0.05$) different among all four species, except for larvae of *M. hyostoma* and *N. oxyrhynchus*, which were not significantly ($P > 0.05$) different (Figure 11, Table 5). There was no correlation, $r = -0.3 \pm 0.2$, between specific gravity and length of Day 1 post-hatch larvae across all species (Figure 12).

Specific gravity of Day 3 post-hatch larvae was measured for two species, *N. oxyrhynchus* and *N. buccula*. Specific gravity of Day 3 post-hatch *N. oxyrhynchus* and *N.*

buccula larvae was not significantly ($P > 0.05$) different (Table 1, Figure 13). Lengths of day 3 post-hatch *N. oxyrhynchus* and *N. buccula* larvae were also not significantly ($P > 0.05$) different (Figure 14). There was no correlation, $r = 0.3 \pm 0.3$, between specific gravity and length of Day 3 post-hatch larvae across both species (Figure 15).

Specific gravity of *N. buccula* larvae significantly ($P < 0.05$) decreased from Day 1 to Day 3 post-hatch (Figure 16). Lengths of *N. buccula* larvae significantly ($P < 0.05$) increased from Day 1 to Day 3 post-hatch (Figure 17).

Specific gravity of *N. oxyrhynchus* larvae did not significantly differ ($P > 0.05$) from Day 1 to Day 3 post-hatch, but decreased significantly ($P < 0.05$) from Day 3 to Day 5 post-hatch most likely due to the development of the swim bladder (Figure 18, Table 6). Lengths of *N. oxyrhynchus* larvae significantly increased ($P < 0.05$) from Day 1 to Day 3 to Day 5 post-hatch (Figure 19, Table 7).

CHAPTER IV

DISCUSSION

Moore (1944) hypothesized that *N. girardi* ova required high flows to stay suspended in the water column and avoid abrasion and burial by shifting sediment. Mueller (2013) used a shaded flow through raceway system to measure the minimum flow required to suspend *N. girardi* ova and found that ova required less than 0.01 m/s of flow to stay in suspension. The general observation that ova require flow for survival has become well accepted; however, the flow required to keep ova suspended is slow enough that they are unlikely to fall out of suspension unless the river stops flowing. I found that ova were semi-buoyant and will float with very low flow, which is in agreement with the results of Mueller (2013) and other studies in which specific gravity of ova was measured (Cowley 2005; Alleman 2008). I found also larvae were negatively buoyant and require higher flow than ova to stay in suspension. Therefore, Moore's hypothesis that pelagophilic broadcast-spawning cyprinids require high flow to stay suspended in the water column is supported by my results, but since ova require very little flow to remain in suspension, Moore's hypothesis is supported more by the larvae than the ova.

Ova and larvae of pelagophilic broadcast-spawning cyprinids with sufficient flow, stay suspended within the water column and can be dispersed passively downstream. As adults, they migrate back upstream to restore populations in the original spawning area and provide future offspring with enough distance to disperse back downstream (Cross et al. 1985). A number of studies have used artificial beads to

simulate passive dispersal of ova (Dudley and Platania 2007; Medley et al. 2007; Widmer et al. 2012). These studies tested dispersal distances of ova related to flow magnitude and channel morphology, yet did not test variation in specific gravity of artificial beads. Because the specific gravity of larvae is greater than that of ova, the rate of dispersal may be different for larvae than ova. A decrease in buoyancy after hatching may slow dispersal. Conversely, the instinctive swimming movement of larvae may limit the effects of density on dispersal rates by keeping them higher within the water column.

After rivers are fragmented by the construction of an impoundment, pelagophilic broadcast-spawning cyprinids often are negatively affected, leading to a decrease in abundance (Bestgen and Platania 1990; Winston et al. 1991; Moss and Mayes 1993; Luttrell et al. 1999; Wilde and Ostrand 1999; Bonner and Wilde 2000; Perkins and Gido 2011). Although cyprinids with other reproductive strategies not adapted to highly variable flow often increase in distribution and abundance with the construction of an impoundment (Wilde and Ostrand 1999; Bonner and Wilde 2000; Taylor 2010). Dudley and Platania (2007) found that most river reaches in the Rio Grande Basin that were less than 100 km in length no longer support members of this guild. They suggested 100 km may not be a sufficient river length for ova and larvae to drift and develop horizontal movement to swim out of the flow. If the river segment is shorter than the required length for larvae to develop, then larvae may flow into a reservoir and become prey to abundant non-native piscivores or settle to the bottom to suffocate under a layer of sediment (Moore 1944; Winston et al. 1991; Platania and Altenbach 1998;

Dodds et al. 2004; Dudley and Platania 2007). Platania and Altenbach (1998) suggested that over 290 km of continuous river was necessary before larvae were capable of maintaining buoyancy. Although this suggestion assumes that ova and larvae drift at the same rates, the distance required for survival is supported by several assemblage studies and distribution models (Dudley and Platania 2007; Perkins and Gido 2011; Wilde and Urbanczyk 2013; Worthington et al. 2014). Though exceptions to Platania and Altenbach's prediction do exist (Dudley and Platania 2007; Perkins and Gido 2011), the idea that these cyprinids require long river reaches to maintain viable populations is widely accepted (Wilde and Urbanczyk 2014).

Platania and Altenbach (1998) found the Speckled Chub *Macrhybopsis aestivalis*, was the only broadcast-spawning cyprinid native to the Pecos River to persist in a relatively short section of river (89 km). They suggested the persistence of *M. aestivalis* must be due to some aspect of life-history that differed from the other species. I found specific gravity of *M. hyostoma* ova, a sister species of *M. aestivalis*, was lower than that of the other study species. The greater buoyancy of *M. hyostoma* ova would presumably require longer dispersal distances than required by the ova of the other study species, thus indicating that *M. hyostoma* ova might require a longer reach of river for development. However, Urbanczyk and Wilde (unpublished) observed *M. hyostoma* ova hatch at a faster rate than those of other broadcast-spawning cyprinids. If ova of *M. aestivalis* develop faster than those of other broadcast-spawning cyprinids, similar to *M. hyostoma*, this may shorten the length of river needed to support development and might explain their persistence in the 89 km section of the Pecos River.

My results are generally consistent with those of Alleman (2008) who also measured specific gravity of ova of five pelagophilic broadcast spawning cyprinids. Alleman (2008) did not provide evidence of converting the standardized beads used for creating the salt gradient from density to specific gravity. I assumed her measurements were densities and converted them to specific gravities. My estimates of specific gravity of *N. buccula, N. girardi, and N. oxyrhynchus* ova were similar to Alleman (2008}'s estimates for *N. girardi and N. simus pecosensis* ova. Conversely, my estimates of specific gravity of *M. hyostoma* ova were less than Alleman (2008)'s estimates for *M. aestivalis* ova. Also, my estimates of specific gravity of *H. placitus* ova were greater than Alleman (2008)'s estimates for both *H. placitus and H. amarus* ova. The difference in specific gravity of *H. placitus* ova from the two studies makes me suspect the *H. placitus* ova in my study did not fully hydrate. Fish may have been sensitive to the spawning (stripping) procedure, including the injection of hormones and extensive handling, or ova were sensitive to being transferred to a different location. More recent trials by Wilde (unpublished) found the specific gravity of *H. placitus* ova to be more comparable to Alleman's (2008) estimates.

Durham and Wilde (2006) found that the only flow condition of the Canadian River in which no young of pelagophilic broadcast-spawning cyprinids were produced was when flow had ceased. Successful production of young during low flow conditions conflicts with Moore's (1944) hypothesis that these cyprinids require high flows to prevent abrasion and suffocation from the shifting sediment. Although successful production of young occurs in low flow conditions, low flows may not be sufficient to

produce enough young to maintain viable populations. Wilde and Durham (2008) found population growth rate of Peppered Chub *Macrhybopsis tetranema* in the Canadian River was directly related to discharge. Population growth did not occur if mean summer discharge did not exceed 11.9 m^3/s. For 28 of the last 40 years the mean summer discharge has not done so (Wilde and Durham 2008) and the population is now considered to be in decline (Perkins and Gido 2011).

Pelagophilic broadcast-spawning cyprinids are of conservation concern across the US Great Plains. At least seven species of this guild are federally endangered (Jelks et al. 2008; U.S. Fish and Wildlife Service 2014) and two are extinct (Chernoff et al.1982; Bestgen and Platania 1990; Platania and Altenbach 1998). The extinction of the Phantom Shiner *Notropis orca* and the Rio Grande Bluntnose Shiner *N. simus simus* have been attributed to declines in annual discharge from irrigation demand and altered natural discharge patterns (Bestgen and Platania 1990). An increase in low and no flow conditions or lack of high flow events from human water use and fragmentation have led to a decrease in abundance for many of these cyprinids (Cross et al. 1983; Bestgen et al. 1989; Bonner and Wilde 2000; Taylor 2010, Perkins and Gido 2011, Wilde and Urbanczyk 2013). My results show that larvae are denser than ova and, consequently, are likely to require higher flows to keep them suspended within the water column. This is consistent with Moore's (1944) hypothesis and the observations of Wilde and Durham (2008) who showed population growth rate is related to flow. Therefore, it seems that survival through the larval stage may limit reproductive success and further studies of larvae survival and dispersal may be required before we have an adequate

understanding of the flow requirements needed for the maintenance of viable populations of pelagophilic broadcast-spawning cyprinids.

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APPENDIX

Table 1: Mean specific gravity of ova and larvae of *Hybognathus placitus*, *Macrhybopsis hyostoma*, *Notropis buccula*, *Notropis girardi*, and *Notropis oxyrhynchus*.

Species	Life Stage	Day Post-Hatch	Mean Specific Gravity	Standard Error	*N*
Hybognathus placitus	Ova		1.0080	0.0001	73
Macrhybopsis hyostoma	Ova		1.0050	0.0001	30
	Larvae	1	1.0459	0.0013	30
Notropis buccula	Ova		1.0078	0.0001	39
	Larvae	1	1.0446	0.0003	39
	Larvae	3	1.0435	0.0003	37
Notropis girardi	Ova		1.0075	0.0001	10
	Larvae	1	1.0460	0.0005	10
Notropis oxyrhynchus	Ova		1.0078	0.0001	73
	Larvae	1	1.0440	0.0003	47
	Larvae	3	1.0434	0.0004	39
	Larvae	5	1.036	0.001	19

Table 2: Analysis of specific gravity of ova approximately 24 hours post-fertilization for *Hybognathus placitus*, *Macrhybopsis hyostoma*, *Notropis buccula*, *Notropis girardi*, and *Notropis oxyrhynchus* using Fishers LSD test. Table shows the relationship between species and indicates statistical significance. If zero was not within the 95% confidence limits, species comparison was considered to be statistically significant (α=0.05).

Species - Comparison	Difference Between Means	95% Confidence Limits		Significant
		Lower	Upper	
Hybognathus placitus - Macrhybopsis hyostoma	0.0029 ± 0.0002	0.0025	0.0033	*
Hybognathus placitus - Notropis buccula	0.0002 ± 0.0002	−0.0002	0.0006	
Hybognathus placitus - Notropis girardi	0.0005 ± 0.0003	−0.0001	0.0011	
Hybognathus placitus - Notropis oxyrhynchus	0.0002 ± 0.0001	−0.0001	0.0005	
Macrhybopsis hyostoma - Notropis buccula	−0.0027 ± 0.0002	−0.0031	−0.0023	*
Macrhybopsis hyostoma - Notropis girardi	−0.0024 ± 0.0003	−0.0031	−0.0018	*
Macrhybopsis hyostoma - Notropis oxyrhynchus	−0.0027 ± 0.0002	−0.0031	−0.0023	*
Notropis buccula - Notropis girardi	0.0003 ± 0.0003	−0.0004	0.0009	
Notropis buccula - Notropis oxyrhynchus	−0.0000 ± 0.0002	−0.0004	0.0003	
Notropis girardi - Notropis oxyrhynchus	−0.0003 ± 0.0003	−0.0009	0.0003	

Table 3: Analysis of diameter of ova approximately 24 hours post-fertilization for *Hybognathus placitus*, *Macrhybopsis hyostoma*, *Notropis buccula*, *Notropis girardi*, and *Notropis oxyrhynchus* using Fishers LSD test. Table shows the relationship between species and indicates statistical significance. If zero was not within the 95% confidence limits, species comparison was considered to be statistically significant (α=0.05).

Species - Comparison	Difference Between Means	95% Confidence Limits		Significant
		Lower	Upper	
Hybognathus placitus - Macrhybopsis hyostoma	− 0.52 ± 0.01	−0.59899	−0.43828	*
Hybognathus placitus - Notropis buccula	0.09 ± 0.01	0.02696	0.16005	*
Hybognathus placitus - Notropis girardi	0.436 ± 0.002	0.33057	0.54216	*
Hybognathus placitus - Notropis oxyrhynchus	0.46 ± 0.01	0.40225	0.51715	*
Macrhybopsis hyostoma - Notropis buccula	0.61 ± 0.02	0.52588	0.69841	*
Macrhybopsis hyostoma - Notropis girardi	0.96 ± 0.02	0.83581	1.07419	*
Macrhybopsis hyostoma - Notropis oxyrhynchus	0.98 ± 0.01	0.89887	1.05779	*
Notropis buccula - Notropis girardi	0.34 ± 0.02	0.23251	0.45321	*
Notropis buccula - Notropis oxyrhynchus	0.37 ± 0.01	0.30074	0.43165	*
Notropis girardi - Notropis oxyrhynchus	0.02 ± 0.02	−0.08178	0.12845	

Table 4: Analysis of specific gravity of larvae within 24 hours post-hatch for *Macrhybopsis hyostoma*, *Notropis buccula*, *Notropis girardi*, and *Notropis oxyrhynchus* using Fishers LSD test. Table shows the relationship between species and indicates statistical significance. If zero was not within the 95% confidence limits, species comparison was considered to be statistically significant (α=0.05).

Species - Comparison	Difference Between Means	95% Confidence Limits		Significant
		Lower	Upper	
Macrhybopsis hyostoma - Notropis buccula	0.0013 ± 0.005	0.0004	0.0023	*
Macrhybopsis hyostoma - Notropis girardi	− 0.0001 ± 0.0007	−0.0015	0.0013	
Macrhybopsis hyostoma - Notropis oxyrhynchus	0.0019 ± 0.0005	0.0010	0.0028	*
Notropis buccula - Notropis girardi	− 0.0014 ± 0.0007	−0.0028	0.0000	*
Notropis buccula - Notropis oxyrhynchus	0.0006 ± 0.0004	−0.0002	0.0014	
Notropis girardi - Notropis oxyrhynchus	0.0020 ± 0.0007	0.0007	0.0034	*

Table 5: Analysis of length of larvae within 24 hours post-hatch for *Macrhybopsis hyostoma*, *Notropis buccula*, *Notropis girardi*, and *Notropis oxyrhynchus* using Fishers LSD test. Table shows the relationship between species and indicates statistical significance. If zero was not within the 95% confidence limits, species comparison was considered to be statistically significant (α=0.05).

Species - Comparison	Difference Between Means	95% Confidence Limits		Significant
		Lower	Upper	
Macrhybopsis hyostoma - Notropis buccula	− 0.79 ± 0.03	−0.92	−0.67	*
Macrhybopsis hyostoma - Notropis girardi	0.30 ± 0.04	0.12	0.49	*
Macrhybopsis hyostoma - Notropis oxyrhynchus	− 0.06 ± 0.02	−0.19	0.06	
Notropis buccula - Notropis girardi	1.1 ± 0.1	0.92	1.28	*
Notropis buccula - Notropis oxyrhynchus	0.73 ± 0.02	0.61	0.85	*
Notropis girardi - Notropis oxyrhynchus	− 0.37 ± 0.04	−0.54	−0.19	*

Table 6: Analysis of specific gravity of larvae for *Notropis oxyrhynchus* using Fishers LSD test. Table shows the relationship between larvae Day 1, Day 3, and Day 5 post-hatch and indicates statistical significance. If zero was not within the 95% confidence limits, age comparison was considered to be statistically significant (α=0.05).

Notropis oxyrhynchus	Difference Between Means	95% Confidence Limits		Significant
		Lower	Upper	
Day 1 - Day 3 Post-Hatch	0.0006 ± 0.0006	−0.0007	0.0018	
Day 1 - Day 5 Post-Hatch	0.0075 ± 0.0008	0.0059	0.0091	*
Day 3 - Day 5 Post-Hatch	0.0069 ± 0.0008	0.0053	0.0086	*

Table 7: Analysis of length of larvae for *Notropis oxyrhynchus* using Fishers LSD test. Table shows the relationship between larvae Day 1, Day 3, and Day 5 post-hatch and indicates statistical significance. If zero was not within the 95% confidence limits, age comparison was considered to be statistically significant (α=0.05).

Notropis oxyrhynchus	Difference Between Means	95% Confidence Limits		Significant
		Lower	Upper	
Day 1 - Day 3 Post-Hatch	0.857 ± 0.002	0.7463	0.9680	*
Day 1 - Day 5 Post-Hatch	1.128 ± 0.003	0.9846	1.2707	*
Day 3 - Day 5 Post-Hatch	0.270 ± 0.003	0.1274	0.4135	*

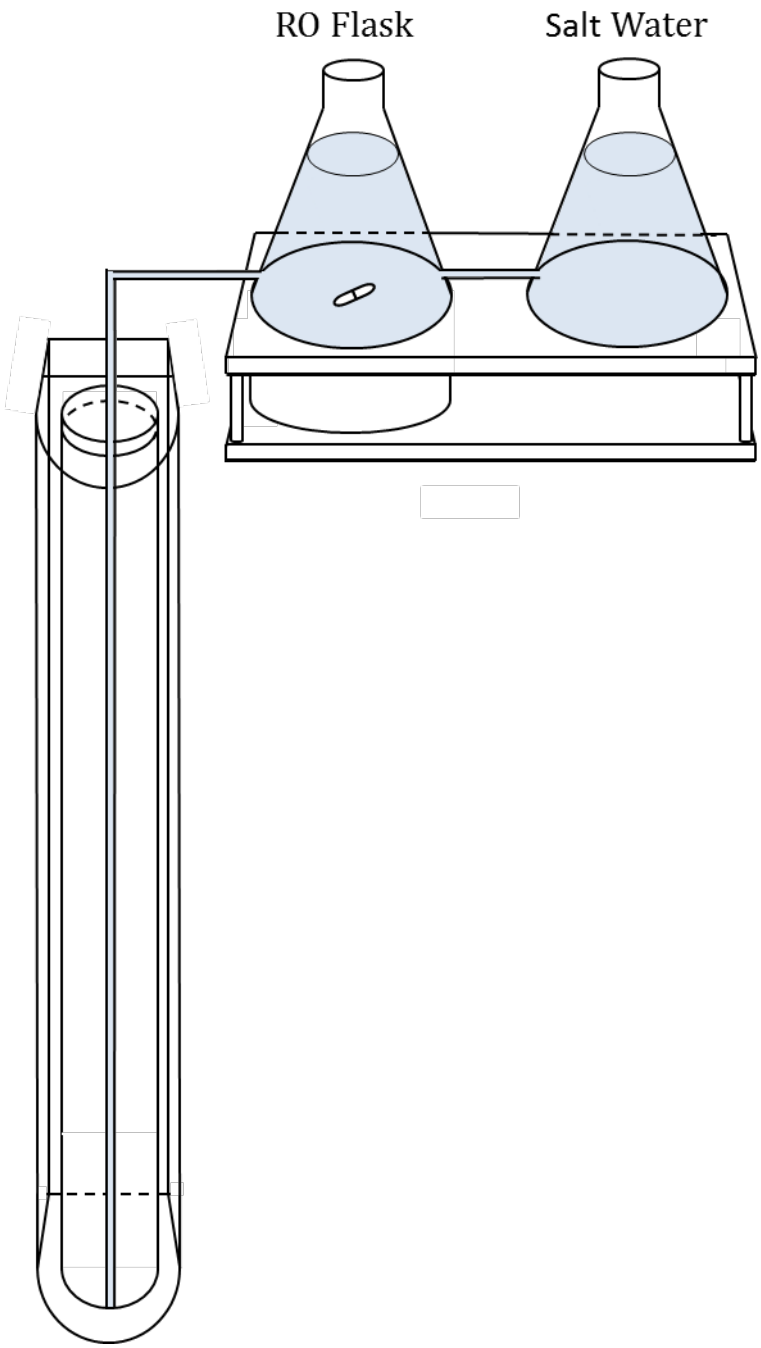

Figure 1: A drawing of the density gradient column used in this study.

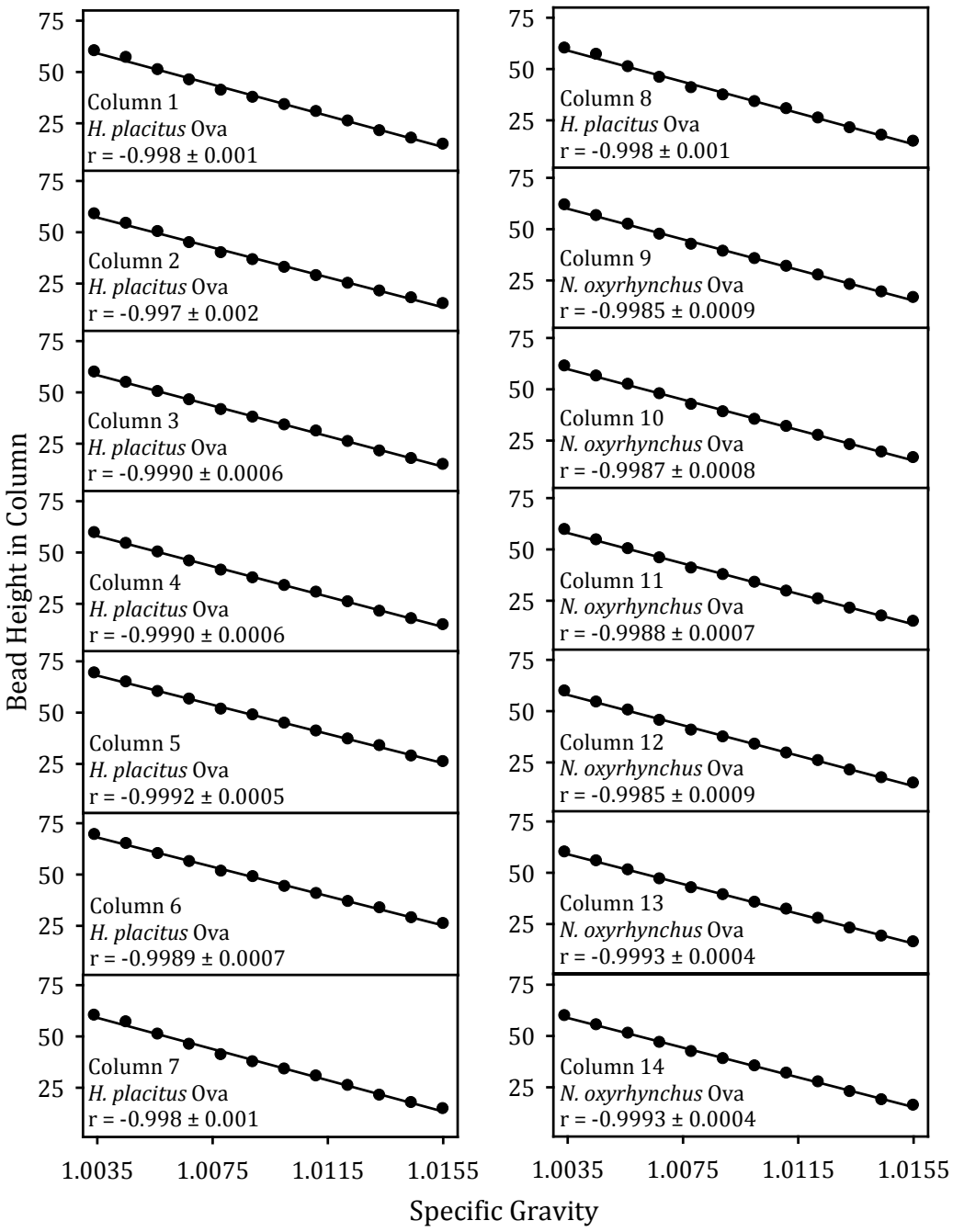

Figure 2: Linear regressions for density gradient columns 1 through 14 were used to estimate specific gravity of ova. Black dots represent 12 standardized specific gravity beads and respective heights within the column.

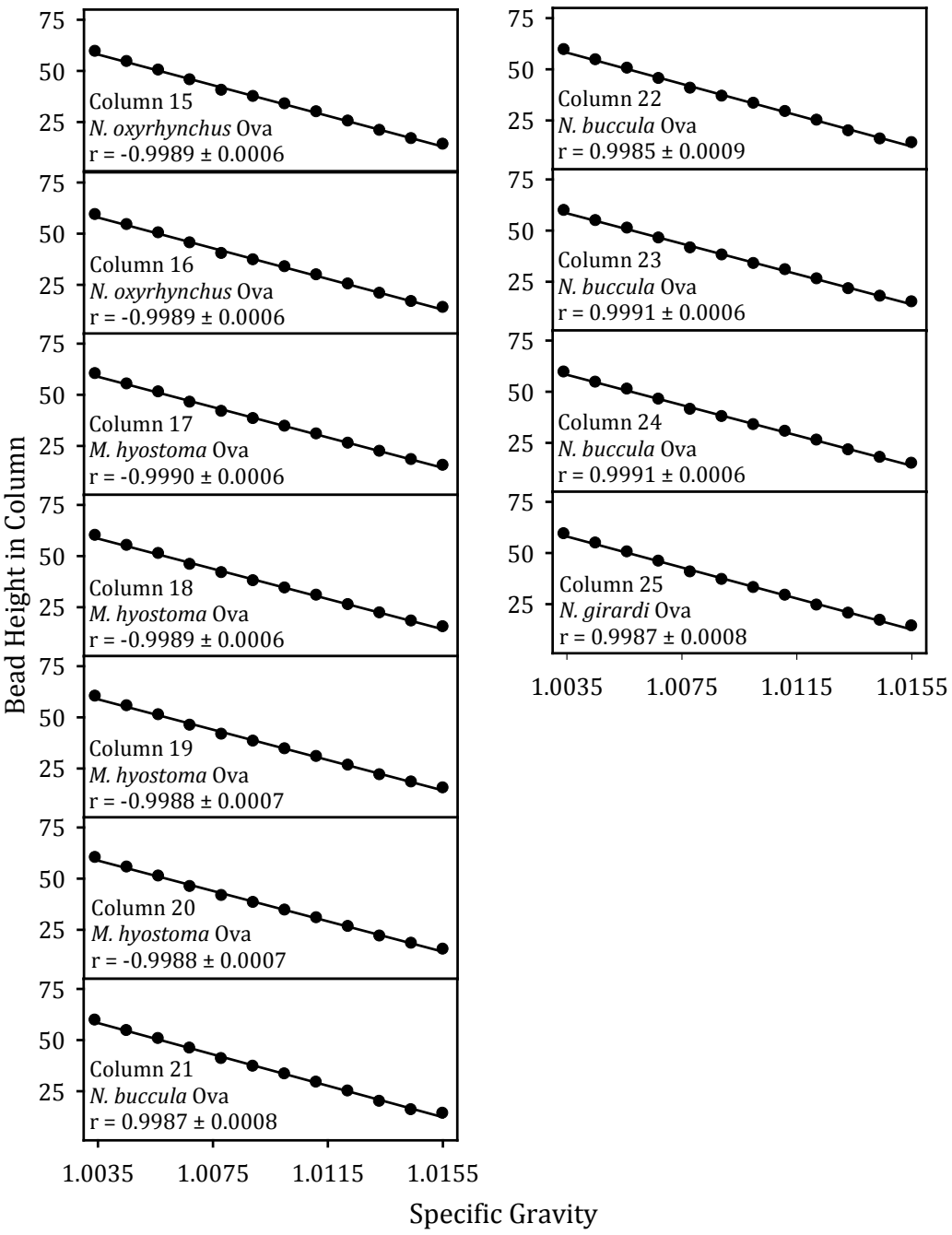

Figure 3: Linear regressions for density gradient columns 15 through 25 were used to estimate specific gravity of ova. Black dots represent 12 standardized specific gravity beads and respective heights within the column.

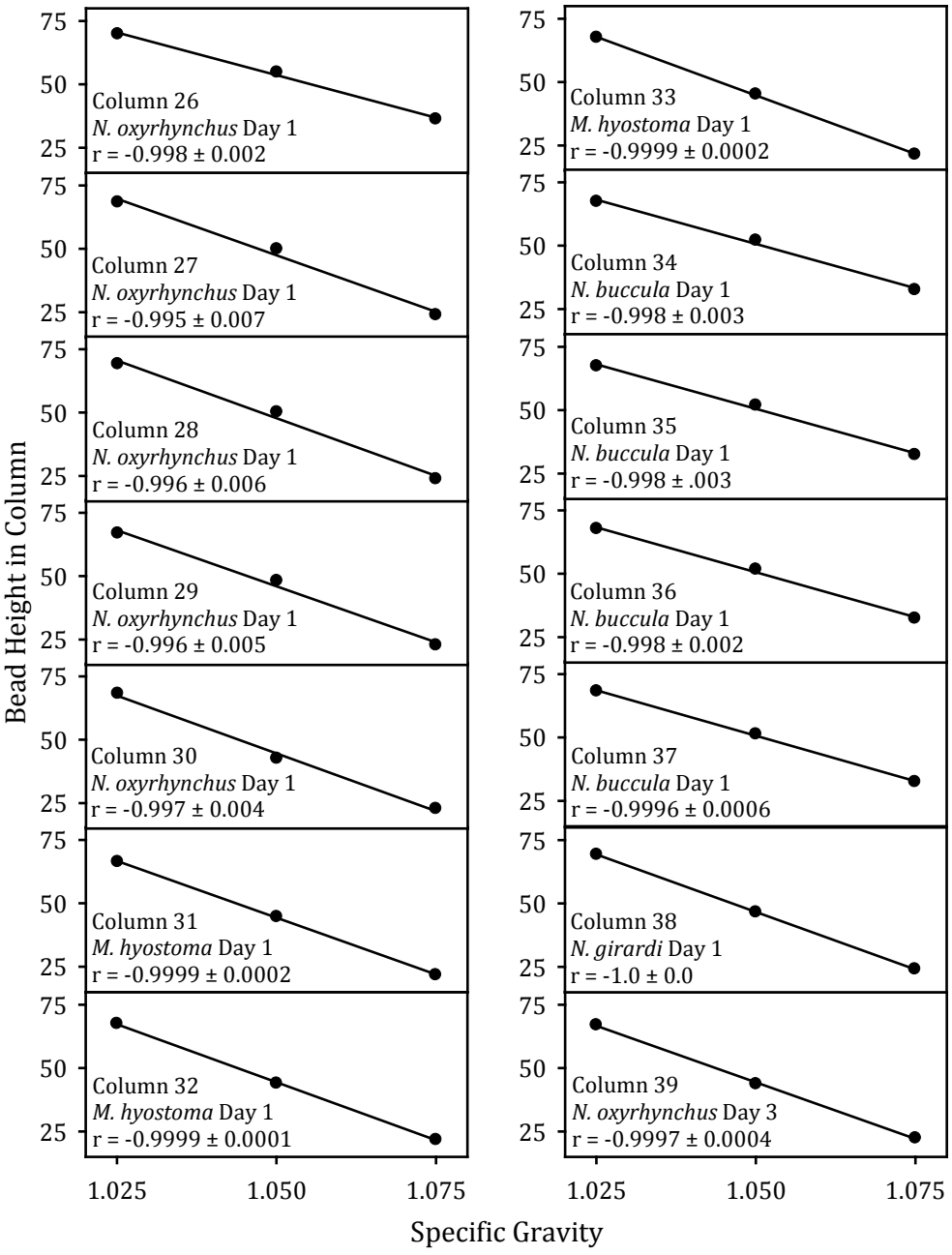

Figure 4: Linear regressions for density gradient columns 26 through 39 used to estimate specific gravity of larvae. Black dots represent three standardized specific gravity beads and respective heights within the column.

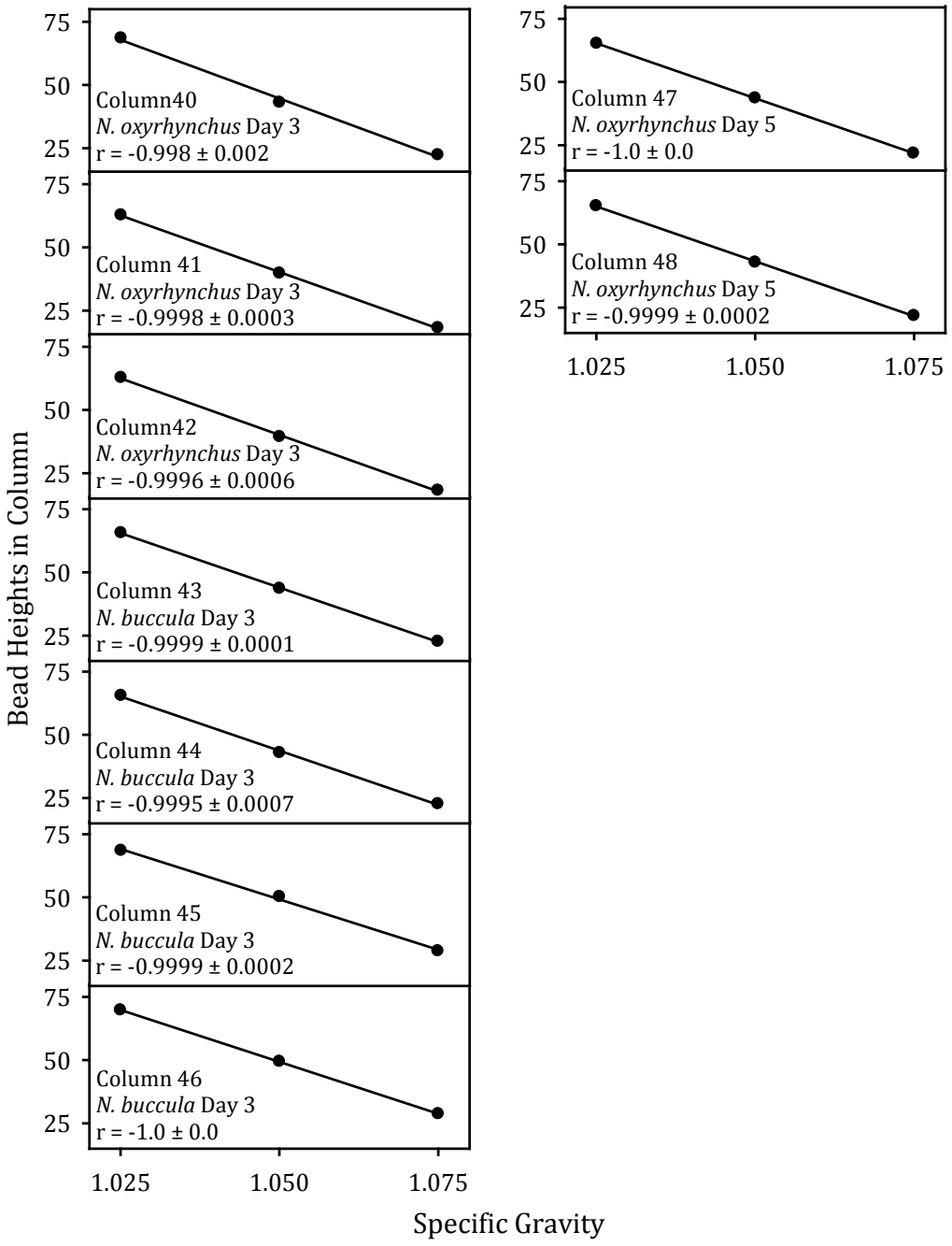

Figure 5: Linear regressions for density gradient columns 40 through 48 used to estimate specific gravity of larvae. Black dots represent three standardized specific gravity beads and respective heights within the column.

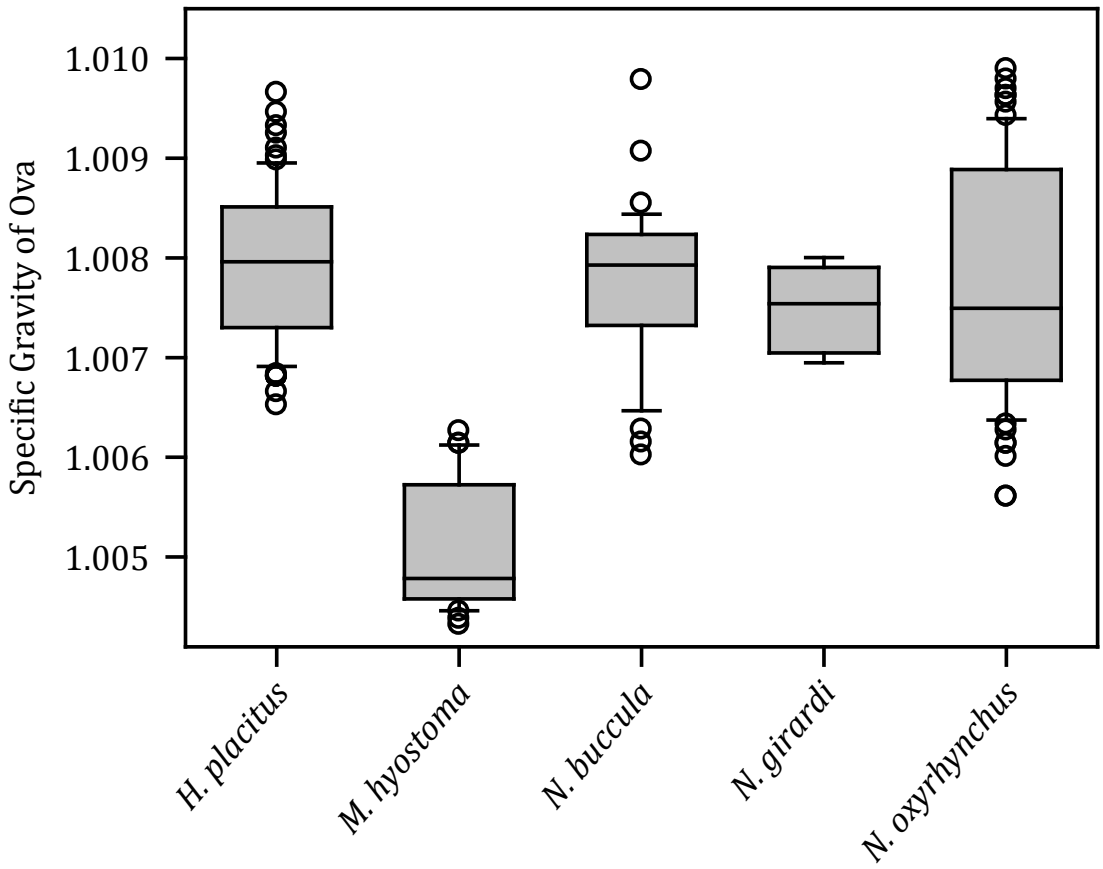

Figure 6: Box plot showing specific gravity of ova (24 hours post-fertilization) for *Hybognathus placitus*, *Macrhybopsis hyostoma*, *Notropis buccula*, *Notropis girardi*, and *Notropis oxyrhynchus*. For each species the box includes the median (denoted by a horizontal line through the box), the 25th percentile, and the 75th percentile. Whiskers above and below the boxes represent 1.5 times the interquartile range. Hollow circles represent outliers.

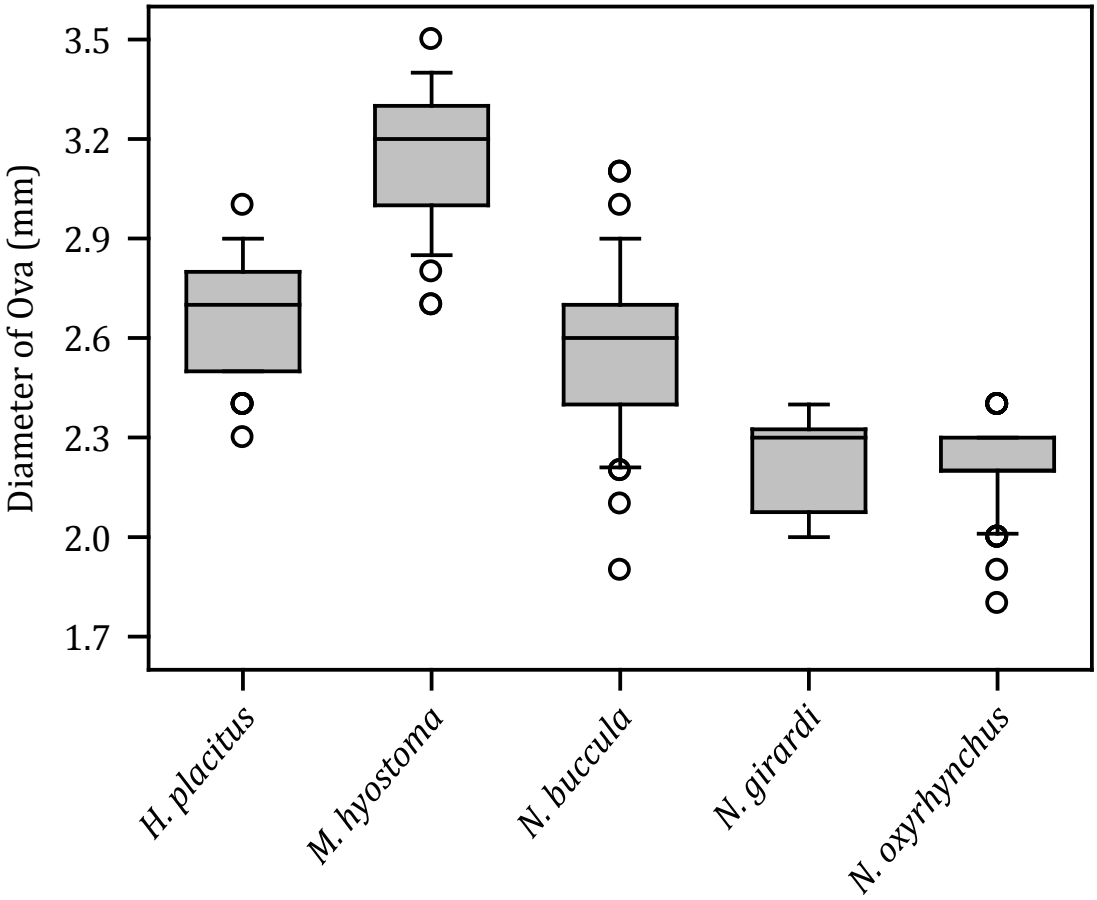

Figure 7: Box plot showing diameter of ova (24 hours post-fertilization) for *Hybognathus placitus*, *Macrhybopsis hyostoma*, *Notropis buccula*, *Notropis girardi*, and *Notropis oxyrhynchus*.

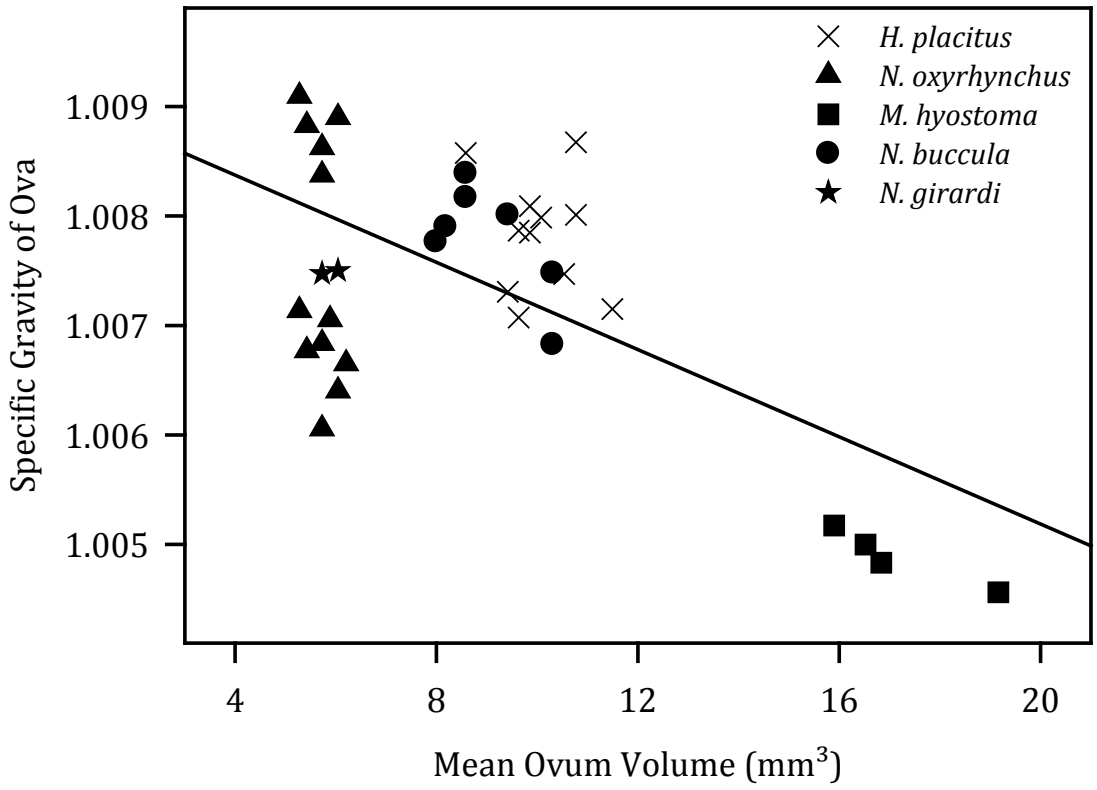

Figure 8: Linear regression describing the relationship between specific gravity of ova and mean ovum volume for *Hybognathus placitus*, *Macrhybopsis hyostoma*, *Notropis buccula*, *Notropis girardi*, and *Notropis oxyrhynchus*.

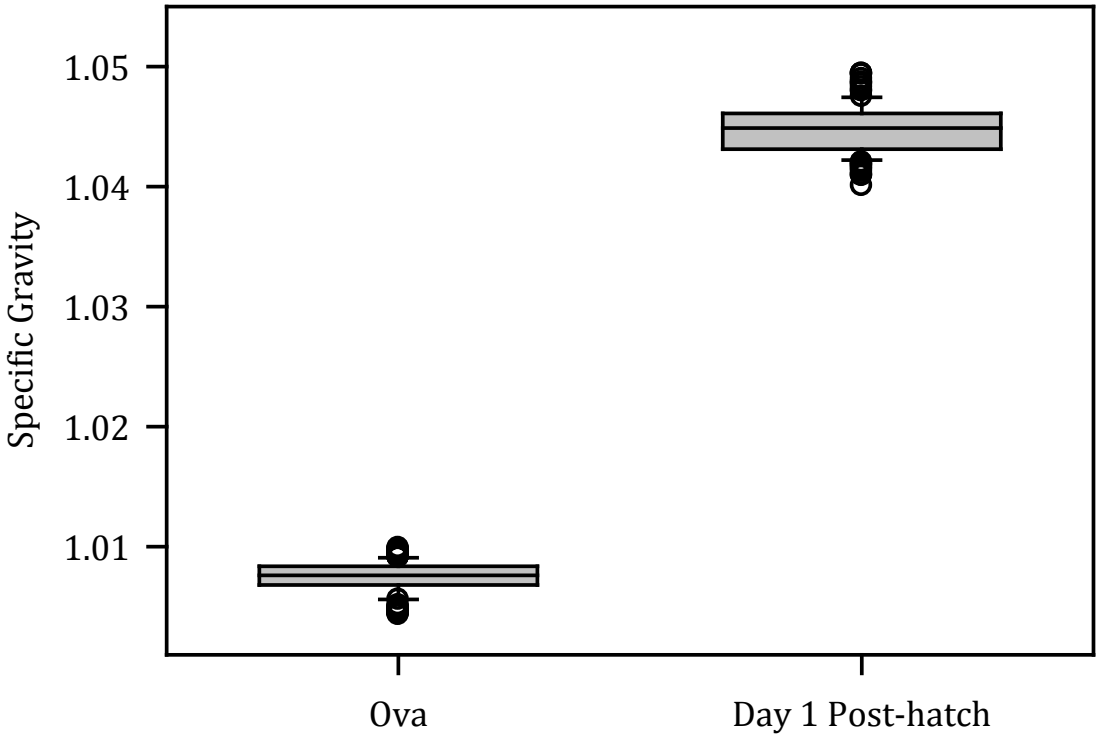

Figure 9: Box plot showing specific gravity of ova (24 hours post-fertilization) and larvae Day 1 post-hatch (24 hours post-hatch) for *Hybognathus placitus*, *Macrhybopsis hyostoma*, *Notropis buccula*, *Notropis girardi*, and *Notropis oxyrhynchus*.

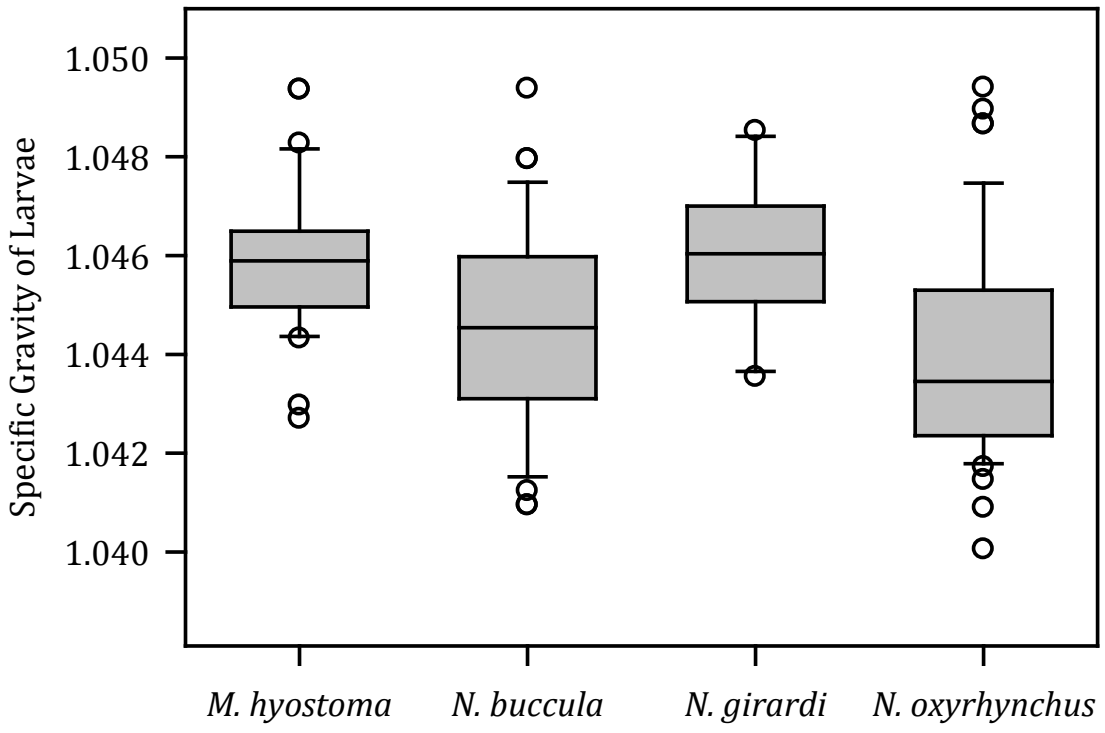

Figure 10: Box plot showing specific gravity of larvae Day 1 post-hatch (24 hours post-hatch) for *Macrhybopsis hyostoma*, *Notropis buccula*, *Notropis girardi*, and *Notropis oxyrhynchus*.

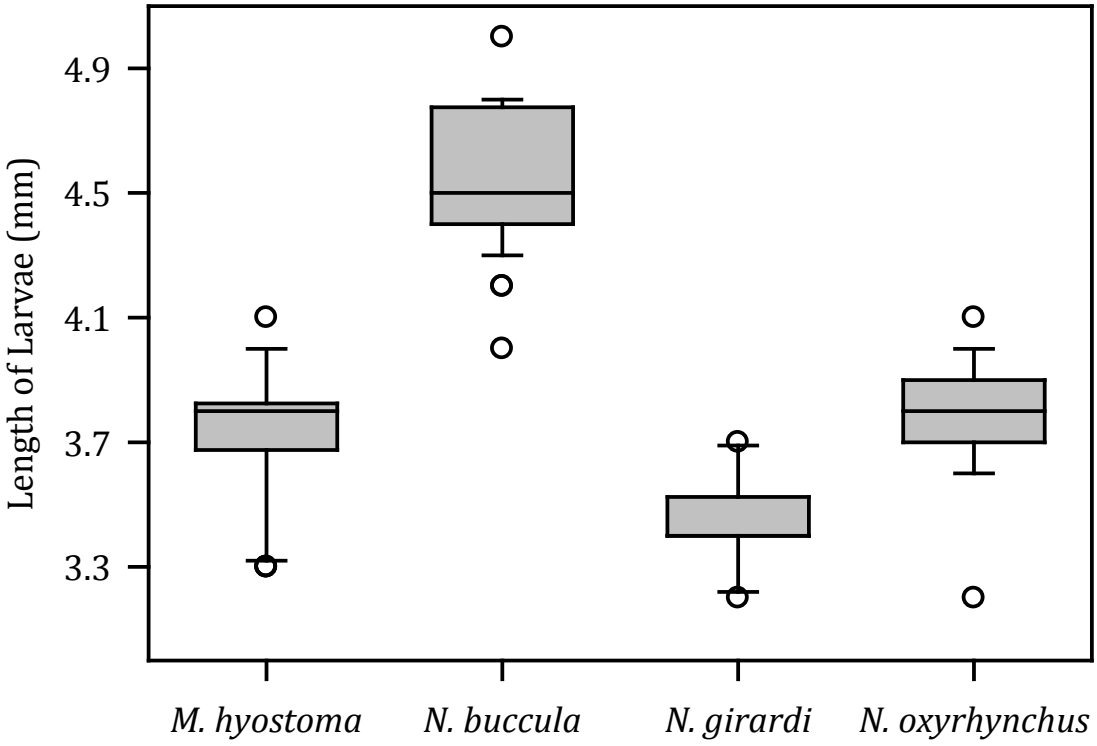

Figure 11: Box plot showing length of larvae Day 1 post-hatch (24 hours post-hatch) for *Macrhybopsis hyostoma*, *Notropis buccula*, *Notropis girardi*, and *Notropis oxyrhynchus*.

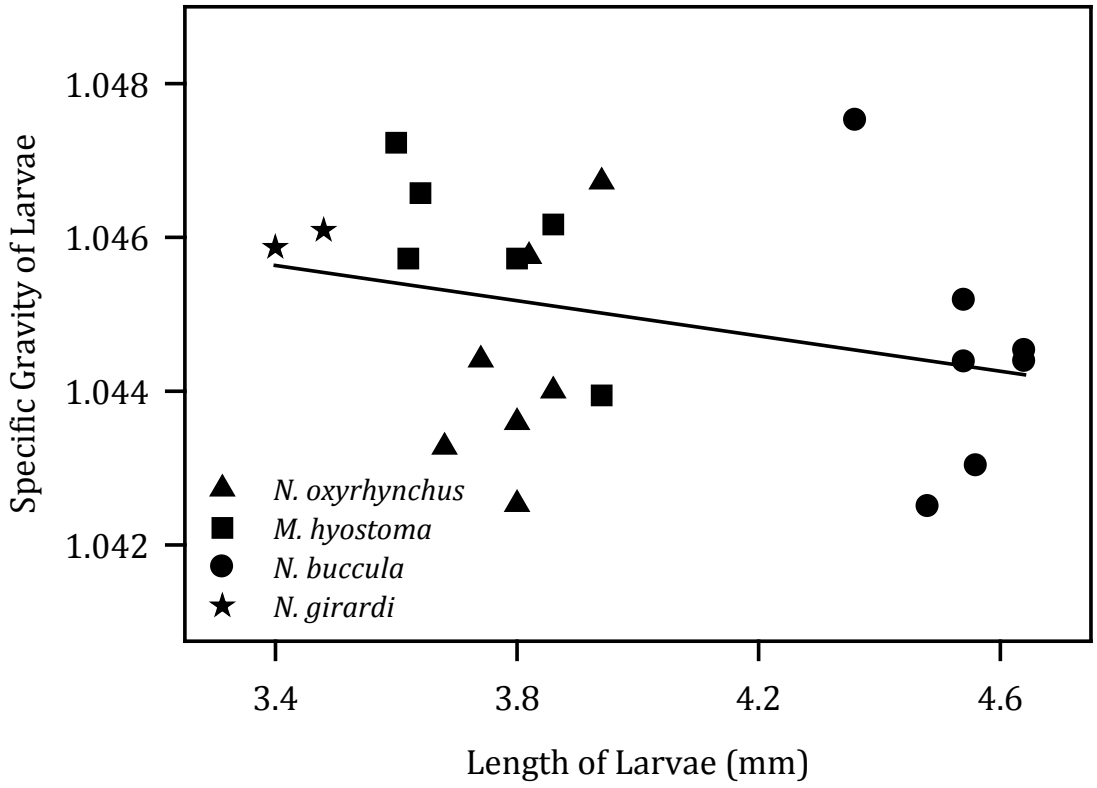

Figure 12: Linear regression describing the relationship between specific gravity and length of larvae Day 1 post-hatch for *Macrhybopsis hyostoma*, *Notropis buccula*, *Notropis girardi*, and *Notropis oxyrhynchus*.

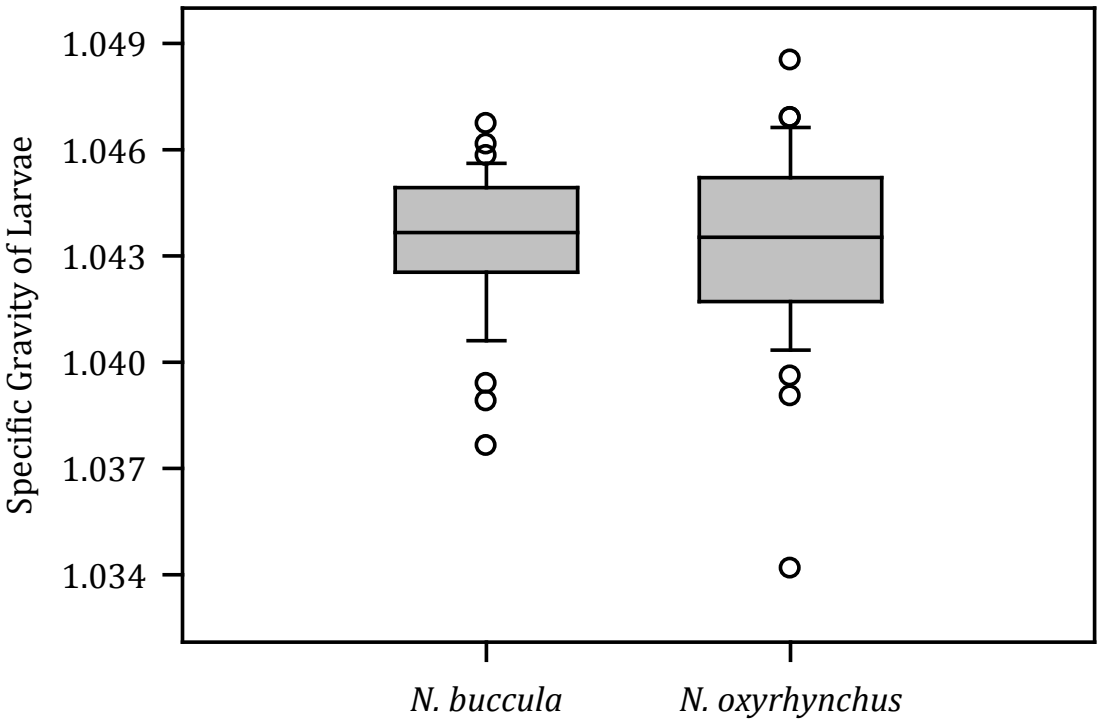

Figure 13: Box plot showing specific gravity of larvae Day 3 post-hatch (72 hours post-hatch) for *Notropis buccula* and *Notropis oxyrhynchus*.

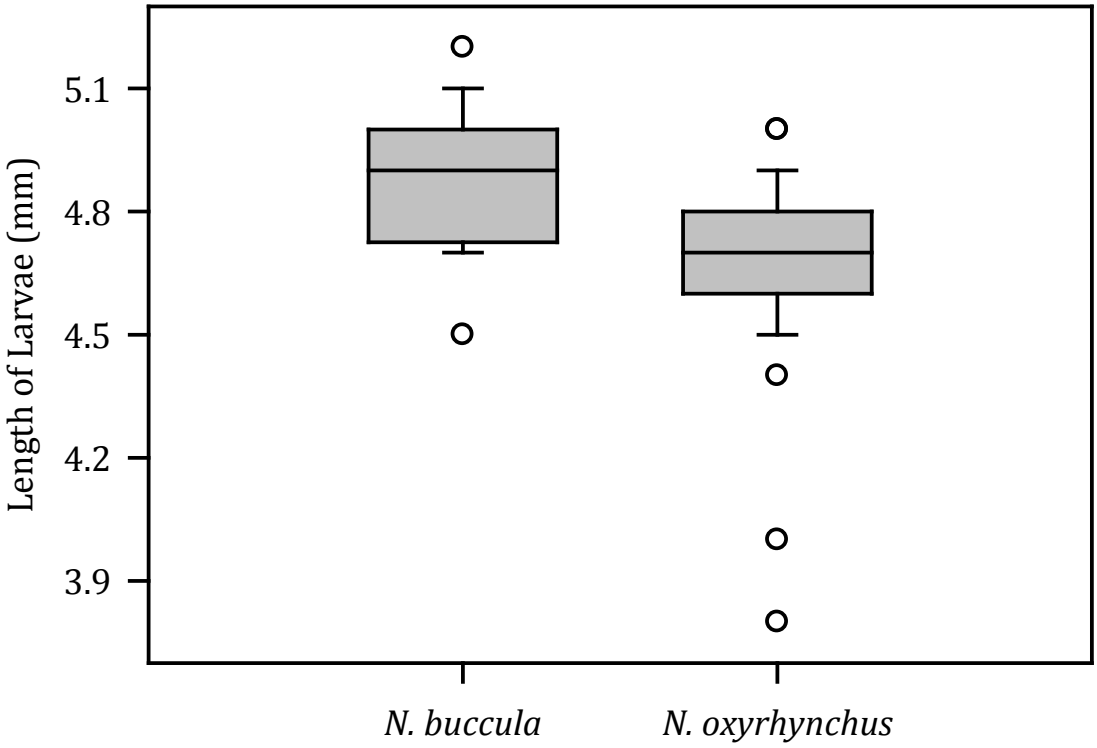

Figure 14: Box plot showing length of larvae Day 3 post-hatch (72 hours post-hatch) for *Notropis buccula* and *Notropis oxyrhynchus*.

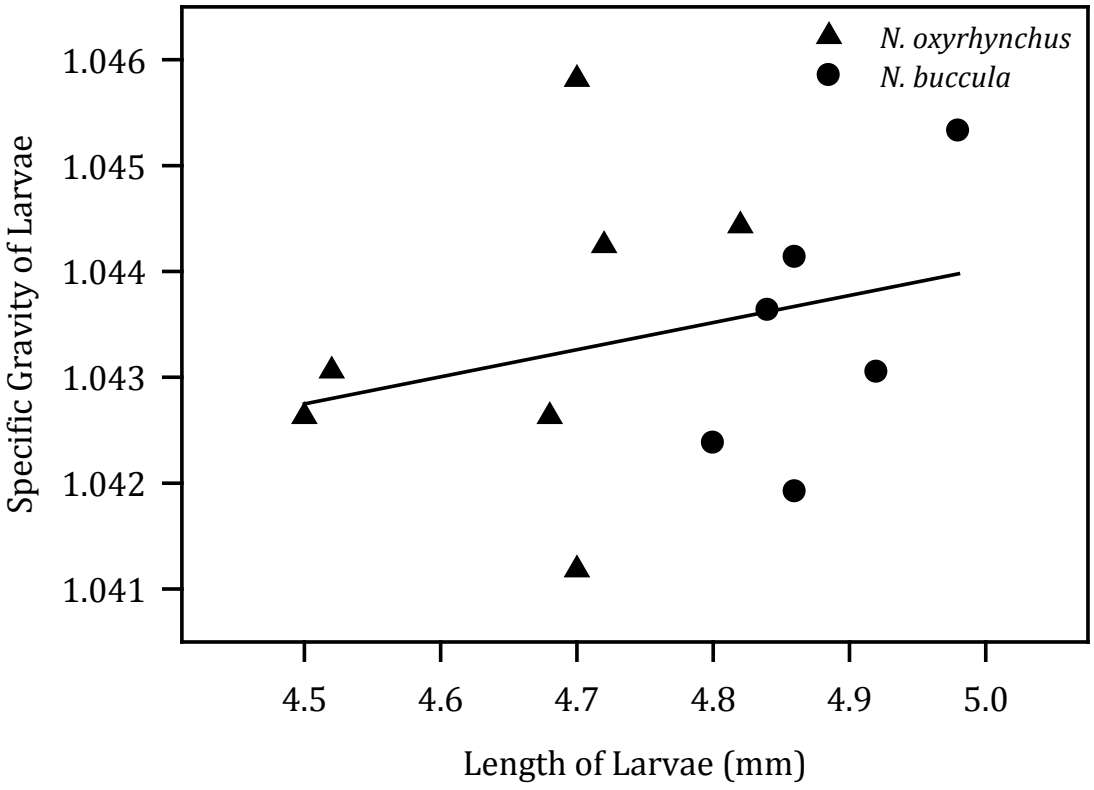

Figure 15: Linear regression describing the relationship between specific gravity and length of larvae Day 3 post-hatch for *Notropis buccula* and *Notropis oxyrhynchus*.

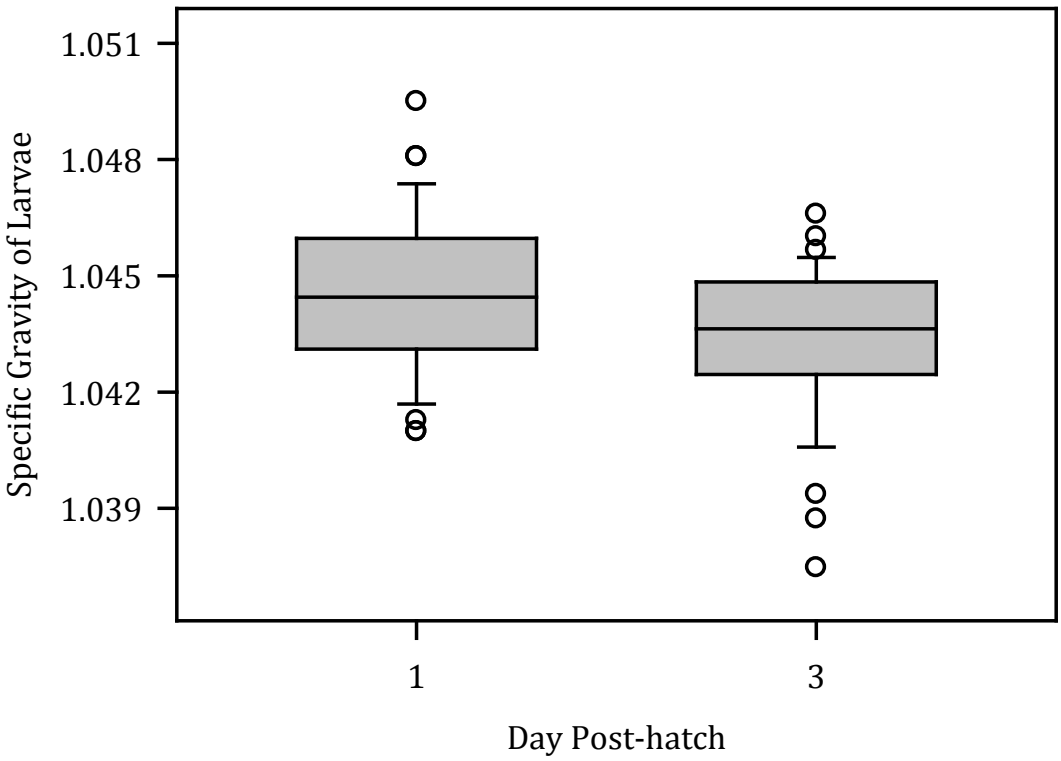

Figure 16: Box plot showing specific gravity of larvae and Day post-hatch for *Notropis buccula*.

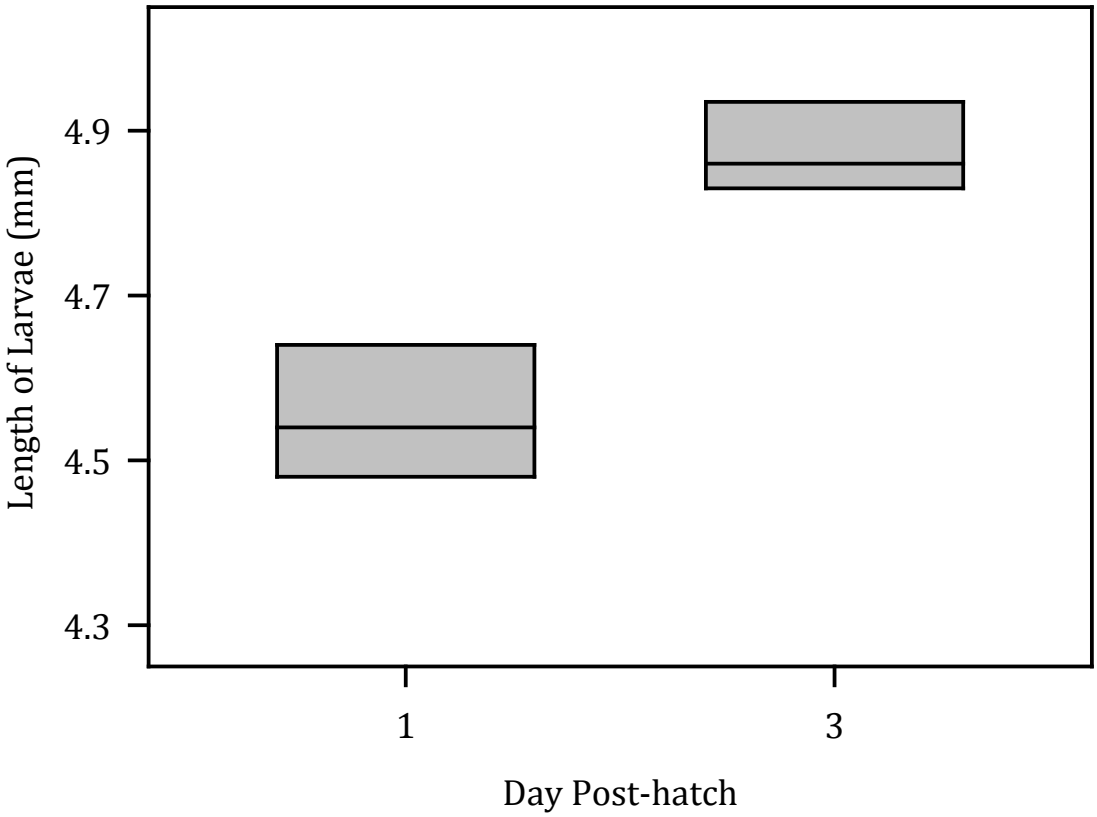

Figure 17: Box plot showing length of larvae and Day post-hatch for *Notropis buccula*.

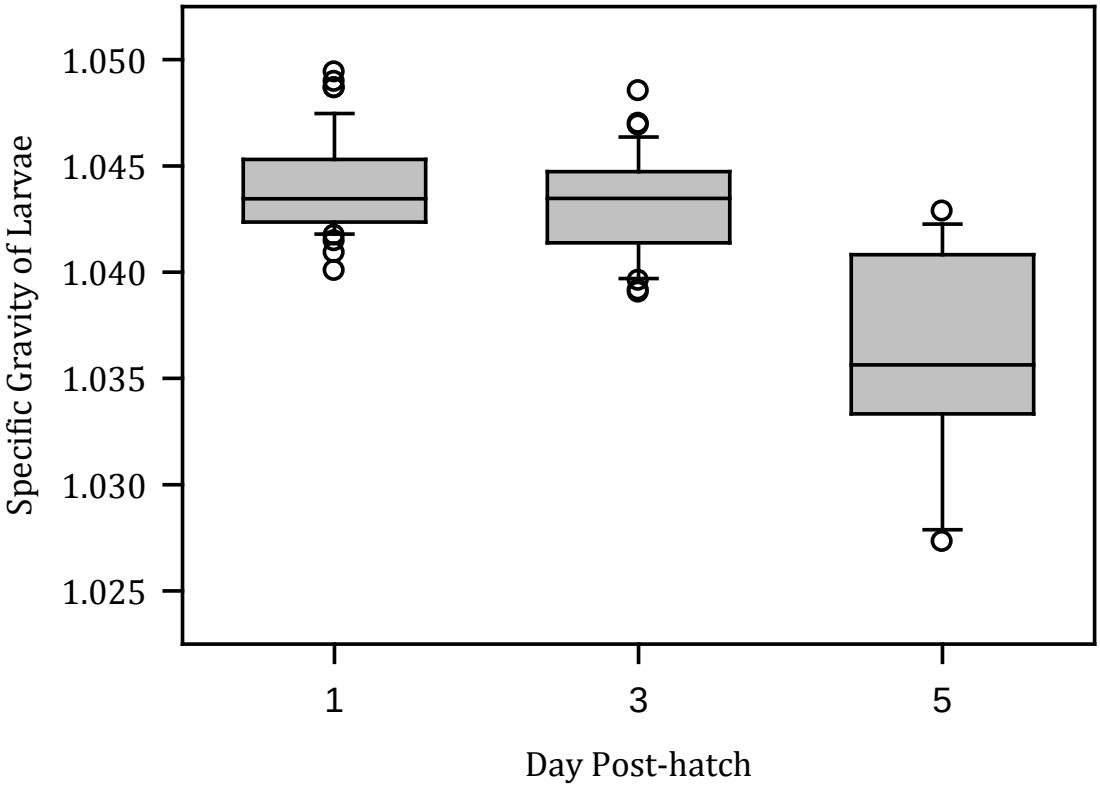

Figure 18: Box plot showing specific gravity of larvae and Day post-hatch for *Notropis oxyrhynchus*.

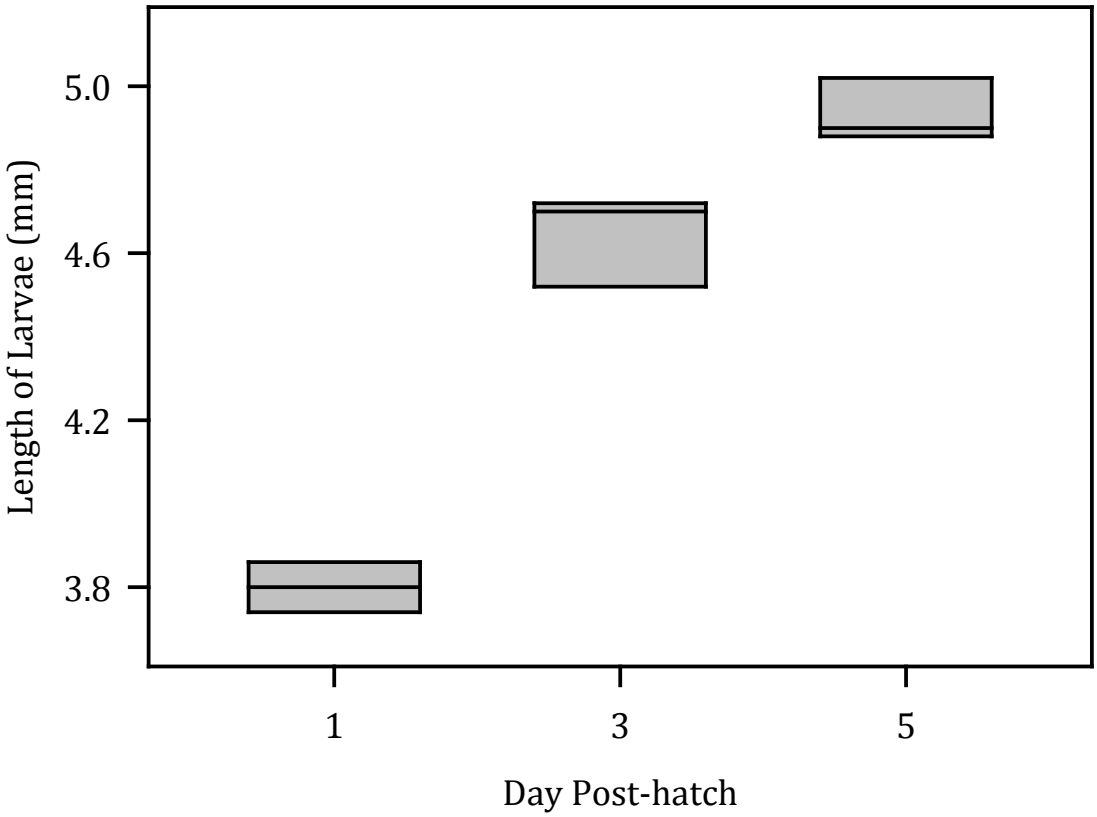

Figure 19: Box plot showing length of larvae and Day post-hatch for *Notropis oxyrhynchus*.