Effects of Temperature and Dissolved Oxygen on Development and Survival of Embryos in Nests of Bluehead Chub *Nocomis leptocephalus*

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ABSTRACT

Temperature and dissolved oxygen (DO) are two fundamental environmental factors that shape metabolic demand, developmental rate, and survival in fish embryos. Because embryos rely entirely on diffusion for oxygen uptake and cannot move away from stressful conditions, even small shifts in temperature or oxygen availability can strongly influence early survival. Despite their importance, the combined effects of temperature and DO remain understudied in non-model species and in natural systems where embryos occur at high densities. The communal nesting system of the Bluehead Chub *Nocomis leptocephalus* and its nest associates provides an ecologically realistic setting in which these stressors may interact, as many species deposit eggs within gravel mounds where oxygen levels fluctuate and embryos develop in close proximity. This thesis experimentally examined how temperature and dissolved oxygen influence embryo physiology and performance within this communal nesting context. Using controlled laboratory experiments across multiple temperature and oxygen conditions, I quantified oxygen consumption, developmental progression, and survival for the combined embryos (Bluehead Chub and nest associates) within the multi-species reproductive aggregation**.** Oxygen use increased consistently with warming, reflecting higher metabolic demand at elevated temperatures, but did not differ with ambient oxygen level, indicating that embryos maintained metabolic activity even when oxygen availability was reduced. Developmental rate also accelerated with increasing temperature, yet this rapid growth came at a cost: survival declined sharply at the warmest conditions, and low oxygen further amplified mortality under high temperatures. Cooler environments supported slower development rate but produced the highest overall survival. Together, the results show that warming is the primary driver of embryo stress, with oxygen availability offering only a limited buffering effect. These patterns highlight that embryos in communal nests are highly sensitive to modest environmental changes, particularly because they cannot behaviorally avoid unfavorable conditions. As stream temperatures rise and oxygen levels fluctuate with climate change, the narrow environmental window required for successful embryo development may constrain reproductive success and ultimately the population resilience of Bluehead Chub and its diverse nest associates.

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GENERAL AUDIENCE ABSTRACT

Fish embryos experience the world very differently from adults. Because they cannot move, they depend completely on the conditions around them, especially water temperature and the amount of oxygen available. If the water becomes too warm or oxygen levels drop, embryos cannot escape, and their chances of surviving can quickly change. With climate change causing streams to warm and oxygen levels to fluctuate more than before, the environments where fish lay their eggs may no longer provide the stable conditions embryos need. Bluehead Chub males build large gravel mound nests that attract many other fish species to lay their eggs alongside them. This creates crowded "neighborhoods" of embryos that share the same space and depend on the same limited supply of oxygen. In these tight conditions, even small environmental changes could make the difference between healthy development and high mortality. To understand how these changes might affect fish, I conducted experiments to see how different temperatures and oxygen levels influenced the development, oxygen use, and survival of the aggregation of embryos found on Bluehead Chub nests. The results showed clear and important patterns. Warmer water helped embryos grow and develop faster, but it also made them use much more oxygen. At the same time, warmer water naturally holds less oxygen, creating a stressful situation for embryos. When oxygen levels were low, survival dropped even more, especially at high temperatures. Interestingly, embryos used about the same amount of oxygen whether oxygen levels were high or low. This suggests that they were working hard to keep up with the demands of development, possibly pushing close to their physiological limits in low-oxygen environments. Overall, the study showed that fish embryos are highly sensitive to changes in temperature and oxygen, and that small shifts in these conditions can ripple through entire nesting communities. As climate change continues to alter stream environments, the narrow window of conditions that embryos require for healthy development may become harder to find. These changes could influence the reproductive success of Bluehead Chub and many of the species that rely on their nests, ultimately shaping fish populations in freshwater ecosystems.

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ATTRIBUTION

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Introduction

Freshwater stream ecosystems are increasingly threatened by human activities such as land-use change, water extraction, and climate change (Oberdorff 2022). Rising temperatures and shifting precipitation patterns contribute to warmer stream water, more frequent extreme weather events, and altered flow regimes, all of which influence oxygen availability and metabolic demands in aquatic organisms (Capon et al. 2021; Rajesh and Rehana 2022). Early life stages of fishes, including embryos and larvae, are particularly sensitive because they rely entirely on diffusive oxygen uptake through the egg membrane (Braga Goncalves et al. 2015; Del Rio et al. 2021) and developing gills in larvae (Rombough 2007). As highlighted in a recent review, embryos depend almost completely on diffusion, making them highly vulnerable to low-oxygen stress, especially under confined or low-flow conditions (Hasan et al. 2023).

Temperature regulates metabolic rate, while oxygen availability sets the upper limit of aerobic capacity. Even small increases in temperature can substantially raise metabolic oxygen demand while reducing oxygen solubility, resulting in physiological imbalance during development (Pörtner and Farrell 2008; Hasan et al. 2023). These effects are especially concerning in early development when embryos cannot escape stressful conditions and mortality risk is highest (Chen et al. 2020; Martin et al. 2020; Cowan et al. 2024). Vulnerability tends to peak during late embryonic development, when oxygen demand accelerates and diffusion may no longer meet metabolic requirements (Hasan et al. 2023). Exposure to warm, oxygen-poor conditions has been associated with premature hatching and developmental deformities, reflecting strong interactions between temperature and oxygen during development (Hasan et al. 2023).

Most studies on fish embryo development have investigated temperature or dissolved oxygen separately, often using salmonids and other model species (Martin et al. 2020; Del Rio et al. 2021; Kupren et al. 2011). Although oxygen availability clearly influences survival in clustered or confined environments (Einum et al. 2002), few studies have assessed how temperature and dissolved oxygen interact under realistic incubation conditions (Hasan et al. 2023). Environmental stressors also vary within microhabitats due to changes in water flow, substrate composition, and embryo density (Bakker and Mundwiler 2021; Moran and Woods 2010). Yet, to date, very few studies have explored these interactions in non-model fish species.

An ecologically relevant system is the communal nesting Bluehead Chub *Nocomis leptocephalus*. Males construct gravel mounds that provide nesting sites for conspecific females and multiple nest-associate fish species (Kim and Kanno 2020). These nests typically form in low-flow microhabitats with high embryo density (Peoples and Frimpong 2011), which may intensify competition for oxygen. Microbial decomposition of organic matter may further reduce oxygen levels, particularly during advanced developmental stages when metabolic demand is high (Overstreet and Hawkins 2017). Communal nesting may protect embryos from predators (selfish-herd theory) (Betts et al. 2024), but this advantage likely depends on suitable environmental conditions. Low oxygen may reduce this benefit and alter embryo positioning (Green and McCormick 2005). Yet, no study has directly tested how temperature and oxygen jointly affect embryo development and survival in this nest-associate system.

Classical selfish-herd theory predicts that individuals lower their risk of predation by positioning themselves near the geometric center of a group, where exposure to predators is lowest (Hamilton, 1971). This idea has been widely applied to mobile groups such as fish schools and bird flocks, where spatial position mainly determines safety from biotic threats. However, in spatially fixed reproductive aggregations, such as communal fish nests, individuals are influenced not only by predation risk but also by the physical environment in which development occurs. Recent work on bluehead chub nesting behavior shows that adult hosts gain selfish-herd benefits by occupying central, safer positions within spawning aggregations, thereby reducing predation risk (Betts et al. 2024). For embryos developing within these dense aggregations, however, the location that minimizes predation risk may not provide the best physiological conditions. In gravel mound nests constructed by *Nocomis leptocephalus*, oxygen availability can vary at very small spatial scales due to differences in embryo density, water flow, and microbial activity. Embryos depend entirely on passive diffusion for oxygen uptake, making local oxygen supply a key factor determining developmental success. As a result, the optimal location within a nest likely reflects a trade-off between protection from predators and access to sufficient oxygen. Under this expanded framework, selfish-herd dynamics are shaped not only by biotic risk but also by abiotic constraints, such that the best position within an aggregation may shift away from the geometric center when oxygen becomes limiting.

Understanding these dynamics is essential because early survival plays a major role in recruitment success, and even small declines in early life-stage survival can strongly impact population trajectories (Maunder and Thorson 2019; Downes et al. 2021). As climate change progresses, stream temperatures are expected to rise and oxygen availability may decrease. This could affect nest functionality and reproductive success of both host and associate species. Identifying the environmental conditions at which embryos become oxygen-limited is vital for predicting developmental outcomes under future climate scenarios (Hasan et al. 2023).

This thesis addresses these gaps by experimentally examining how temperature and dissolved oxygen jointly influence oxygen consumption, developmental rate, and survival for the combined embryos in the multi-species reproductive aggregation. Using controlled laboratory conditions that reflect natural environments, I measured oxygen consumption across developmental stages and assessed development rate and mortality under a range of environmentally realistic treatment combinations. By combining physiological measurements with ecological relevance, this research provides insight into how environmental stressors influence embryo performance in an iconic communal nesting system of fishes and may ultimately explain reproductive behaviors and population resilience in the participating species.

Chapter 1 focuses on examining how temperature, dissolved oxygen, embryo size, and developmental stage influence oxygen use in the combined embryos within the multi-species reproductive aggregation. Specifically, this chapter quantifies how oxygen consumption changes with temperature and across embryonic developmental stages in both Bluehead Chub and its associated species as grouped by embryo size.

Chapter 2 investigates how temperature and dissolved oxygen affect embryo developmental progression and survival of embryos collected from Bluehead Chub nests. The first objective is to quantify how temperature, expressed as degree days, and dissolved oxygen influence the rate of embryo development. The second objective evaluates how survival varies under different combinations of temperature and dissolved oxygen.

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Chapter 1

Effect of Temperature and Developmental Stage on Oxygen Consumption in Bluehead Chub *Nocomis leptocephalus* and Nest-Associate Embryos.

Abstract

Early developmental stages of fish are strongly influenced by environmental and biological factors that regulate oxygen supply and metabolic demand. Among these factors, temperature, ambient dissolved oxygen (DO), embryo size, and developmental stage play critical roles in determining growth trajectories, survival, and recruitment success. Although these relationships have been widely investigated in model species, they have not been systematically evaluated in leuciscid fishes. A nest-building, communal breeding species such as Bluehead Chub *Nocomis leptocephalus* which spawn in dense aggregations with nest associate species provides a valuable model for understanding DO consumption dynamics of embryos that develop within oxygen-limited microhabitats.

In this study, embryos collected from Bluehead Chub nests and incubated under controlled laboratory conditions at three temperatures (18 °C, 21 °C, and 24 °C) using a closed respirometry system. Embryos were classified into size-based groups (large – primarily Bluehead Chub and Central Stoneroller, and small – primarily smaller nest associates) and staged across pre-hatching, hatching, and post-hatching phases. Oxygen consumption per embryo per hour was quantified and modeled as a function of temperature, ambient DO, embryo size, and developmental stage. Oxygen consumption per embryo per hour was quantified and modeled as a function of temperature, ambient DO, embryo size, and developmental stage.

Results showed oxygen consumption values ranging from 0.05 to 219 mg O_2 embryo^{-1} hr^{-1}, with a bimodal distribution reflecting both near-anoxic conditions and elevated metabolic demand. Temperature had the strongest effect on oxygen use. Embryos kept at 24 °C used the most oxygen, while those at 18 °C and 21 °C used less. Embryo size showed a marginal positive effect. Ambient oxygen level had no significant effect. These results show that temperature is the main factor controlling embryo metabolism. Overall, Bluehead Chub and their nest associates may be at risk as water warms, because higher temperatures increase oxygen needs in already low-oxygen habitats.

Introduction

Early life stages of fishes are especially vulnerable to environmental variation, with conditions during incubation directly influencing individual survival, population recruitment, and ecosystem stability (Connell et al. 1999; Hamdoun and Epel 2007; Melzner et al. 2009; Leung and McAfee 2020; Dong and Tian 2023). Among these conditions, dissolved oxygen (DO) and temperature are the most critical. Oxygen availability governs embryonic growth, survival, and metabolism, while temperature accelerates developmental rate but simultaneously reduces oxygen solubility (von Bertalanffy 1960; Bradford 1990; Wetzel 2001). The interaction between DO and temperature creates a physiological bottleneck: as water warms, embryos require more oxygen when less is available, a mismatch that can reduce developmental success and recruitment (Shang and Wu 2004; Mohammed 2013)

Evolutionarily, oxygen and temperature have also shaped reproductive strategies in fishes. Embryos rely entirely on passive diffusion for oxygen uptake (Kranenbarg et al. 2000; de Salvo Souza et al. 2001). As embryo size increases, oxygen demand rises faster than surface area for diffusion, reducing efficiency (Martin et al. 2020). Elevated temperatures further exacerbate this imbalance by intensifying metabolic demand (Brett, 1972).

The Bluehead Chub (*Nocomis leptocephalus*: Leuciscidae) reproductive system provides a natural model for studying oxygen-limited reproduction and how aquatic, egg-laying species could optimize increasing oxygen supply and decreasing predation to embryos, which requires brood-hiding (Betts et al. 2024). Male Bluehead Chub build large gravel mound nests in streambeds to hide their brood. As these patches of concentrated gravel represent some of the cleanest and well protected spawning habitats, a chub nest attracts a diverse assemblage of confamiliar species who also mostly spawn on the nest, collectively known as “nest associates”. While communal spawning reduces predation risk, it also creates dense aggregations of embryos buried beneath gravel, where interstitial flow is reduced and hypoxic microhabitats may arise (Chapman 1988; Peterson and Quinn 1996). Because embryos cannot relocate to oxygen-rich environments, their survival depends on how environmental and biological factors together shape oxygen availability and consumption.

Experimental work in various fish species, including model species, highlights the interplay of temperature, DO availability, embryo developmental stage, embryo oxygen consumption rate, and aspects of development and survival. In zebrafish, hypoxia reduces critical thermal maxima, alters blood physiology, and disrupts metabolic stability (Jannat et al. 2024). Oxygen consumption was highest in zebrafish (*Danio rerio*) embryos compared to larvae, juveniles, and adults, peaking near 40 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at day 10 of development before declining to 4–5

$\mu mol \cdot g^{-1} \cdot h^{-1}$ by day 100, with early stages showing the weakest tolerance to hypoxia (Barrionuevo and Burggren 1999). Critical oxygen thresholds (P_{crit}) also declined with age: embryos regulate oxygen poorly (P_{crit} ~80 mmHg), while adults tolerate much lower levels (P_{crit} ~20 mmHg) (Barrionuevo and Burggren 1999). Similarly, in channel catfish *Ictalurus punctatus*, oxygen consumption increased from eggs through fry, with sensitivity to hypoxia peaking just before hatching; premature hatching was triggered when oxygen availability approached limiting thresholds, and survival to swim-up was reduced by 16.4% under low oxygen (Torrans and Steeby 2008). Temperature and dissolved oxygen interactively affected development in hybrid yellow catfish (*Tachysurus fulvidraco* × *Pseudobagrus vachellii*), with optimal growth and antioxidant capacity occurring at 26.8 °C and 7.3 mg L^{-1} DO (Qiang et al. 2019). Collectively, these studies show that temperature, oxygen availability, and developmental stage strongly shape metabolic performance and survival.

Early developmental research on annual killifish genera further demonstrates that oxygen availability regulates developmental timing and hatching, with reduced oxygen slowing growth or triggering early emergence (Myers 1942, 1952; Wourms 1972a, 1972b, 1972c). Oxygen consumption is widely used as a proxy for metabolic rate in fish embryos (Hamdorf 1961; Blaxter 1969), and oxygen tension is a known physiological cue for hatching across multiple species (Milkman 1954; DiMichele and Taylor 1980; Yamagami et al. 1983; DiMichele and Powers 1984). At the cellular level, mitochondrial respiration underlies these patterns, with developmental stage, sex, and age all influencing oxygen use (Mishra and Chan 2016; Wallace 2011; Wang et al. 2019; Konadu et al. 2023; San-Millán 2023).

Taken together, these studies show that oxygen consumption during early development is determined by the combined influence of temperature, oxygen availability, embryo size, and developmental stage. However, despite extensive work in model species, no study has quantified how these factors affect non model species, like early life of Bluehead Chub and its nest-associate species. The communal nesting system of Bluehead Chub creates a natural context in which environmental and biological constraints converge, providing a unique opportunity to understand how parents choose to locate their offspring to optimize the tradeoff between protecting them from predation and giving them access to oxygen and how embryos balance oxygen demand with limited supply. This chapter therefore investigates the influence of temperature, dissolved oxygen, embryo size, and developmental stage on oxygen consumption in Bluehead Chub and nest-associate embryos.

Methods

Study Site

Field collections and laboratory experiments were carried out during the summers of 2021 and 2022 using embryos obtained from approximately a 1 km section of Toms Creek, with the midpoint of this reach located at 37.261955° N, −80.436715° W (Figure 1.1). Toms Creek is a third-order stream within the New River drainage in Montgomery County, Virginia (Bustamante and Frimpong 2025). The creek flows through the Valley and Ridge physiographic region and supports a robust, well-established population of Bluehead Chub *Nocomis leptocephalus*, which I used to obtain study embryos (Bustamante and Frimpong 2025).

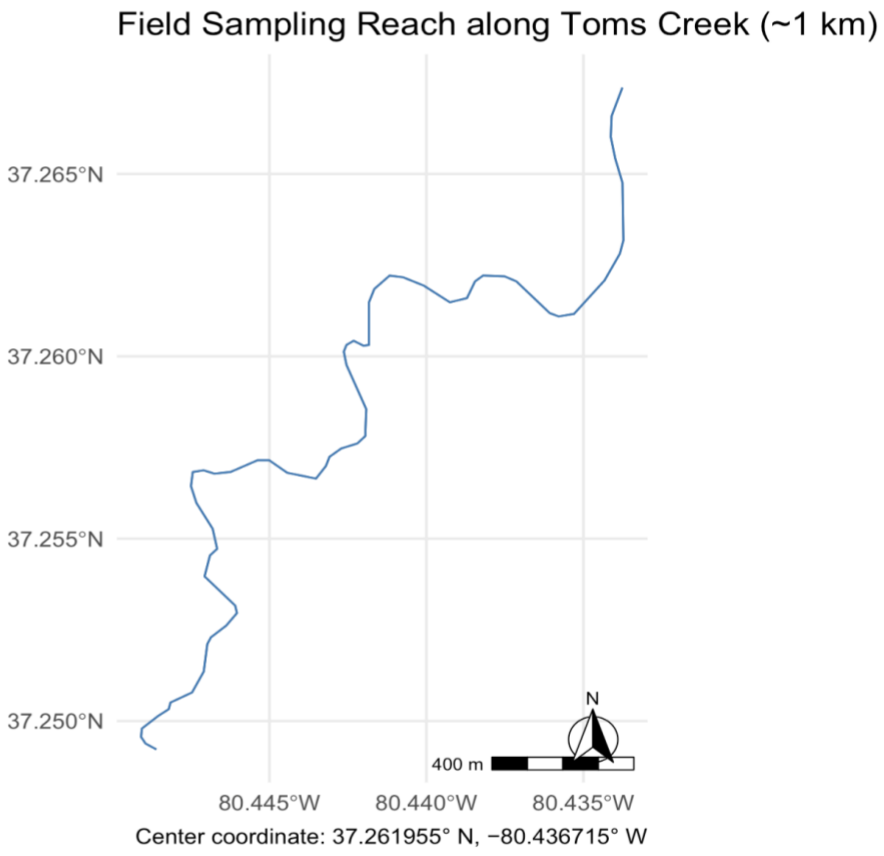

Figure 1.1. Map showing the ~1 km field sampling reach along Toms Creek near Blacksburg, Virginia, centered at 37.261955° N and −80.436715° W.

Bluehead Chub and Nest Associate Study System

The Bluehead Chub *Nocomis leptocephalus* is a member of the genus *Nocomis* (family Leuciscidae), a group of pebble-nest–building minnows whose biology and systematics were first described by Lachner (1946) and later organized into species-groups by Lachner and Jenkins (1971). Maurakis et al. (1991) emphasized that all *Nocomis* species share three key reproductive synapomorphies: “the three-stage process of nest construction (excavating a concavity, forming a platform and building a mound), pit fanning of spawning troughs and pits, and circle swims," behavioral traits that collectively support the monophyly of the genus. These gravel mounds function as important reproductive structures for many other leuciscid fishes, forming diverse nest-associate assemblages across the *Nocomis* range. Maurakis et al. documented that mound construction begins with a male excavating a concavity "ca. 0.5 m in diam by removing stones with its mouth," followed by forming a platform from stones scraped from the concavity margins, and finally depositing pebbles collected "as far as 25 m from the nest" to build the completed mound with a central trough (Reighard 1943; Vives 1990; Maurakis et al. 1991). During the breeding season, male Bluehead Chubs construct large gravel mounds that frequently exceed 40 cm in diameter, formed through mouth transport of gravel, and containing shallow spawning pits on the upstream side where eggs are deposited and subsequently buried (Bustamante and Frimpong 2025; Betts et al. 2024). In Toms Creek, nest structures attract a diverse assemblage of nest associates, including Mountain Redbelly Dace *Chrosomus oreas*, Rosyside Dace *Clinostomus funduloides*, Crescent Shiner *Luxilus cerasinus*, White Shiner *Luxilus albeolus*, Rosefin Shiner *Lythrurus ardens*, and Central Stoneroller *Campostoma anomalum* (Betts et al., 2024). At peak spawning, many individuals may simultaneously spawn within a single nest, forming one of the most striking examples of reproductive mutualism among North American stream fishes (Betts et al. 2024) (Figure 1.2). This communal nesting system produces dense aggregations of embryos with high local oxygen demand, creating conditions where the balance of oxygen supply and demand may be low in at least parts of the nest during embryo development.

Figure 1.2. A male Bluehead Chub *Nocomis leptocephalus* transports a pebble during mound construction, surrounded by a diverse assemblage of nest-associating leuciscids, including Mountain Redbelly Dace *Chrosomus oreas*, Rosyside Dace *Clinostomus funduloides*, and Central Stoneroller *Campostoma anomalum*. Photo by Todd Pusser.

Embryo Collection and Classification

Fertilized eggs were collected from freshly constructed nests or existing nests observed to have new spawning activity and transported to the Virginia Tech laboratory on ice within a few hours of sampling to maintain viability. Upon arrival, eggs were gently rinsed with aerated stream water and examined under a dissecting microscope. A subsample of about 120 embryos (60 for each of two visually separated size groups based on egg diameter – large vs. small) was taken, and each embryo was staged according to developmental features. The actual diameters of a subsample of 10 large and 10 small embryos were taken under high-resolution magnification using Leica Microsystems Imaging Software. Large embryos were assumed to primarily represent Bluehead Chub *Nocomis leptocephalus* and Central Stoneroller *Campostoma anomalum*, which produce embryos of equal or greater diameter, whereas small embryos were assumed to primarily represent the remaining nest-associate species which produce smaller eggs (i.e., *Chrosomus, Lythrurus, Clinostomus, and Luxilus* spp.). This classification allowed me to compare oxygen consumption across developmental stages and between embryo size-based species groups.

The timing of larval departure from nests was not directly observed in this study. I therefore assumed that embryos remained within the nest until yolk sac absorption, after which larvae must leave the nest to initiate exogenous feeding and survive. Consequently, oxygen consumption and survival were evaluated through embryonic development and the early larval stage, which represents the biologically relevant period during which embryos and larvae remain associated with the nest environment.

Experimental Design and Respirometry

Oxygen consumption was quantified using a custom-built closed respirometry system that also served as an incubator. Three temperature-controlled water baths maintained experimental treatments of 18 °C, 21 °C, and 24 °C. The 18 °C bath was cooled with a chiller, while the 21 °C and 24 °C baths were heated with aquarium heaters controlled with a thermostat. Within each bath, airtight glass jars served as sealed respirometry chambers, paired with open jars that remained unsealed to allow continuous gas exchange and thus served as controls. Prior to incubation, bath water was equilibrated to the target temperature and aerated to near saturation.

Each sealed chamber contained a magnetic stir bar operated at low speed to ensure uniform oxygen distribution without disturbing embryos. At the beginning of each approximate 8-hour incubation cycle, dissolved oxygen (DO) concentration and temperature were measured using a calibrated YSI 556 MPS multi-probe system. A subset of embryos were removed from each treatment and photographed under high power microscope at 8-hour intervals. Temperature and DO were also measured before chambers were resealed. To prevent stress under severe hypoxia, chambers were gently refreshed with oxygenated bath water if DO levels fell below 3 mg L^{-1}. At the end of multiple 8-hour incubation cycles, all surviving embryos from each treatment were counted and photographed. Photographs were later used for determining the distribution and modal developmental stage of embryos in each treatment. When the total number of embryo at the end of the experiment was less than the number incubated at the beginning, I assumed a linear rate of mortality across the total incubation period and calculated the number of surviving embryos, rounded to the nearest whole number, for each 8-hour interval. Oxygen consumption was calculated as the decline in DO over the incubation time, standardized by the number of embryos in each chamber during that interval. Because oxygen demand changes dynamically as embryos progress through development, we documented and classified embryonic stages throughout the experiment. These stages, further grouped into three phases, are described in detail below.

Embryonic Developmental Stages and Phases

Developmental stages were classified based on external morphological features observed under high resolution microscopy using Leica Microsystems Imaging Systems. Although accepted ichthyological terminology distinguishes protolarval, mesolarval, and metalarval stages, almost all laboratory incubations in this study were terminated before complete yolk sac absorption or the appearance of fin rays. Consequently, embryos did not progress into the mesolarval or metalarval stages. The developmental observations in this study therefore represent embryogenesis and early larval development up to the end of the protolarval stage.

Fish embryos pass through a series of predictable developmental phases, each associated with characteristic morphological and physiological changes that influence oxygen use. We categorized embryos into three broad phases:

- Pre-hatching Phase (Stages 1–26; Appendix A): Embryonic development occurs entirely within the egg, encompassing cleavage, blastula, and gastrula stages, followed by formation of key structures such as the neural tube, somites, and early heart.

- Hatching Phase (Stage 27; Appendix A): Marks the emergence of the embryo from the egg, a transition accompanied by major shifts in oxygen uptake and activity.

- Post-hatching Phase (Stages 28–39; Appendix A): Involves larval growth and continued organ development, including tail separation, pigmentation, and active swimming behavior.

Within these phases, specific developmental milestones were used to refine staging: fertilization, cell cleavage (2-cell, 4-cell, 8-cell, up to 64-cell), blastula and gastrula formation, neural tube and somite development, heart formation, tail separation, eye and pigment development, and eventually larval motility. A full list of these developmental stages, with time and visual characteristics, is provided in Appendix A.

Data Analysis

I used linear regression to evaluate dissolved oxygen (DO) availability, oxygen-consumption rates, and their relationships with temperature, ambient DO, embryo size, and developmental stage. These variables served as independent predictors, and I modeled DO consumption rate per embryo per hour as the dependent variable. I ensured that all statistical assumptions for linear regression were met before interpreting results. All analyses were conducted in R version 4.3.2 (R Core Team, 2024).

Equation 1. The full model was:

$$
\begin{aligned}
Oxygen\ Consumption_{\mathrm{i}} &= \beta_0 + \beta_1(Temperature_{\mathrm{i}}) + \beta_2(DissolvedOxygen_{\mathrm{i}}) + \beta_3(EmbryoSize_{\mathrm{i}}) \\
&\quad + \beta_4(DevelopmentalStage_{\mathrm{i}}) + \varepsilon_{\mathrm{i}}
\end{aligned}
$$

Where:

O_i = dissolved oxygen used (mg O_2 L^{-1} per interval)

β^0 = intercept

β^1 = effect of incubation temperature (°C)

β^2 = effect of dissolved oxygen level (mg/L)

β^3 = effect of embryo size (large/small or continuous size value)

β^4 = effect of developmental stage (1–39)

ε_i = random error term

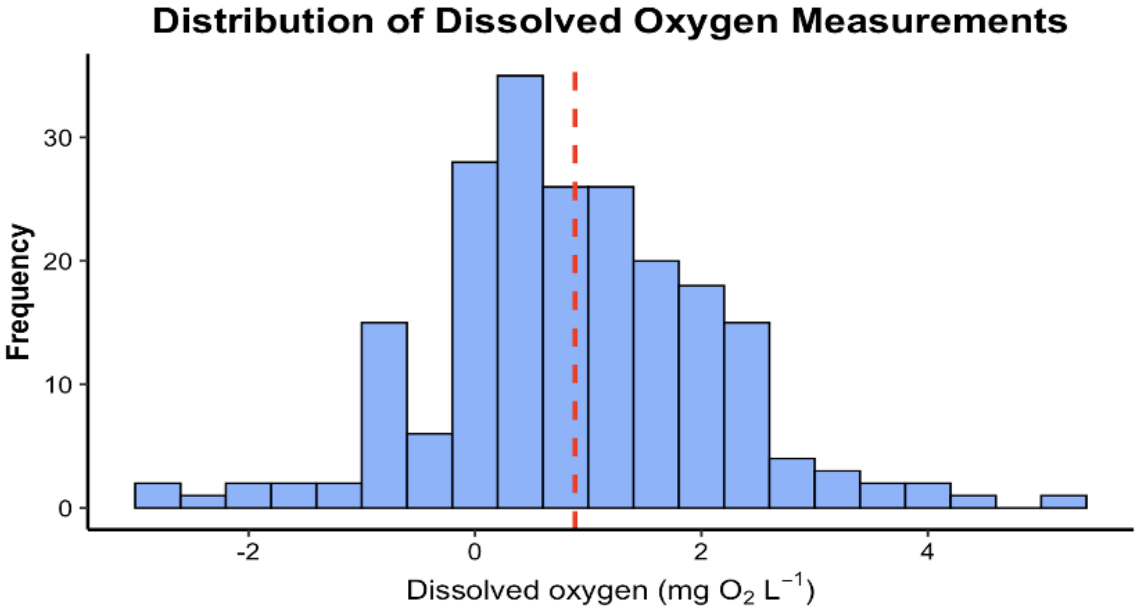

Figure 1.3. Histogram of dissolved oxygen (DO) measurements. This figure shows the distribution of raw dissolved oxygen (DO) differences recorded in the incubation chambers. Each value represents begin DO minus end DO for an incubation interval (mg O_2 L^{-1}), measured before any normalization by embryo count or interval duration.

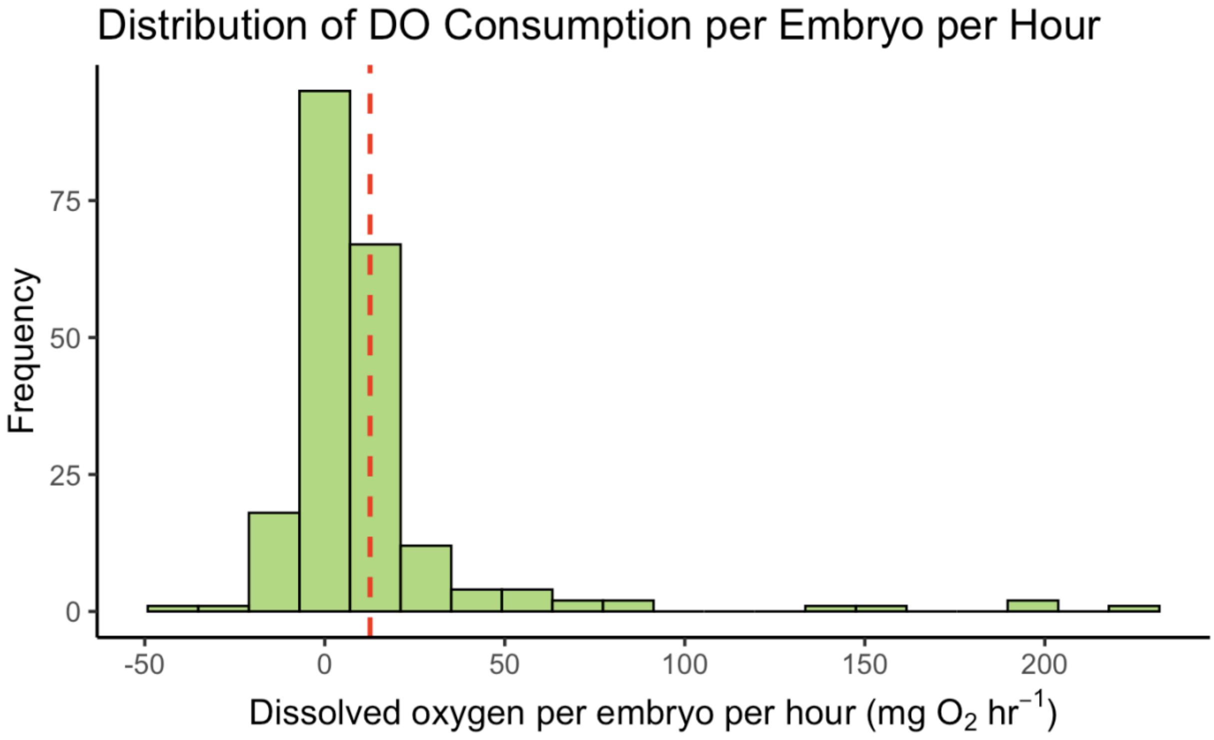

Figure 1.4. Histogram of dissolved oxygen (DO) consumption per embryo per hour. This figure shows the distribution of dissolved oxygen (DO) consumption rates calculated for each incubation interval. Each value represents (begin DO – end DO) divided by the number of embryos and the duration of the interval (decimal hours), expressed in mg O_2 per embryo per hour. The red dashed line indicates the mean DO-consumption rate across all intervals.

To support the regression analyses, I first examined the distribution of the raw dissolved oxygen (DO) measurements and the calculated per-embryo DO-consumption rates. Figures 1.3 and 1.4 present these exploratory visualizations, showing the raw DO differences recorded in the incubation chambers and the corresponding consumption rates derived from them. To meet linear regression assumptions, several transformations of per-embryo oxygen-consumption values were evaluated during exploratory analysis. A Box–Cox procedure identified an optimal transformation parameter (λ = 0.1; Figure 1.5), which was applied in all subsequent analyses.

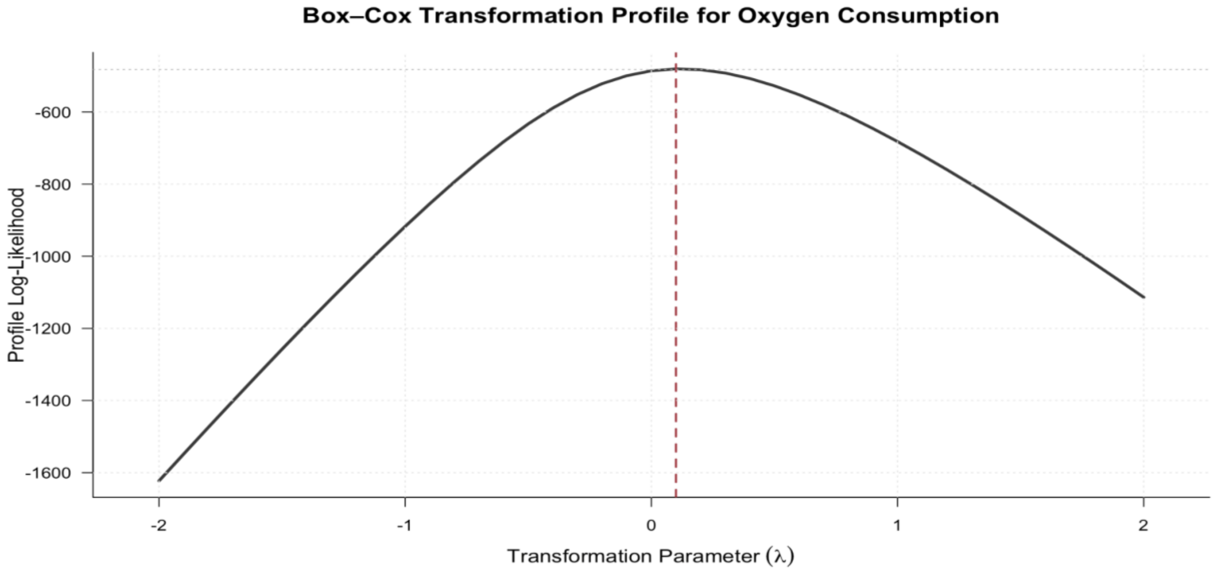

Figure 1.5. Box–Cox transformation profile for dissolved oxygen (DO) consumption. This figure shows the Box–Cox transformation profile used to identify an appropriate transformation for the dissolved oxygen (DO) consumption data. The solid line represents the profile log-likelihood across a range of λ values, and the red dashed line marks the λ that maximizes the likelihood. The procedure identified an optimal λ of 0.1, which was used as the transformation applied in the regression analysis.

Results

A total of 211 dissolved oxygen–consumption trials were included in the regression analyses. Observation intervals averaged 8.80 ± 2.56 hours. Each trial contained an average of 15.29 ± 8.85 embryos, with 7.90 ± 5.09 large and 7.40 ± 5.32 small individuals. Developmental stages varied widely across trials; combined stages averaged 26.28 ± 7.70, while large and small embryos averaged 23.46 ± 11.08 and 21.78 ± 12.01, respectively. Incubation temperature averaged 20.58 ± 2.25 °C, and dissolved oxygen concentration at the start of intervals averaged 4.88 ± 2.01 mg L^{-1}. Large embryos were significantly larger than small embryos (mean ± SD: 2.75 ± 0.44 mm vs. 1.82 ± 0.16 mm), indicating substantial variability in embryo density within multi-species reproductive aggregations.

Given the range of oxygen-consumption values observed, the distribution of raw dissolved oxygen differences (Figure 1.3) and per-embryo oxygen-consumption rates (Figure 1.4) showed right-skewed patterns, which motivated the use of data transformations. A Box–Cox analysis identified an optimal λ of 0.1 (Figure 1.5), and this transformation was applied in all regression models.

Multiple regression models were then fitted to identify predictors of dissolved oxygen consumption per embryo per hour. Initial models that separated large and small embryos did not show significant effects of embryo size, developmental stage, dissolved oxygen, or their interactions; therefore, large and small embryos were combined in all subsequent analyses.

The final model included average incubation temperature and combined developmental stage. I used a Box–Cox procedure to identify an appropriate transformation for the response variable. The Box–Cox profile indicated an optimal transformation parameter of λ = 0.1 (Figure 1.5).

Standardized regression coefficients indicated that incubation temperature and embryo size were the two strongest predictors of embryo oxygen consumption, with large positive effects (temperature: β = 0.41, 95% CI = 0.02–0.80; embryo size: β = 0.43, 95% CI = 0.04–0.82).In contrast, developmental stage exhibited a weaker and statistically uncertain effect (β = 0.11, 95% CI = −0.29–0.50), while dissolved oxygen had no detectable effect (β = 0.03, 95% CI = −0.39–0.45). Figure 1.6 illustrates the relative strength and uncertainty of these standardized effects.

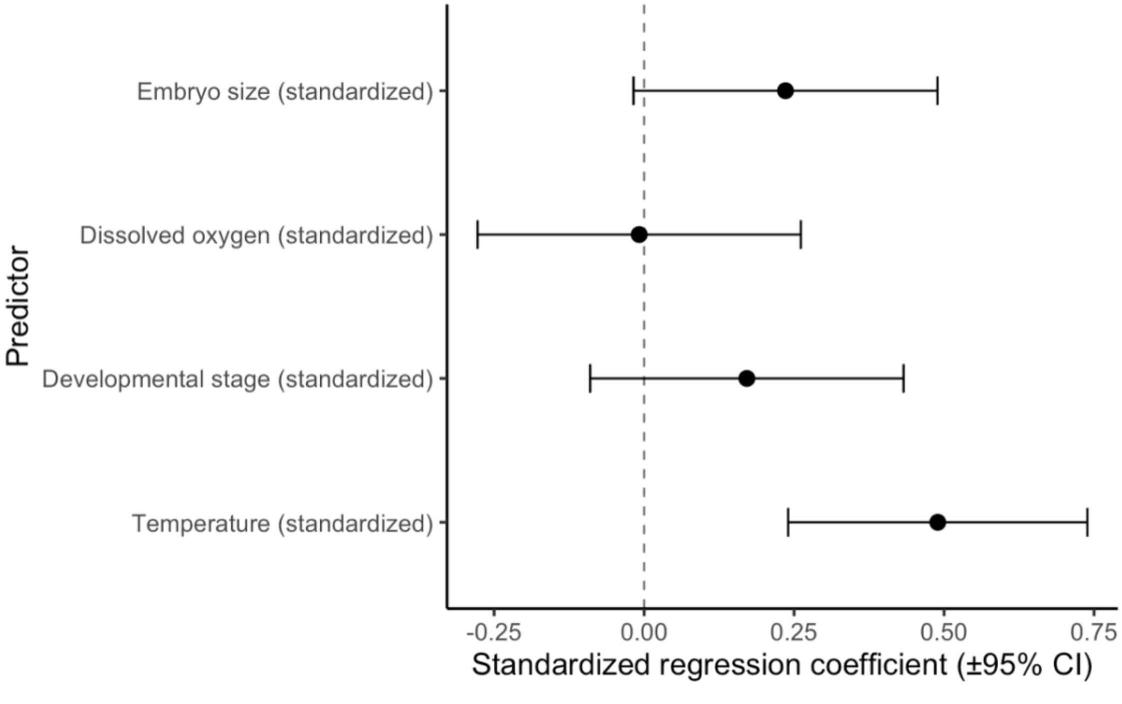

Figure 1.6 Standardized regression coefficients (±95% confidence intervals) for predictors of per-embryo dissolved oxygen consumption, including incubation temperature, developmental stage, dissolved oxygen concentration, and embryo size.

Discussion

This study set out to quantify how temperature and dissolved oxygen shape the metabolic demands of embryos developing within communal Bluehead Chub nests. The results demonstrate that temperature was the strongest predictor of embryo oxygen consumption, with metabolic demand increasing steadily as incubation temperature rose. Embryo number was not evaluated as an independent predictor because it was used to standardize oxygen consumption rates. Developmental stage and embryo size showed only marginal effects, and their contributions to variation in oxygen consumption were much smaller than that of temperature. Together, these findings indicate that thermal conditions are the primary driver of oxygen demand during early development for both large and small embryos within this multi-species nesting system.

The relationship between temperature and metabolic rate in fishes is well established. As water warms, biochemical reaction rates increase, elevating metabolic demand (von Bertalanffy 1960; Bradford 1990), while oxygen solubility simultaneously declines (Wetzel 2001). Developing fish embryos rely entirely on passive diffusion across the egg membrane for oxygen uptake (Kranenbarg et al. 2000; de Salvo Souza et al. 2001), making them particularly sensitive to changes in oxygen availability. As embryos grow, volume increases faster than surface area, reducing the surface-area-to-volume ratio and further constraining diffusion (Martin et al. 2020). The consistent rise in oxygen consumption between 18 °C and 24 °C observed in this study reflects these physiological constraints. Embryos incubated at higher temperatures exhibited faster but more variable metabolic rates, whereas embryos in cooler conditions maintained lower and more stable oxygen use.

The absolute oxygen-consumption rates measured here are comparable to those reported for other freshwater fishes, supporting the ecological relevance of the experimental design. For example, Chinook salmon embryos show increasing oxygen uptake with warming (Martin et al. 2020), and similar temperature-dependent patterns have been reported for zebrafish, Japanese medaka, and totoaba embryos (Marty et al. 1990; Barrionuevo and Burggren 1999; Larios-Soriano et al. 2023). Predicted oxygen-consumption rates for Bluehead Chub embryos at 24 °C fall squarely within the range reported for these taxa, indicating that embryo metabolism in communal nests follows broadly conserved physiological patterns.

The bimodal distribution of dissolved oxygen observed in sealed chambers, with one cluster near 0 mg L^{-1} and another near 2–3 mg L^{-1}, closely matched oxygen gradients previously documented within natural Bluehead Chub nests by the Frimpong Lab. This correspondence suggests that the respirometry setup successfully recreated oxygen conditions embryos are likely to experience in the field, particularly in areas of the nest where water exchange is limited.

Embryo size exhibited a weak positive effect on oxygen consumption, with larger embryos tending to consume slightly more oxygen than smaller embryos. This pattern is consistent with previously documented spatial structure within Bluehead Chub nests, where host embryos are concentrated near the center of the spawning aggregation and spawning pit, while smaller nest-associate embryos occur more frequently toward the periphery (Betts et al. 2024). Central placement may reduce predation risk but may also increase exposure to oxygen depletion, highlighting a trade-off between protection and physiological stress within the nest environment.

Rather than drawing direct conclusions about the effects of warming, the results of this study provide a physiological baseline that can be integrated with long-term field observations of nest timing and seasonal spawning patterns. In Toms Creek, nest construction begins early in spring and may persist into mid- to late summer, meaning embryos experience different temperature regimes depending on when spawning occurs. Although nest construction and spawning began earlier than typically observed in 2025, this single year should not be interpreted as evidence of a long-term shift in reproductive timing. Long-term observations from 2016–2024 indicate that nesting in this system generally begins later in the season. The early onset observed in 2025 may therefore reflect short-term environmental variability rather than directional change. These observations suggest that Bluehead Chub may exhibit some flexibility in spawning timing, which could partially buffer embryos from peak summer temperatures in some years. However, nesting activity extending into mid- to late summer indicates that embryos can still be exposed to warm conditions, emphasizing that temperature-related stress remains a potential risk. A formal statistical analysis of long-term spawning phenology would be required to assess whether reproductive timing is shifting in response to climate change.

This approach may also help refine estimates of larval departure from nests. Larval exit was not directly observed in this study, but larvae are assumed to leave nests once yolk sac reserves are depleted and exogenous feeding becomes necessary. Combining developmental rates measured under controlled conditions with long-term observations of nest persistence could help narrow down estimates of the timing of larval exit and improve our understanding of early survival in this system.

This study has some limitations. Although temperature was the strongest predictor of oxygen consumption, the final model explained a modest proportion of the total variation (adjusted $R^2 \approx 0.10$). This likely reflects biological variability inherent to mixed-species embryo assemblages and dynamic changes in metabolism across developmental stages. Embryos within communal nests differ in species identity, size, developmental rate, and spatial position within the nest, all of which may contribute additional unexplained variation. Future work that combines controlled laboratory experiments with long-term field observations of nest timing, embryo density, and spatial structure would help further resolve how these sources of variability influence oxygen demand during development.

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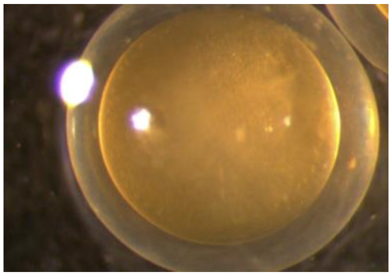
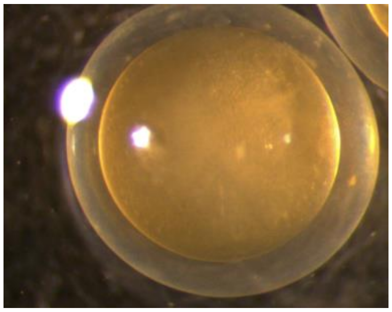
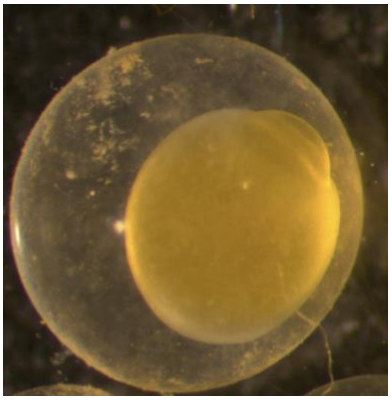
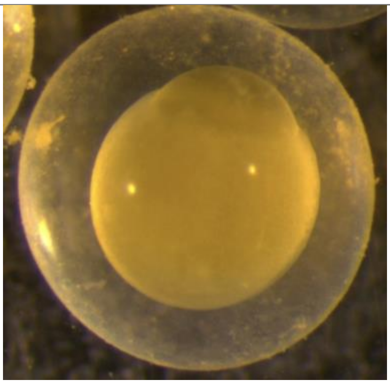
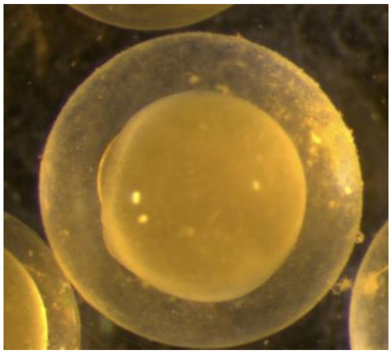
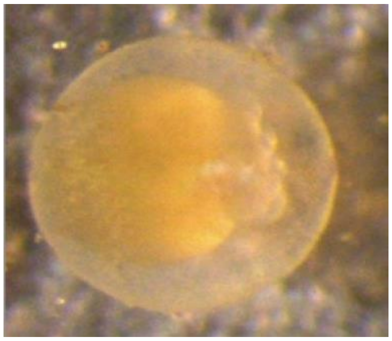
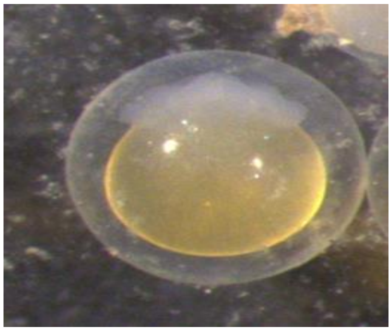
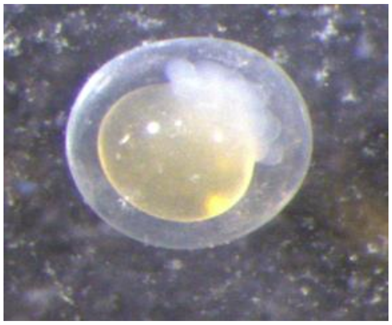
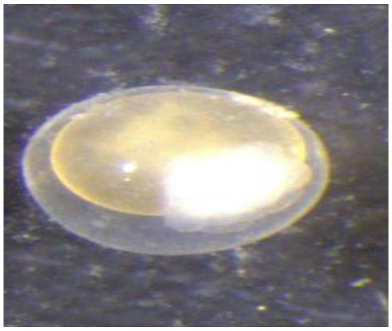
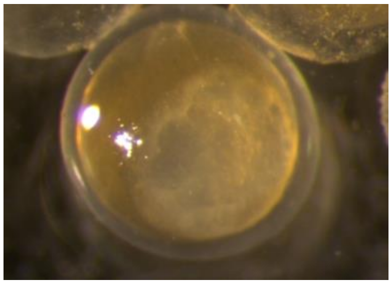
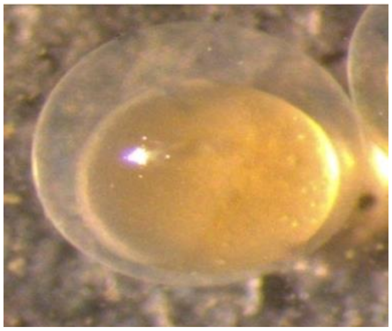
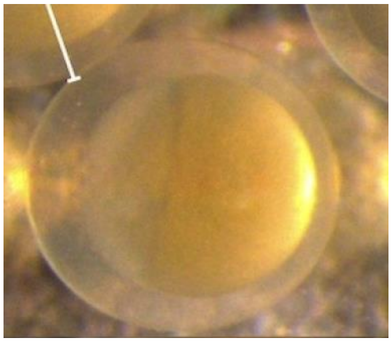
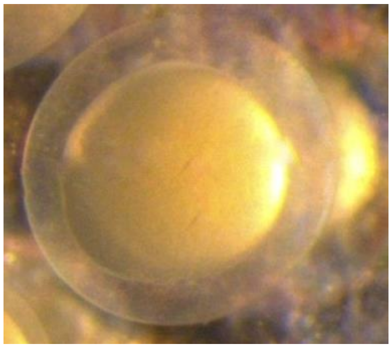
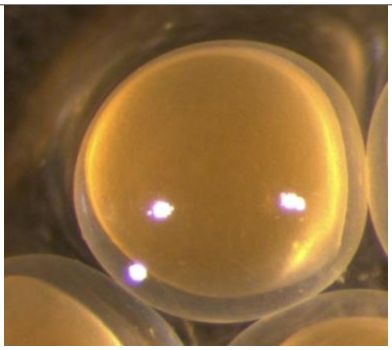
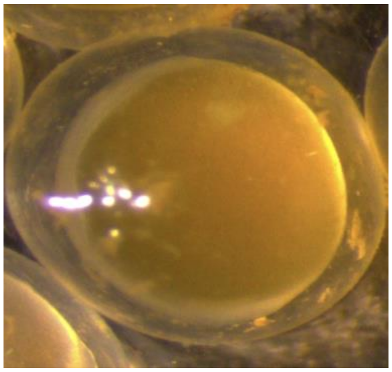
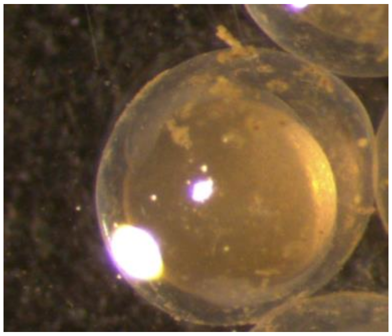
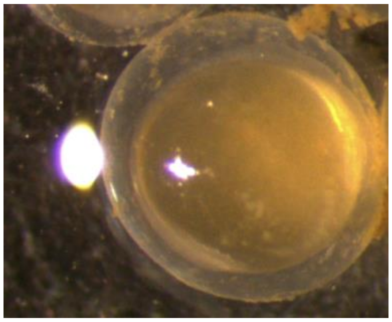
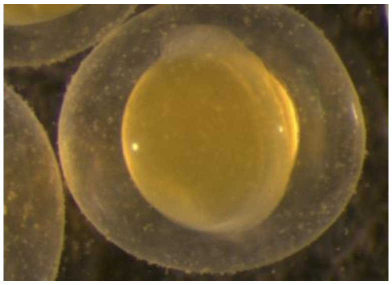
Appendix A:

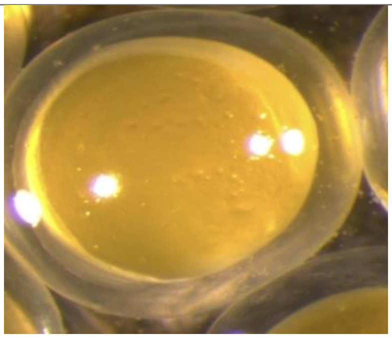
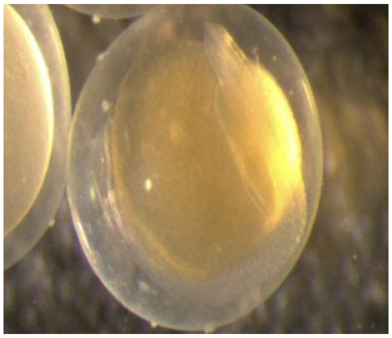
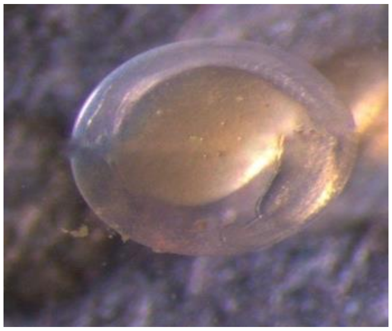
Description of the 39 embryo developmental stages used in this study. Stages were categorized based on observable morphological characteristics

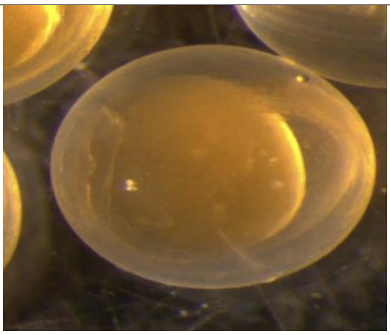
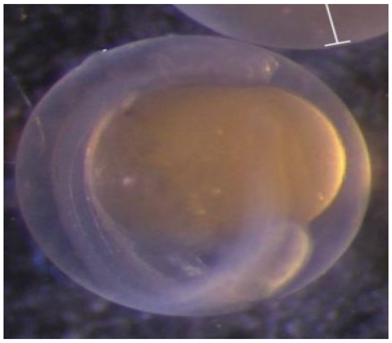
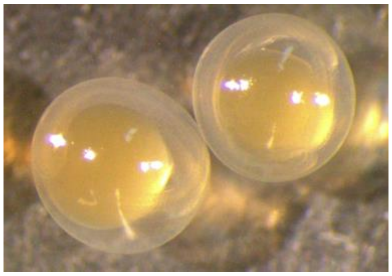
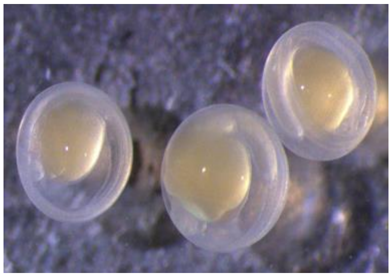
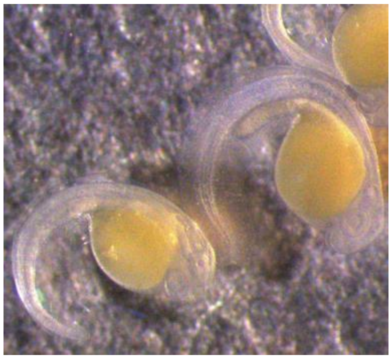
No.	Stage	Description	Image
1	Newly Fertilized egg	The membrane is clear, sticky, and oval-shaped. It has two layers and holds a lot of yolk inside.	
2	The Fully-Swollen Egg	The egg starts to absorb water and grow bigger. Then, a small space called the perivitelline space forms around the yolk.	
3	Blastodisc formation	A distinct disc of cells forms on top of the yolk. This disc, known as the blastodisc, is the foundational structure for the developing embryo.	

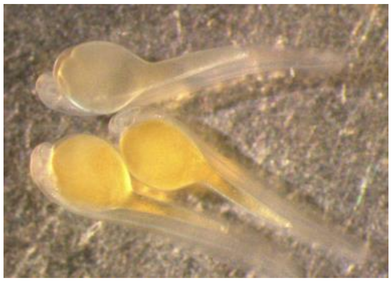
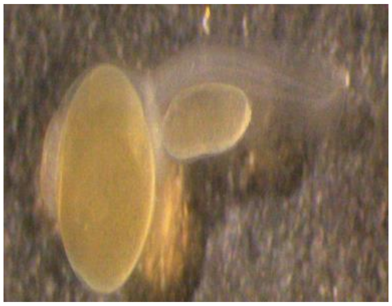
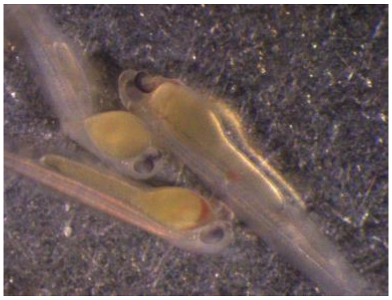
4	First Cleavage Furrow	The first cleavage furrow is the initial division of the fertilized egg (zygote) into two separate cells. It is a visible indentation on the surface of the egg, marking the plane of division between the two blastomeres.	
5	2 cell phase	The 2-cell phase is an early stage in embryonic development following the first cleavage division of the fertilized egg (zygote). During this phase, the embryo consists of two blastomeres, which are similar in size and contain identical genetic material.	
6	4 cell phase	The 4-cell phase follows the second cleavage division of the fertilized egg. At this stage, the embryo consists of four blastomeres, each derived from the division of the two blastomeres formed during the 2-cell phase. This phase occurs after two rounds of mitotic cell division.	
7	8 cell phase	In the 8-cell stage, the embryo consists of eight smaller cells (blastomeres), which are arranged in a compact formation.	
8	16 cell phase	At this stage, the embryo, known as the morula, consists of 16 cells, which are increasingly compacted together. It sets the stage for the formation of the blastocyst, a more complex structure that will eventually develop into the	

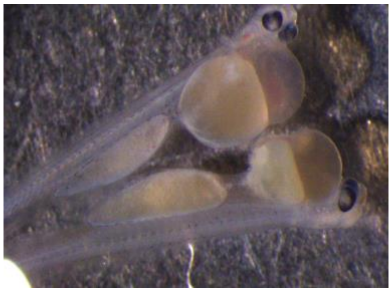
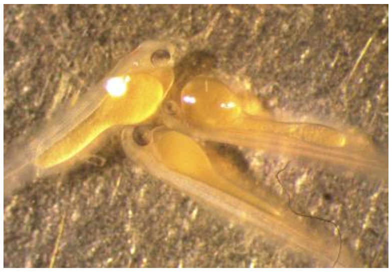
		embryo's body plan.	
9	32 cell phase	At this stage, the embryo, still referred to as a morula, consists of approximately 32 tightly packed cells. During this phase, the embryo starts to form a central cavity, signaling the beginning of the transition to the blastocyst stage.	
10	64 cell phase	The 64-cell phase is a pivotal stage in early embryonic development, occurring as the embryo transitions from the morula to the blastocyst stage. At this point, the embryo consists of approximately 64 cells.	
11	Early Blastula Phase	During the early blastula phase, the embryo forms a fluid-filled cavity in the center, known as the blastocoel.	
12	Mid Blastula Phase	At this stage, the blastocoel, or fluid-filled cavity, is more prominent, and the blastula structure becomes more defined. Blastoderm flattened out and began to wrap around the yolk sac.	
13	Late Blastula Phase	During this stage, the blastocoel, the fluid-filled cavity within the blastoderm, reaches its maximum size. The blastoderm, which consists of a single layer of cells, thins out and flattens as the blastocoel expands.	

14	Early Gastrula Phase	The germ ring formed all around the edge of the blastoderm. This showed that the gastrula stage had started. In the early part of this stage, the blastoderm covered about half of the yolk sac.	
15	MidGastrula Phase	The blastoderm expanded to include ⅔ of the yolk sac, blastoderm involution thickened at the germ ring's edge, and the embryonic shield appeared, signaling the start of the middle gastrula stage.	
16	Late Gastrula Phase	In this stage, the blastoderm covers about 3/4 of the yolk sac.	
17	Neural embryo formation	During this stage, the blastoderm covered about 4/5 of the yolk sac. Simultaneously, the blastopore and the yolk plug appeared.	
18	Closure of Blastopore	As the epiboly of blastoderm proceeded, the blastopore closed (encircled the yolk) completely in this stage	

19	Somite appearance	In the somite appearance stage, the fish embryo begins to develop segmented blocks of cells called somites along its length. These somites will eventually form important structures like the vertebrae, muscles, and skin.	
20	Optical vesicle Formation	Optic vesicle formation happens when two small bumps grow from the sides of the embryo's brain. These bumps, called optic vesicles, move toward the outside and begin to form parts of the eye.	
21	Tail Bud appearance	The embryo starts forming a small, tail-like structure at its rear end.	
22	Tail separates from the Yolk	The tail bud, which was connected to the yolk for food, begins to pull away as the embryo gets bigger.	

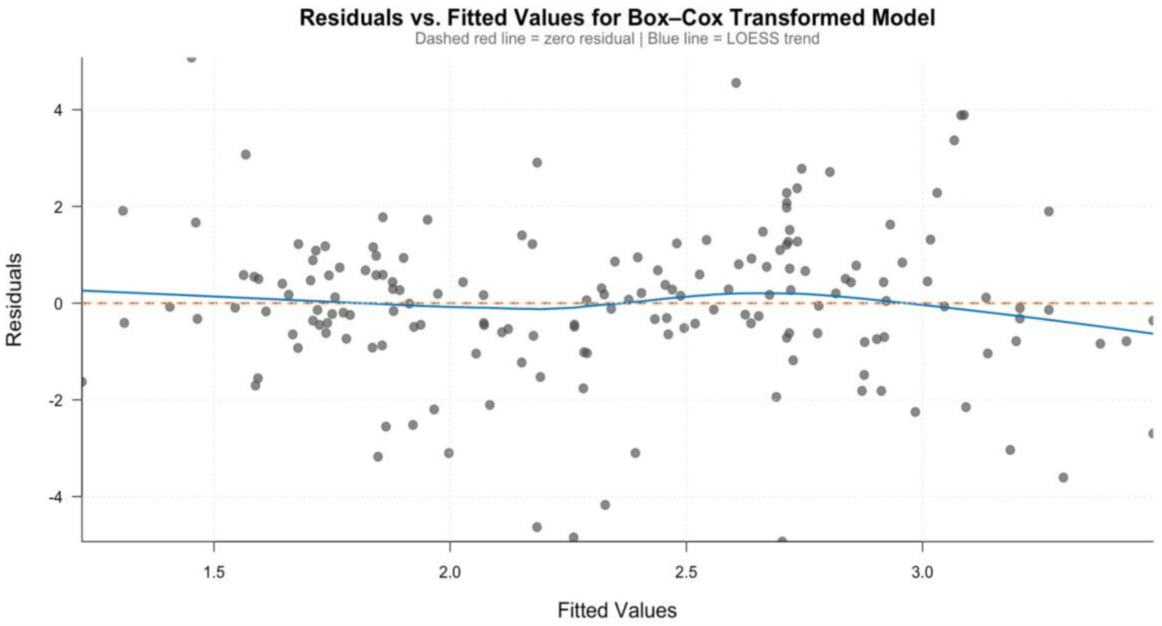
23	Otic vesicle appearance	Small, paired bulges called otic vesicles form on either side of the embryo's head, near the developing brain. These vesicles will eventually develop into the inner ear structures responsible for hearing and balance.	
24	Flexure of the tail, brain differentiation	During the tail flexure stage, the embryo's tail begins to curve. Simultaneously, brain differentiation occurs.	
25	Elongation of the yolk sac, Urogenital duct forms	At this stage, the yolk sac typically appears as an elongated to support nutrient absorption	
26	Heart Formation	A colorless liquid flowing slowly in the embryo could also be observed at this stage.	
27	Protolarva, Recently hatched	The proto-larva stage is the first stage of a fish's life after it hatches from its egg. Proto larvae are typically only a few millimeters long. A large yolk sac: This is the fish's main food source during this stage. The fish's digestive system is not fully developed yet, so it cannot eat solid food. The fish's fins are starting to grow, but they still need to be fully functional. A transparent	

		body, allows us to see the fish's internal organs.	
28	Streamlined body, Elongated yolk sac	During this stage, the fish's body begins to take on a more streamlined shape, allowing for greater efficiency in swimming. The yolk sac is gradually absorbed and the fish's internal organs begin to develop and take on a more defined form.	
29	Starting of somite development	The primary feature of this stage is the regular, sequential arrangement of somites	
30	Looks like divided yolk sac	At this stage, the yolk sac becomes increasingly segmented and more closely associated with the developing embryo. The yolk sac may appear as smaller, more discrete lobes or compartments.	
31	Starting of heart development	A red color liquid flowing slowly in the embryo could also be observed at this stage.	
32	Starting of eye pigmentation	During this stage, the lens of the eye begins to form, and the retina starts to develop. The pigment cells in the retina also begin to develop during this stage.	

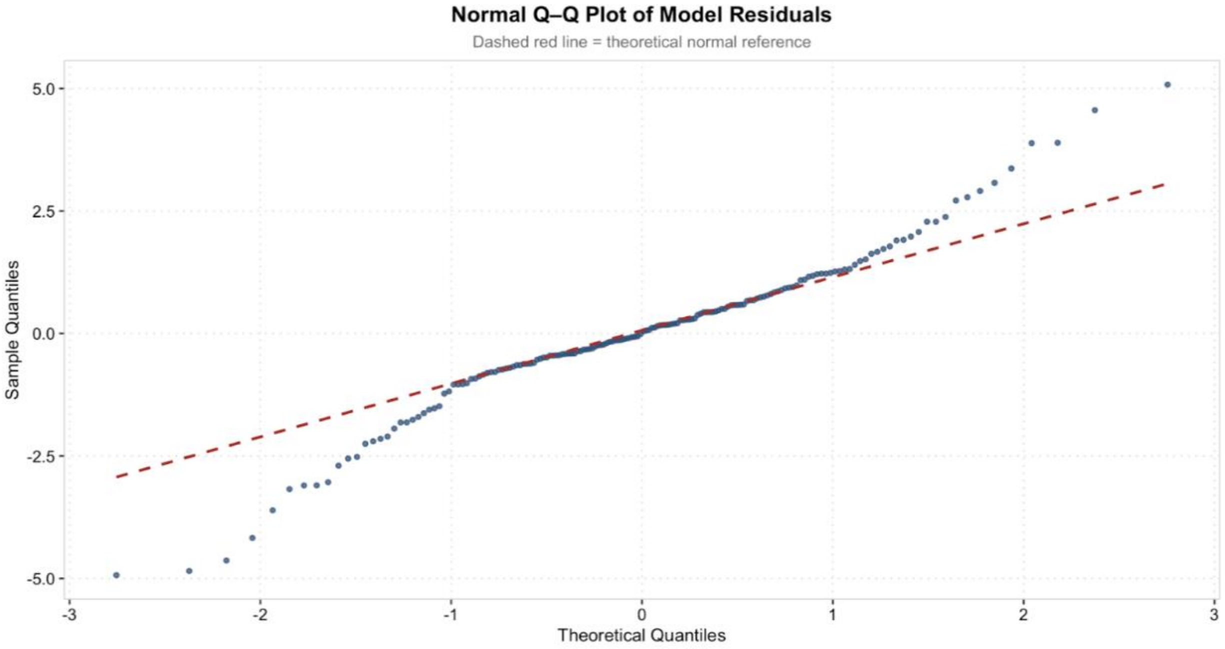
33	Starting of body development	During this stage, the fish's body begins to take on a more defined shape, and the major organs and systems start to form. The notochord, a rod-like structure that runs the length of the embryo, begins to differentiate into the vertebral column. The muscles also begin to develop.	
34	Starting of body pigmentation	During this stage, the fish's body begins to develop color. The pigment cells, called melanophores, start to produce melanin, which is the pigment that gives the fish its color.	
35	Blood flowed posteriorly on the dorsal surface	In this stage Blood, carrying oxygen and nutrients, starts to flow posteriorly on the dorsal surface.	
36	Developed and pigmented eyes	The eyes, now visibly pigmented, are becoming functional.	
37	Developed Somite	The somites in fish embryos continue to grow and mature. These somites, which are blocks of tissue along the embryo's back, start to form specific parts of the body. The sclerotome part of the somites develops into the bones of the spine and ribs.	

38	Pigmented body and eye	During this stage, the embryo's body acquires distinct pigmentation. Simultaneously, the eyes develop and begin to function, allowing the embryo to perceive light and navigate its environment	
39	Species-specific pattern of pigmentation forming	During this stage, the pigmentation becomes more defined and distributed across the body, creating the characteristic appearance of the fish. These patterns can include stripes, spots, or other markings	

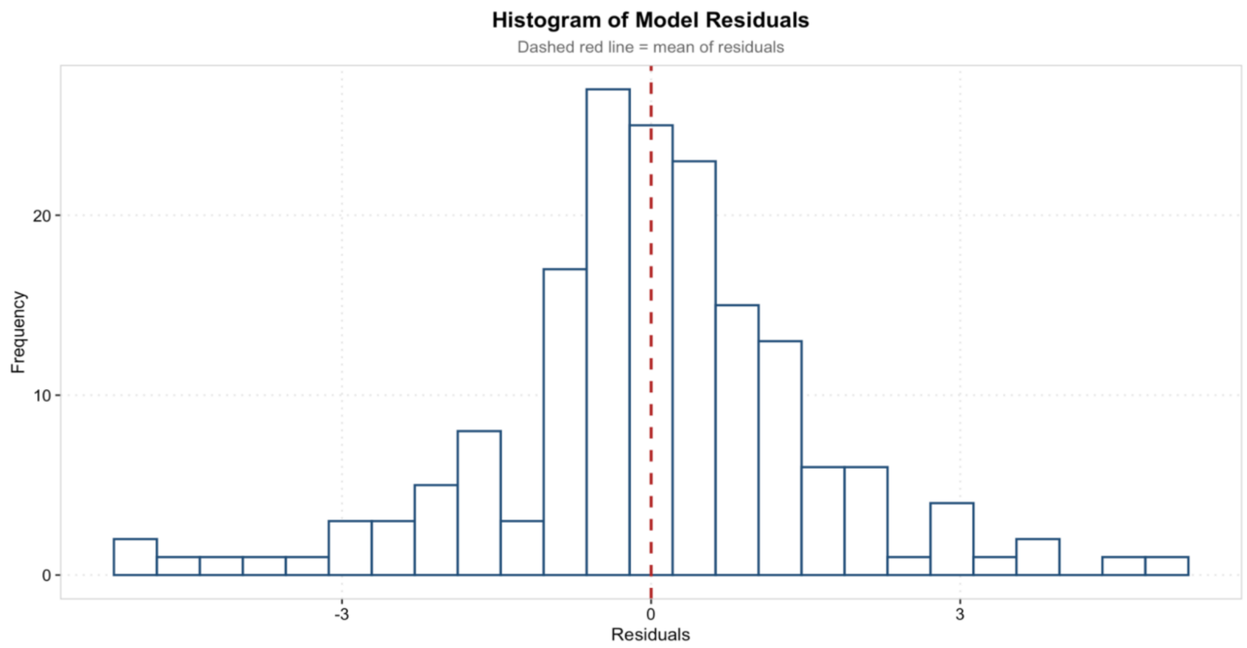
Appendix Figures:



Appendix Figure A1.1. Residuals versus fitted values for the Box–Cox transformed model. This diagnostic plot shows the residuals (model errors) plotted against the fitted values after applying the Box–Cox transformation (λ = 0.1) to dissolved oxygen (DO) consumption data. The red dashed horizontal line represents the zero-residual reference, while the blue curve shows the smoothed LOESS trend indicating the general pattern of residuals.



Appendix Figure A1.2. Normal Q–Q plot of model residuals after Box–Cox transformation. This figure assesses whether the residuals from the Box–Cox transformed regression model follow a normal distribution, an important assumption for valid statistical inference. The blue points represent the ordered sample quantiles of residuals, while the red dashed line shows the theoretical quantiles expected under perfect normality.



Appendix Figure A1.3. Histogram of model residuals after Box–Cox transformation.

This histogram displays the distribution of residuals from the Box–Cox transformed model of dissolved oxygen (DO) consumption. The red dashed vertical line represents the mean of the residuals, which is centered near zero.

Tables

Table A1.1 Oxygen Consumption Rates in Embryos of Different Fish Species Across Developmental Stages and Temperatures.

Species	Developmental Stage	Temp (°C)	Oxygen Consumption (µg/h/embryo)	Citation
Danio rerio	Stage 3 (day 10)	25	~0.54	(Barrionuevo & Burggren, 1999)
Danio rerio	Stage 3 (day 10)	28	~0.15	(Barrionuevo & Burggren, 1999)
Danio rerio	Stage 3 (day 10)	31	~2.24	(Barrionuevo & Burggren, 1999)
Totoaba macdonaldi	Stage 1	21	99.2 ± 0.3	(Larios-Soriano et al., 2023)
Totoaba macdonaldi	Stage 1	24	118.4 ± 0.3	(Larios-Soriano et al., 2023)
Totoaba macdonaldi	Stage 1	27	137.6 ± 0.3	(Larios-Soriano et al., 2023)
Oncorhynchus tshawytscha	Stage 3 (15-18 dph)	8	~1.5–3.0	(Martin et al., 2020)
Oncorhynchus tshawytscha	Stage 3 (15-18 dph)	12	~2.0–3.5	(Martin et al., 2020)
Oncorhynchus tshawytscha	Stage 3 (15-18 dph)	14.5	~2.5–4.5	(Martin et al., 2020)
Oncorhynchus tshawytscha	Stage 3 (15-18 dph)	17	~3.5–6.0	(Martin et al., 2020)
Oryzias latipes	Stage 1	20	0.10 ± 0.2	(Marty et al., 1990)
Oryzias latipes	Stage 1	23	0.14 ± 0.5	(Marty et al., 1990)
Oryzias latipes	Stage 1	26	0.08 ± 1.0	(Marty et al., 1990)
Oryzias latipes	Stage 1	29	0.17 ± 0.5	(Marty et al., 1990)
Oryzias latipes	Stage 1	20	0.18 ± 0.7	(Marty et al., 1990)
Oryzias latipes	Stage 1	23	0.23 ± 1.9	(Marty et al., 1990)
Oryzias latipes	Stage 1	26	0.17 ± 1.4	(Marty et al., 1990)
Oryzias latipes	Stage 1	29	0.20 ± 0.8	(Marty et al., 1990)
Oryzias latipes	Stage 1	20	0.22 ± 1.0	(Marty et al., 1990)
Oryzias latipes	Stage 1	23	0.29 ± 3.7	(Marty et al., 1990)
Oryzias latipes	Stage 1	26	0.25 ± 6.5	(Marty et al., 1990)
Oryzias latipes	Stage 1	29	0.52 ± 7.7	(Marty et al., 1990)
Danio rerio	Stage 1 (0–24 h)	26±0.5	0.221	(Strecker et al., 2011).
Danio rerio	Stage 1 (24–48 h)	26±0.5	0.326	(Strecker et al., 2011).
Danio rerio	Stage 1 (48–72 h)	26±0.5	0.352	(Strecker et al., 2011).
Oncorhynchus mykiss	Stage 1	9.5	0.27	Carter, 2005
Nocomis leptocephalus	Stage 1	18	1.04-1.75	Predicted Do Table
Nocomis leptocephalus	Stage 2	18	1.78	Predicted Do Table
Nocomis leptocephalus	Stage 3	18	1.81-2.15	Predicted Do Table
Nocomis leptocephalus	Stage 1	21	1.70-2.41	Predicted Do Table
Nocomis leptocephalus	Stage 2	21	2.44	Predicted Do Table
Nocomis leptocephalus	Stage 3	21	2.47-2.81	Predicted Do Table
Nocomis leptocephalus	Stage 1	24	2.37-3.10	Predicted Do Table
Nocomis leptocephalus	Stage 2	24	3.13	Predicted Do Table
Nocomis leptocephalus	Stage 3	24	3.16-3.47	Predicted Do Table

Table A1.2 Model-predicted dissolved oxygen consumption rates (mg O_2 h^{-1} per embryo) across incubation temperatures and developmental stages

Temp (˚C)	Embryo Developmental Stage																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
17.20	0.86	0.89	0.92	0.95	0.98	1.01	1.03	1.06	1.09	1.12	1.15	1.18	1.20	1.23	1.26	1.29	1.32	1.35	1.38	1.40
17.40	0.91	0.94	0.97	0.99	1.02	1.05	1.08	1.11	1.14	1.16	1.19	1.22	1.25	1.28	1.31	1.33	1.36	1.39	1.42	1.45
17.60	0.95	0.98	1.01	1.04	1.07	1.09	1.12	1.15	1.18	1.21	1.24	1.26	1.29	1.32	1.35	1.38	1.41	1.43	1.46	1.49
17.80	1.00	1.03	1.05	1.08	1.11	1.14	1.17	1.20	1.22	1.25	1.28	1.31	1.34	1.37	1.39	1.42	1.45	1.48	1.51	1.54
18.00	1.04	1.07	1.10	1.13	1.15	1.18	1.21	1.24	1.27	1.30	1.32	1.35	1.38	1.41	1.44	1.47	1.49	1.52	1.55	1.58
18.20	1.09	1.11	1.14	1.17	1.20	1.23	1.26	1.28	1.31	1.34	1.37	1.40	1.43	1.45	1.48	1.51	1.54	1.57	1.60	1.62
18.40	1.13	1.16	1.19	1.21	1.24	1.27	1.30	1.33	1.36	1.38	1.41	1.44	1.47	1.50	1.53	1.55	1.58	1.61	1.64	1.67
18.60	1.17	1.20	1.23	1.26	1.29	1.32	1.34	1.37	1.40	1.43	1.46	1.49	1.51	1.54	1.57	1.60	1.63	1.66	1.68	1.71
18.80	1.22	1.25	1.27	1.30	1.33	1.36	1.39	1.42	1.44	1.47	1.50	1.53	1.56	1.59	1.61	1.64	1.67	1.70	1.73	1.76
19.00	1.26	1.29	1.32	1.35	1.38	1.40	1.43	1.46	1.49	1.52	1.55	1.57	1.60	1.63	1.66	1.69	1.72	1.74	1.77	1.80
19.20	1.31	1.33	1.36	1.39	1.42	1.45	1.48	1.50	1.53	1.56	1.59	1.62	1.65	1.67	1.70	1.73	1.76	1.79	1.82	1.85
19.40	1.35	1.38	1.41	1.44	1.46	1.49	1.52	1.55	1.58	1.61	1.63	1.66	1.69	1.72	1.75	1.78	1.80	1.83	1.86	1.89
19.60	1.39	1.42	1.45	1.48	1.51	1.54	1.56	1.59	1.62	1.65	1.68	1.71	1.73	1.76	1.79	1.82	1.85	1.88	1.90	1.93
19.80	1.44	1.47	1.50	1.52	1.55	1.58	1.61	1.64	1.67	1.69	1.72	1.75	1.78	1.81	1.84	1.86	1.89	1.92	1.95	1.98
20.00	1.48	1.51	1.54	1.57	1.60	1.62	1.65	1.68	1.71	1.74	1.77	1.79	1.82	1.85	1.88	1.91	1.94	1.96	1.99	2.02
20.20	1.53	1.56	1.58	1.61	1.64	1.67	1.70	1.73	1.75	1.78	1.81	1.84	1.87	1.90	1.92	1.95	1.98	2.01	2.04	2.07
20.40	1.57	1.60	1.63	1.66	1.68	1.71	1.74	1.77	1.80	1.83	1.85	1.88	1.91	1.94	1.97	2.00	2.02	2.05	2.08	2.11
20.60	1.61	1.64	1.67	1.70	1.73	1.76	1.79	1.81	1.84	1.87	1.90	1.93	1.96	1.98	2.01	2.04	2.07	2.10	2.13	2.15
20.80	1.66	1.69	1.72	1.74	1.77	1.80	1.83	1.86	1.89	1.91	1.94	1.97	2.00	2.03	2.06	2.08	2.11	2.14	2.17	2.20
21.00	1.70	1.73	1.76	1.79	1.82	1.85	1.87	1.90	1.93	1.96	1.99	2.02	2.04	2.07	2.10	2.13	2.16	2.19	2.21	2.24
21.20	1.75	1.78	1.80	1.83	1.86	1.89	1.92	1.95	1.97	2.00	2.03	2.06	2.09	2.12	2.14	2.17	2.20	2.23	2.26	2.29
21.40	1.79	1.82	1.85	1.88	1.91	1.93	1.96	1.99	2.02	2.05	2.08	2.10	2.13	2.16	2.19	2.22	2.25	2.27	2.30	2.33
21.60	1.84	1.86	1.89	1.92	1.95	1.98	2.01	2.03	2.06	2.09	2.12	2.15	2.18	2.20	2.23	2.26	2.29	2.32	2.35	2.37
21.80	1.88	1.91	1.94	1.97	1.99	2.02	2.05	2.08	2.11	2.14	2.16	2.19	2.22	2.25	2.28	2.31	2.33	2.36	2.39	2.42
22.00	1.92	1.95	1.98	2.01	2.04	2.07	2.09	2.12	2.15	2.18	2.21	2.24	2.26	2.29	2.32	2.35	2.38	2.41	2.43	2.46
22.20	1.97	2.00	2.02	2.05	2.08	2.11	2.14	2.17	2.20	2.22	2.25	2.28	2.31	2.34	2.37	2.39	2.42	2.45	2.48	2.51
22.40	2.01	2.04	2.07	2.10	2.13	2.15	2.18	2.21	2.24	2.27	2.30	2.32	2.35	2.38	2.41	2.44	2.47	2.49	2.52	2.55
22.60	2.06	2.08	2.11	2.14	2.17	2.20	2.23	2.26	2.28	2.31	2.34	2.37	2.40	2.43	2.45	2.48	2.51	2.54	2.57	2.60
22.80	2.10	2.13	2.16	2.19	2.21	2.24	2.27	2.30	2.33	2.36	2.38	2.41	2.44	2.47	2.50	2.53	2.55	2.58	2.61	2.64
23.00	2.14	2.17	2.20	2.23	2.26	2.29	2.32	2.34	2.37	2.40	2.43	2.46	2.49	2.51	2.54	2.57	2.60	2.63	2.66	2.68
23.20	2.19	2.22	2.25	2.27	2.30	2.33	2.36	2.39	2.42	2.44	2.47	2.50	2.53	2.56	2.59	2.61	2.64	2.67	2.70	2.73
23.40	2.23	2.26	2.29	2.32	2.35	2.38	2.40	2.43	2.46	2.49	2.52	2.55	2.57	2.60	2.63	2.66	2.69	2.72	2.74	2.77
23.60	2.28	2.31	2.33	2.36	2.39	2.42	2.45	2.48	2.50	2.53	2.56	2.59	2.62	2.65	2.67	2.70	2.73	2.76	2.79	2.82
23.80	2.32	2.35	2.38	2.41	2.44	2.46	2.49	2.52	2.55	2.58	2.61	2.63	2.66	2.69	2.72	2.75	2.78	2.80	2.83	2.86
24.00	2.37	2.39	2.42	2.45	2.48	2.51	2.54	2.56	2.59	2.62	2.65	2.68	2.71	2.73	2.76	2.79	2.82	2.85	2.88	2.90
24.20	2.41	2.44	2.47	2.49	2.52	2.55	2.58	2.61	2.64	2.67	2.69	2.72	2.75	2.78	2.81	2.84	2.86	2.89	2.92	2.95
24.40	2.45	2.48	2.51	2.54	2.57	2.60	2.62	2.65	2.68	2.71	2.74	2.77	2.79	2.82	2.85	2.88	2.91	2.94	2.96	2.99
24.60	2.50	2.53	2.55	2.58	2.61	2.64	2.67	2.70	2.73	2.75	2.78	2.81	2.84	2.87	2.90	2.92	2.95	2.98	3.01	3.04
24.80	2.54	2.57	2.60	2.63	2.66	2.68	2.71	2.74	2.77	2.80	2.83	2.85	2.88	2.91	2.94	2.97	3.00	3.02	3.05	3.08
25.00	2.59	2.61	2.64	2.67	2.70	2.73	2.76	2.79	2.81	2.84	2.87	2.90	2.93	2.96	2.98	3.01	3.04	3.07	3.10	3.13

Temp (°C)	Embryo Developmental Stage																			
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
17.20	1.43	1.46	1.49	1.52	1.55	1.57	1.60	1.63	1.66	1.69	1.72	1.74	1.77	1.80	1.83	1.86	1.89	1.91	1.94	1.97
17.40	1.48	1.50	1.53	1.56	1.59	1.62	1.65	1.67	1.70	1.73	1.76	1.79	1.82	1.84	1.87	1.90	1.93	1.96	1.99	2.02
17.60	1.52	1.55	1.58	1.61	1.63	1.66	1.69	1.72	1.75	1.78	1.80	1.83	1.86	1.89	1.92	1.95	1.97	2.00	2.03	2.06
17.80	1.56	1.59	1.62	1.65	1.68	1.71	1.73	1.76	1.79	1.82	1.85	1.88	1.90	1.93	1.96	1.99	2.02	2.05	2.08	2.10
18.00	1.61	1.64	1.67	1.69	1.72	1.75	1.78	1.81	1.84	1.86	1.89	1.92	1.95	1.98	2.01	2.03	2.06	2.09	2.12	2.15
18.20	1.65	1.68	1.71	1.74	1.77	1.79	1.82	1.85	1.88	1.91	1.94	1.96	1.99	2.02	2.05	2.08	2.11	2.14	2.16	2.19
18.40	1.70	1.73	1.75	1.78	1.81	1.84	1.87	1.90	1.92	1.95	1.98	2.01	2.04	2.07	2.09	2.12	2.15	2.18	2.21	2.24
18.60	1.74	1.77	1.80	1.83	1.85	1.88	1.91	1.94	1.97	2.00	2.02	2.05	2.08	2.11	2.14	2.17	2.20	2.22	2.25	2.28
18.80	1.79	1.81	1.84	1.87	1.90	1.93	1.96	1.98	2.01	2.04	2.07	2.10	2.13	2.15	2.18	2.21	2.24	2.27	2.30	2.32
19.00	1.83	1.86	1.89	1.91	1.94	1.97	2.00	2.03	2.06	2.08	2.11	2.14	2.17	2.20	2.23	2.25	2.28	2.31	2.34	2.37
19.20	1.87	1.90	1.93	1.96	1.99	2.02	2.04	2.07	2.10	2.13	2.16	2.19	2.21	2.24	2.27	2.30	2.33	2.36	2.38	2.41
19.40	1.92	1.95	1.97	2.00	2.03	2.06	2.09	2.12	2.14	2.17	2.20	2.23	2.26	2.29	2.31	2.34	2.37	2.40	2.43	2.46
19.60	1.96	1.99	2.02	2.05	2.08	2.10	2.13	2.16	2.19	2.22	2.25	2.27	2.30	2.33	2.36	2.39	2.42	2.44	2.47	2.50
19.80	2.01	2.03	2.06	2.09	2.12	2.15	2.18	2.20	2.23	2.26	2.29	2.32	2.35	2.37	2.40	2.43	2.46	2.49	2.52	2.55
20.00	2.05	2.08	2.11	2.14	2.16	2.19	2.22	2.25	2.28	2.31	2.33	2.36	2.39	2.42	2.45	2.48	2.50	2.53	2.56	2.59
20.20	2.09	2.12	2.15	2.18	2.21	2.24	2.26	2.29	2.32	2.35	2.38	2.41	2.43	2.46	2.49	2.52	2.55	2.58	2.61	2.63
20.40	2.14	2.17	2.20	2.22	2.25	2.28	2.31	2.34	2.37	2.39	2.42	2.45	2.48	2.51	2.54	2.56	2.59	2.62	2.65	2.68
20.60	2.18	2.21	2.24	2.27	2.30	2.32	2.35	2.38	2.41	2.44	2.47	2.49	2.52	2.55	2.58	2.61	2.64	2.66	2.69	2.72
20.80	2.23	2.26	2.28	2.31	2.34	2.37	2.40	2.43	2.45	2.48	2.51	2.54	2.57	2.60	2.62	2.65	2.68	2.71	2.74	2.77
21.00	2.27	2.30	2.33	2.36	2.38	2.41	2.44	2.47	2.50	2.53	2.55	2.58	2.61	2.64	2.67	2.70	2.72	2.75	2.78	2.81
21.20	2.32	2.34	2.37	2.40	2.43	2.46	2.49	2.51	2.54	2.57	2.60	2.63	2.66	2.68	2.71	2.74	2.77	2.80	2.83	2.85
21.40	2.36	2.39	2.42	2.44	2.47	2.50	2.53	2.56	2.59	2.61	2.64	2.67	2.70	2.73	2.76	2.78	2.81	2.84	2.87	2.90
21.60	2.40	2.43	2.46	2.49	2.52	2.55	2.57	2.60	2.63	2.66	2.69	2.72	2.74	2.77	2.80	2.83	2.86	2.89	2.91	2.94
21.80	2.45	2.48	2.50	2.53	2.56	2.59	2.62	2.65	2.67	2.70	2.73	2.76	2.79	2.82	2.84	2.87	2.90	2.93	2.96	2.99
22.00	2.49	2.52	2.55	2.58	2.61	2.63	2.66	2.69	2.72	2.75	2.78	2.80	2.83	2.86	2.89	2.92	2.95	2.97	3.00	3.03
22.20	2.54	2.56	2.59	2.62	2.65	2.68	2.71	2.73	2.76	2.79	2.82	2.85	2.88	2.90	2.93	2.96	2.99	3.02	3.05	3.08
22.40	2.58	2.61	2.64	2.67	2.69	2.72	2.75	2.78	2.81	2.84	2.86	2.89	2.92	2.95	2.98	3.01	3.03	3.06	3.09	3.12
22.60	2.62	2.65	2.68	2.71	2.74	2.77	2.79	2.82	2.85	2.88	2.91	2.94	2.96	2.99	3.02	3.05	3.08	3.11	3.13	3.16
22.80	2.67	2.70	2.73	2.75	2.78	2.81	2.84	2.87	2.90	2.92	2.95	2.98	3.01	3.04	3.07	3.09	3.12	3.15	3.18	3.21
23.00	2.71	2.74	2.77	2.80	2.83	2.85	2.88	2.91	2.94	2.97	3.00	3.02	3.05	3.08	3.11	3.14	3.17	3.19	3.22	3.25
23.20	2.76	2.79	2.81	2.84	2.87	2.90	2.93	2.96	2.98	3.01	3.04	3.07	3.10	3.13	3.15	3.18	3.21	3.24	3.27	3.30
23.40	2.80	2.83	2.86	2.89	2.91	2.94	2.97	3.00	3.03	3.06	3.08	3.11	3.14	3.17	3.20	3.23	3.25	3.28	3.31	3.34
23.60	2.84	2.87	2.90	2.93	2.96	2.99	3.02	3.04	3.07	3.10	3.13	3.16	3.19	3.21	3.24	3.27	3.30	3.33	3.36	3.38
23.80	2.89	2.92	2.95	2.97	3.00	3.03	3.06	3.09	3.12	3.14	3.17	3.20	3.23	3.26	3.29	3.31	3.34	3.37	3.40	3.43
24.00	2.93	2.96	2.99	3.02	3.05	3.08	3.10	3.13	3.16	3.19	3.22	3.25	3.27	3.30	3.33	3.36	3.39	3.42	3.44	3.47
24.20	2.98	3.01	3.03	3.06	3.09	3.12	3.15	3.18	3.20	3.23	3.26	3.29	3.32	3.35	3.37	3.40	3.43	3.46	3.49	3.52
24.40	3.02	3.05	3.08	3.11	3.14	3.16	3.19	3.22	3.25	3.28	3.31	3.33	3.36	3.39	3.42	3.45	3.48	3.50	3.53	3.56
24.60	3.07	3.09	3.12	3.15	3.18	3.21	3.24	3.26	3.29	3.32	3.35	3.38	3.41	3.43	3.46	3.49	3.52	3.55	3.58	3.60
24.80	3.11	3.14	3.17	3.20	3.22	3.25	3.28	3.31	3.34	3.37	3.39	3.42	3.45	3.48	3.51	3.54	3.56	3.59	3.62	3.65
25.00	3.15	3.18	3.21	3.24	3.27	3.30	3.32	3.35	3.38	3.41	3.44	3.47	3.49	3.52	3.55	3.58	3.61	3.64	3.66	3.69

Chapter 2

Effects of Temperature and Dissolved Oxygen on the Developmental Rate and Survival of Embryos of Bluehead Chub *Nocomis leptocephalus* and its Nest Associates

Abstract

Early life stages of freshwater fishes are highly vulnerable to environmental stress because embryos rely entirely on oxygen diffusion and have limited capacity to regulate physiological demand. In stream habitats, temperature increases metabolic rate, while dissolved oxygen (DO) determines available metabolic capacity. When warming elevates oxygen demand under limited supply, developmental stress may occur. Therefore, understanding how temperature and DO influence embryonic development and survival is necessary to assess recruitment potential under changing environmental conditions.

This chapter examines the effects of temperature and DO on development and survival of embryos of Bluehead Chub *Nocomis leptocephalus* and its nest associates. I conducted laboratory experiments at three temperatures (18 °C, 21 °C, and 24 °C) and under two oxygen conditions: high (≈8 mg/L, aerated) and low (≈5 mg/L, sealed). I photographed embryos every eight hours to determine developmental stage, and I recorded survival at hatch, 24 hours, and 48 hours post-hatch. I calculated degree days to represent cumulative thermal exposure. I used ordinal logistic regression to describe developmental progression, and I applied binomial generalized linear models for survival analysis.

Embryos developed faster at warmer temperatures, but survival declined under high temperature and low oxygen. Degree-day accumulation was a reliable predictor of developmental stage, confirming that cumulative thermal exposure better explained development progress than time alone. Oxygen played a larger role during early stages, when metabolic demand and diffusion limits were most pronounced. Later stages showed weaker responses, suggesting greater physiological tolerance once embryos reached advanced development.

Overall, the results indicate that temperature predominantly controls both development and survival, while oxygen primarily modifies its effects during early stages. These findings emphasize that successful reproduction of Bluehead Chub and its nest associates depends on cool, oxygen-rich water and that future increases in stream temperature or hypoxia may reduce recruitment success in these nest-spawning fishes.

Introduction

Environmental conditions during the early life stages of fishes play a key role in shaping their development, growth, and survival, ultimately influencing recruitment and population persistence (Connell et al. 1999; Hamdoun and Epel 2007; Melzner et al. 2009; Leung and McAfee 2020; Dong and Tian 2023). Among these factors, temperature and dissolved oxygen (DO) directly influence metabolic processes in fishes (Shang and Wu 2004; Mohammed 2013). Temperature determines the rate of biochemical reactions, whereas oxygen availability constrains aerobic metabolism (von Bertalanffy 1960; Bradford 1990). As water warms, metabolic oxygen demand rises, but oxygen solubility falls, creating a physiological mismatch that restricts growth, development, and survival (von Bertalanffy 1960; Bradford 1990; Wetzel 2001; Shang and Wu 2004; Mohammed 2013).

High temperature and low oxygen often occur together and interact to limit performance in fishes (Earhart et al. 2022). High temperatures increase metabolic demand and simultaneously increase the prevalence and severity of environmental hypoxia, which limits the ability to supply this demand aerobically (Pörtner 2001; McBryan et al. 2013; Earhart et al. 2022; Deutsch et al. 2015; Schulte 2015). When oxygen demand outpaces supply, physiological systems such as circulation, energy balance, and growth begin to fail (Earhart et al. 2022:317; Schulte 2015; Somero et al. 2016; Little et al. 2020). Temperature and oxygen jointly influence the metabolic capacity of developing embryos. When oxygen availability does not meet metabolic demand, it limits aerobic energy supply and can reduce growth and survival (Pörtner et al. 2017).

With global warming, these combined stressors are expected to intensify. Rising temperatures increase microbial respiration and further reduce oxygen concentrations, expanding hypoxic zones in freshwater habitats (Iriberri et al. 1985; Pörtner and Knust 2007; Schulte 2015; Lim and Bernier 2023). Such changes are predicted to lower growth, reproduction, and overall fish biomass (Díaz and Rosenberg 2011; Jenny et al. 2016; Sampaio et al. 2021; Harvey et al. 2022). Although fish can adjust their development and body functions to some changes, they often only partially compensate, which may not be sufficient to protect them from the combined stress of high temperature and low oxygen (Earhart et al. 2022).

Previous research shows that both temperature and dissolved oxygen strongly influence early development and hatching success in freshwater fishes. Embryos typically develop more slowly and experience higher mortality under low oxygen or extreme temperatures, indicating that these stressors interact and shape growth and survival (Hamdoun and Epel 2007; Leung and McAfee 2020). Laboratory and field studies have also shown that small changes in oxygen availability can delay development, reduce hatching success, and lower post-hatch fitness in many species (Melzner et al. 2009; Dong and Tian 2023). Because metabolic rate increases with temperature, higher thermal conditions raise oxygen demand, which can further stress developing embryos when oxygen supply is limited (Pörtner et al. 2017). Together, these findings highlight the importance of examining temperature–oxygen interactions to understand how embryos respond to changing environmental conditions.

The Bluehead Chub *Nocomis leptocephalus*, a native minnow of southeastern U.S. streams, provides a natural model for studying how temperature and oxygen interact during development. Male Bluehead Chubs build large gravel mounds that serve as communal spawning sites for several other fish species (Hultin 2022; Betts et al. 2024). During peak spawning, hundreds of fish may deposit eggs in a single mound (Betts et al. 2024), creating dense clusters that may reduce water movement through the gravel and limit oxygen availability. Multiple species spawn together on Bluehead Chub nests, where predators such as crayfish (*Cambarus* spp.) and sunfish (*Lepomis* spp.) have been observed stalking or attacking embryo aggregations (Betts et al. 2024). Because embryos from different species develop concurrently under shared nest conditions, this system naturally allows researchers to examine how temperature and dissolved oxygen interact and influence embryo development and survival.

Studies on related minnows show that warmer water speeds up embryo development but can also increase mortality when oxygen levels are low (Cooper 1980; Pepin 1991; Mendiola et al. 2006).

Similarly, higher temperatures raise oxygen demand, which can intensify stress under hypoxic conditions (Motani and Wainwright 2015). These findings suggest that the interaction between temperature and oxygen, rather than either factor alone, determines developmental success.

Although much is known from model species such as zebrafish, little research has examined how these combined stressors affect embryo development in stream-dwelling minnows that spawn communally. In Bluehead Chub nests, the way the nest is built and the number of embryos may create areas with different oxygen levels that can affect how embryos grow and survive. By evaluating temperature and oxygen separately, this study explains how embryos are likely to respond in natural nests, where these differences may intensify with warming and decreased oxygen.

This study investigates how temperature and dissolved oxygen interact to influence the developmental rate and survival of embryos of Bluehead Chub *Nocomis leptocephalus* and its nest associates. Specifically, I test whether higher degree-day totals (representing warmer and longer exposure) accelerate embryo development but also increase mortality; whether embryos reared under oxygen-rich conditions show greater survival and accelerated development than those in oxygen-limited treatments; and whether simultaneous exposure to high temperature and low oxygen intensifies physiological stress and reduces survival. Although I conducted all experiments fully in the laboratory, I collected embryos from natural nests, which makes the results more realistic and relevant to conditions that may occur in the wild. Therefore, this chapter aims to help understand how temperature and dissolved oxygen together may affect embryo development and survival, and what this could mean for fish reproductive success as environmental conditions change.

Methods

Study Site and Sample Collection

I carried out field collections and laboratory experiments during the summers of 2021 and 2022 using embryos obtained from approximately a 1 km section of Toms Creek, with the midpoint of this reach located at 37.261955° N, −80.436715° W (Figure 1.1). Toms Creek is a third-order stream within the New River drainage in Montgomery County, Virginia (Bustamante and Frimpong 2025). The creek flows through the Valley and Ridge physiographic region and supports a robust, well-established population of Bluehead Chub *Nocomis leptocephalus*, which I used to obtain study embryos (Bustamante and Frimpong 2025).

Experimental Design

Experiments were conducted in a temperature-controlled laboratory at Virginia Tech using the same incubation system described in Chapter 1. Three water baths were maintained at 18 °C, 21 °C, and 24 °C. The 18 °C bath was cooled using a chiller, while the 21 °C and 24 °C baths were heated with thermostat-controlled aquarium heaters. To prevent overheating and maintain stable conditions in the 21 °C bath, a small fan was directed continuously across the water surface. Within each temperature bath, embryos were assigned to two dissolved oxygen (DO) treatments designed to simulate natural variation within Bluehead Chub nests. In the high-oxygen treatment, embryos were incubated in open PVC cylinders placed in the water baths and supplied with continuous aeration to maintain DO near saturation. In the low-oxygen treatment, embryos were incubated in identical PVC cylinders placed inside glass jars sealed with airtight rubber stoppers, creating closed respirometry chambers. Dissolved oxygen gradually declined over each 8-hour interval as embryos respired, with gentle circulation provided by a magnetic stir bar. When DO approached approximately 3 mg L^{-1}, chambers were briefly opened and replenished with oxygenated bath water to prevent excessive hypoxia, following procedures described in Chapter 1. Together, these treatments simulated vertical gradients within natural nests, where embryos near the surface experience higher oxygen availability and flow, while embryos in deeper layers experience lower oxygen but increased protection from predators.

After each 8-hour incubation period, chambers were opened, dissolved oxygen was measured, and embryos were examined under a dissecting microscope. Each embryo was photographed and assigned a developmental stage based on morphological features such as eye pigmentation, somite formation, and tail movement, following standard fish embryo development guides. At the end of each trial, the number of hatched embryos was recorded to quantify survival. Degree days were calculated for each treatment to represent cumulative thermal exposure and allow comparisons across temperature groups. To facilitate analysis of developmental progression and variation in responses to temperature and dissolved oxygen, the 39 detailed developmental stages were grouped into four broader phases: cleavage and early blastula (Stages 1–10), gastrulation and early organogenesis (Stages 11–20), hatching and early larval development (Stages 21–32), and advanced larval development (Stages 33–39). This grouping enabled evaluation of how temperature and oxygen effects varied across major developmental transitions.

Statistical Analysis

To understand how temperature, dissolved oxygen (DO), and their interaction influenced embryo development, survival, and growth, three statistical approaches were used: ordinal logistic regression, multiple logistic regression, and two-way analysis of variance (ANOVA). I selected these tests to match the type of data for each biological response. All analyses were performed in

R_version 4.3.2. I used ordinal logistic regression for developmental stage, which is an ordered categorical variable. This model estimates the probability that an embryo has reached or surpassed a given developmental stage while accounting for the ordered nature of the data. I used multiple logistic regression to analyze embryo survival, modeled as a binary (1 = hatched, 0 = unhatched) or proportional outcome. This approach allowed estimation of survival probability as a function of temperature, DO, and their interaction. Together, these statistical methods provided a rigorous and biologically meaningful approach for evaluating how environmental treatments shaped embryo development and survival, and enabled assessment of whether temperature, oxygen, or their interaction exerted stronger influences.

Data Analysis

The developmental dataset consisted of 389 interval observations, representing repeated measurements of embryos incubated under three temperatures (18 °C, 21 °C, 24 °C) and two dissolved oxygen (DO) conditions (high-oxygen and low-oxygen). For regression analysis, average values of temperature, DO, and degree-day measurements for each experimental unit were calculated for the observation period based on values measured at the beginning and the end of that period. The starting developmental stage (“Begin Stage”) was included in all models to account for initial differences among embryos. Because embryos move through stages in a set order, ordinal logistic regression was used to describe how temperature and oxygen affected their developmental rate.

I first explored the dataset to understand general patterns before conducting statistical modeling. Most embryos were in early to mid-developmental stages, and only a few reached advanced stages, which aligns with expected developmental progression under laboratory incubation conditions. In the high-oxygen treatment, dissolved oxygen remained close to saturation throughout the incubation period. In contrast, oxygen levels gradually declined in the low-oxygen treatment as embryos consumed available oxygen, reflecting natural oxygen limitation in deeper or less-ventilated nest microhabitats.

Three ways of describing temperature were tested: mean temperature, Degree Days 1 (DD1), and Degree Days 2 (DD2). Correlation tests showed mean temperature was not related to DD1 ($r = -0.034$, $p = 0.504$) and only moderately related to DD2 ($r = 0.420$, $p < 0.001$). Because these variables were not highly correlated, all were retained for model comparison.

Model Comparison and Selection

Using a hypothesis-driven approach, each statistical model was treated as a biological hypothesis and evaluated against a null model representing no predictor influence. Table 2.1 outlines the candidate model set, including the null model (H0), the model using average temperature (H1), and those using degree-day metrics (H2 and H3), along with their underlying biological interpretation and corresponding regression formulas.

Table: 2.1- Candidate model set for ordinal logistic regression, summarizing each biological hypothesis and its corresponding model formula.

Model	Hypothesis	Formula
Null model (H0)	No predictors influence development (baseline only).	*stage_gain* ~ 1

Model	Hypothesis	Formula
H1: Temperature effect	Development depends on average interval temperature.	*stage_gain* ~ Beginning stage + Dissolved oxygen + Embryo size + Temperature
H2: Degree Days 1 effect	Development depends on cumulative thermal exposure (Degree Days, base1).	*stage_gain* ~ Beginning stage + Dissolved oxygen + Embryo size + Degree Days 1
H3: Degree Days 2 effect	Development depends on cumulative thermal exposure (Degree Days, base2).	*stage_gain* ~ Beginning stage + Dissolved oxygen + Embryo size + Degree Days 2

To determine which temperature measure best explained developmental progression, three initial linear models were compared using the Akaike Information Criterion (AIC; Akaike 1974):

- Temp + DO + Embryo size + Begin Stage
- DD_1 + DO + Embryo size + Begin Stage
- DD_2 + DO + Embryo size + Begin Stage

Because physiological responses to temperature are often nonlinear, a quadratic term ($Temp^2$) was added to test for curvature in developmental responses. This resulted in the following extended models:

- Temp + $Temp^2$ + DO + Embryo size + Begin Stage
- DD_1 + $Temp^2$ + DO + Embryo size + Begin Stage

- DD_2 + $Temp^2$ + DO + Embryo size + Begin Stage

Final Model Specification

The final ordinal logistic regression model estimated the probability that an embryo had reached or not yet reached a certain stage (j):

Where Y is the developmental stage. $P(Y \leq j)$ is the probability that an embryo is at or below stage j, and β_1, β_2, β_3, β_4, β_5, and β_6 are the coefficients representing the effects of temperature, temperature squared, dissolved oxygen (DO), the interaction between temperature and DO, embryo size, and initial developmental stage, respectively.

Model Diagnostics and Visualization

Model performance was assessed using AICc, R^2, and likelihood-ratio. Residual plots and proportional-odds tests showed no pattern problems. I used the ggeffects package in R (Lüdecke 2018) to generate predicted probabilities from the final model and plotted them using ggplot2 (Wickham 2016). These plots help visualize how embryos advanced through development under different conditions. Early stages reacted strongly to heat accumulation, while later stages were more stable patterns explored further in the Results section.

For survival data analysis, Embryo survival was evaluated at three developmental checkpoints: (1) hatching, (2) 24 hours post-hatch, and (3) 48 hours post-hatch. For each incubation jar, survival was calculated as the proportion of embryos alive out of the total stocked at the beginning of the trial. Because survival is a binomial outcome bounded between 0 and 1, analyses were performed using generalized linear mixed-effects models (GLMMs) with a binomial error distribution and a logit link function.

Temperature (18 °C, 21 °C, 24 °C), dissolved oxygen (High vs. Low), and embryo size (Small vs. Large) were treated as fixed effects. A random intercept for experimental Block (incubation run) was included to account for repeated measures and unmeasured variation among independent experimental trials.

Before model fitting, survival data for each developmental checkpoint were explored visually to assess the distribution of outcomes, identify potential non-linear patterns, and evaluate whether treatment combinations differed in variance or baseline mortality. Because these exploratory checks showed clear differences among the three observation times, survival at hatch, 24 h, and 48 h were analyzed separately rather than combined in a single model.

For each time (hatch, 24 h, and 48 h), binomial GLMs were fit to estimate the effects of temperature, oxygen, and size, as well as their interactions, on survival probability. The general model structure followed

$$\begin{aligned} &log(p/1-p) \\ &\qquad = \beta_0 + \beta_1(Temp) + \beta_2(DO) + \beta_3(Size) + \beta_{\mathrm{ij}}(Temp \times DO) \\ &\qquad + \beta_{\mathrm{i}k}(Temp \times Size) + \beta_{\mathrm{j}k}(DO \times Size) + \beta_{\mathrm{ij}k}(Temp \times DO \times Size) + u_{Block} \\ &\qquad \sim N(0, \sigma_{Block}^2) \end{aligned}$$

where *p* denotes the predicted probability of survival and $u_{\text{Block}} \sim \mathcal{N}(0, \sigma_{\text{Block}}^2)$ represents the random effect of experimental run. Survival at each time was modeled separately to capture differences in baseline mortality across developmental stages.

Predicted probabilities from the fitted GLMs were used to generate treatment-wise estimates. Model assessment used the Akaike Information Criterion (AIC); interaction terms were retained when they improved model fit (ΔAIC ≥ 2).

Model selection was based on AICc to balance explanatory power and parsimony. Interaction terms were retained only when they improved model fit according to AICc and were supported by biological reasoning; otherwise, models were simplified by removing noninformative interactions. Model assumptions were evaluated using residual diagnostics, checks for overdispersion, and comparisons of predicted versus observed survival across treatment levels. Confidence intervals for fixed effects were derived from model-based standard errors, and parametric bootstrapping was used when necessary to stabilize mixed-model estimates.

Final models for each developmental checkpoint were used to estimate predicted survival probabilities under each treatment combination, with uncertainty expressed through 95% confidence intervals.

Results

A total of 389 observation intervals were analyzed across all developmental treatments. Average incubation temperatures ranged from 11.5 to 24.5 °C (mean ± SD = 20.6 ± 2.37 °C), and average dissolved oxygen concentrations ranged from 1.27 to 13.86 mg L^{-1} (mean ± SD = 5.61 ± 2.10 mg L^{-1}). Observation intervals varied in length, with an average duration of 9.30 hours.

To examine whether these developmental conditions were linked to embryo survival, survival was analyzed at the jar level. A total of 81 experimental jars were included in the survival analyses. Embryo survival varied across temperature, dissolved oxygen, and embryo size treatments. Survival at hatch ranged from 0 to 100% (mean = 63.3%), decreased by 24 hours post-hatch (range = 0–100%, mean = 48.6%), and was lowest at 48 hours post-hatch (range = 0–91.7%, mean = 34.7%).

Following these summary statistics, model-based analyses were used to examine the effects of temperature, dissolved oxygen, and embryo size on embryo development. Results of model comparisons are presented in Table 2.2.

Table 2.2 Model comparison for predictors of embryo development.

Model	logLik	k	McFadden_R2	dAIC	weight	AICc	dAICc	weight_AICc
Avg Intvl temp	-443.33	10	0.055	0	0.548	907.25	0	0.548
Degree Days 1	-444.16	10	0.053	1.65	0.24	908.9	1.65	0.24
Degree Days 2	-444.28	10	0.053	1.9	0.212	909.15	1.9	0.212

Model comparison using Akaike Information Criterion (AICc) indicated that temperature-related predictors best explained embryo development (Table 2.2). Models based on average interval temperature and cumulative thermal exposure (degree days) received the strongest support (ΔAICc < 2).

Table 2.3. Final model odds ratios (OR) with 95% confidence intervals for temperature, dissolved oxygen, and degree-day predictors (DD1 and DD2).

Term	OR	OR_low	OR_high	std.error	statistic	Track
Begin Stage	0.865	0.829	0.902	0.022	-6.738	Temp+DD1 (final)
do_sc	0.838	0.679	1.032	0.107	-1.659	Temp+DD1 (final)
temp_sc	1.164	0.956	1.418	0.1	1.516	Temp+DD1 (final)
I(temp_sc^2)	1.487	1.14	1.951	0.137	2.9	Temp+DD1 (final)
dd1_sc	0.731	0.568	0.937	0.127	-2.461	Temp+DD1 (final)
Embr_sizeLg	0.945	0.64	1.398	0.199	-0.284	Temp+DD1 (final)
Begin Stage	0.869	0.833	0.906	0.021	-6.568	Temp+DD2 (final)
do_sc	0.861	0.699	1.057	0.105	-1.422	Temp+DD2 (final)
temp_sc	1.287	1.033	1.606	0.112	2.245	Temp+DD2 (final)

I(temp_sc^2)	1.246	1.021	1.517	0.107	2.05	Temp+DD2 (final)
dd2_sc	0.812	0.635	1.039	0.12	-1.738	Temp+DD2 (final)
Embr_sizeLg	0.946	0.641	1.395	0.198	-0.281	Temp+DD2 (final)

In the final model (Table 2.3), initial developmental stage (Begin Stage) had the strongest influence on embryo progression (odds ratio ≈ 0.87, $p < 0.001$), with later-stage embryos developing more slowly, as expected biologically.

Table 2.4. VIF values for temperature and degree-day models (DD1 and DD2). Values above 5–10 suggest multicollinearity.

Term	VIF (Temp + DD1)	VIF (Temp + DD2)
Begin Stage	1.11	1.1
Dissolved oxygen	1.16	1.15
Temperature	1.06	1.26
Degree Days 1	1.02	
Embr size	1	1
Degree Days 2		1.22

My Variance Inflation Factor assessment indicated no multicollinearity (Table 2.4), confirming that the parameter estimates were stable and reliable.

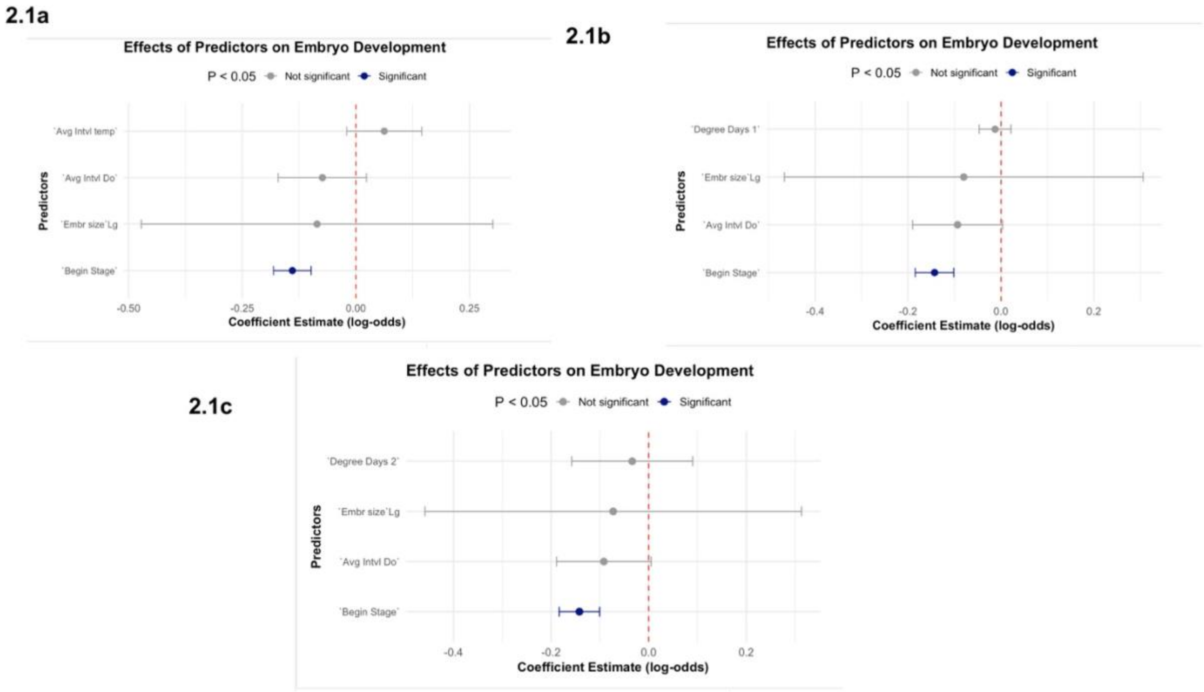


Figure 2.1. Effects of environmental and biological predictors on embryo developmental progression estimated from ordinal logistic regression models. Panels show coefficient estimates (log-odds ±95% confidence intervals) for models using (a) average interval temperature, (b) cumulative thermal exposure calculated as Degree Days 1, and (c) cumulative thermal exposure calculated as Degree Days 2. The vertical dashed line indicates a coefficient of zero (no effect). Filled points denote statistically significant predictors ($p < 0.05$), while open points indicate non-significant predictors.

Initial developmental stage was the only predictor that consistently influenced how far embryos progressed when average interval temperature was included in the model (Figure 2.1a). The same pattern was observed when Degree Days 1 was used (Figure 2.1b) and was further supported by the alternative degree-day formulation (Figure 2.1c). This results suggests that embryos starting at earlier stages were more likely to continue developing within each eight-hour interval. Temperature, DO, and degree days showed weak short-term effects, they became more important when development was viewed across entire phases instead of single time steps.

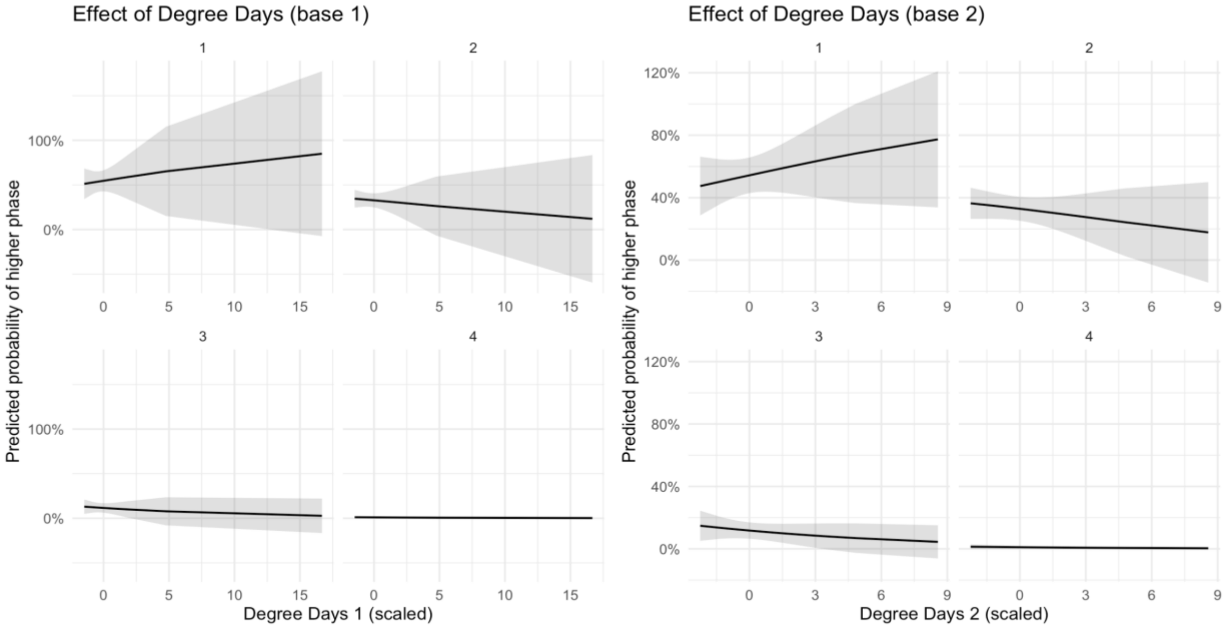


Figure 2.2: Predicted probability of reaching higher developmental phases as a function of accumulated degree days under two base temperature thresholds (Base 1 and Base 2).

Predicted probabilities from the final model showed clear phase-specific differences (Figure 2.2). In Phase 1, the probability of progressing increased sharply from about 50% at low degree-day values to more than 80% at high values, showing that heat strongly promoted early cleavage and blastula formation. In Phase 2, the pattern was reversed: the probability of progressing dropped from roughly 35% to below 15% with higher heat exposure, suggesting that extra warming slowed organ formation, likely due to reduced oxygen availability in warmer water. Phases 3 and

4 showed little change across the temperature range (probabilities below 20 %), indicating that later stages were mostly controlled by internal timing rather than temperature.

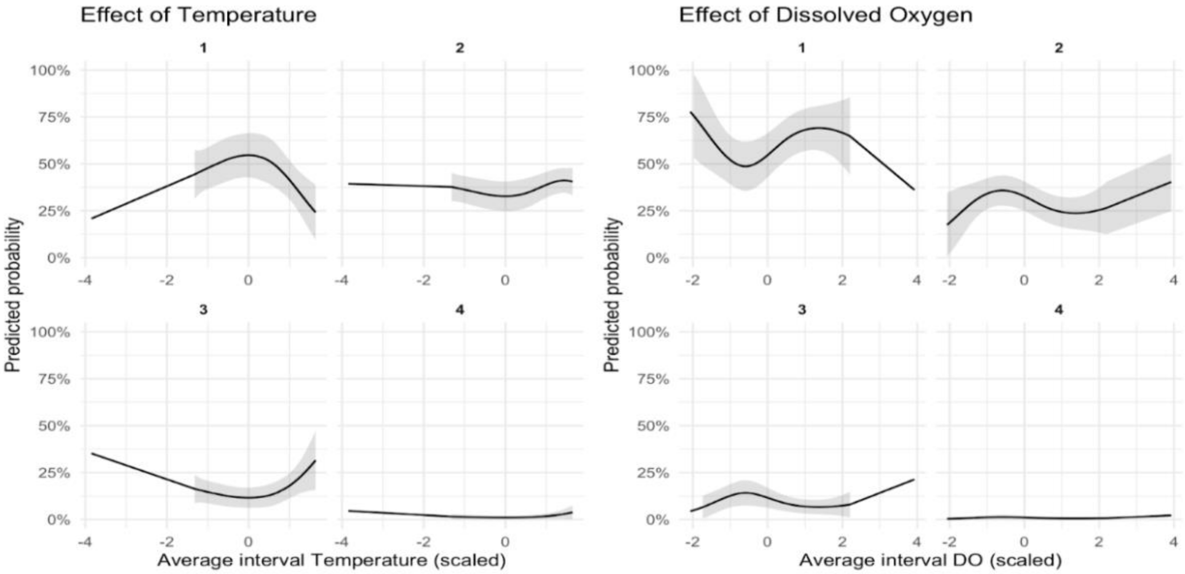


Figure 2.3: Predicted probabilities of reaching higher developmental phases as functions of average interval temperature and dissolved oxygen.

Temperature and oxygen also interacted in ways that changed through development (Figure 2.3). During Phase 1, embryos developed fastest at moderate temperatures and higher DO levels, suggesting an optimal combination of temperature and oxygen for early growth. In Phase 2, both variables had weaker effects, and by Phases 3 and 4, neither had a clear influence. This pattern shows that early embryos are sensitive to external environmental conditions, but later ones rely more on internal control mechanisms.

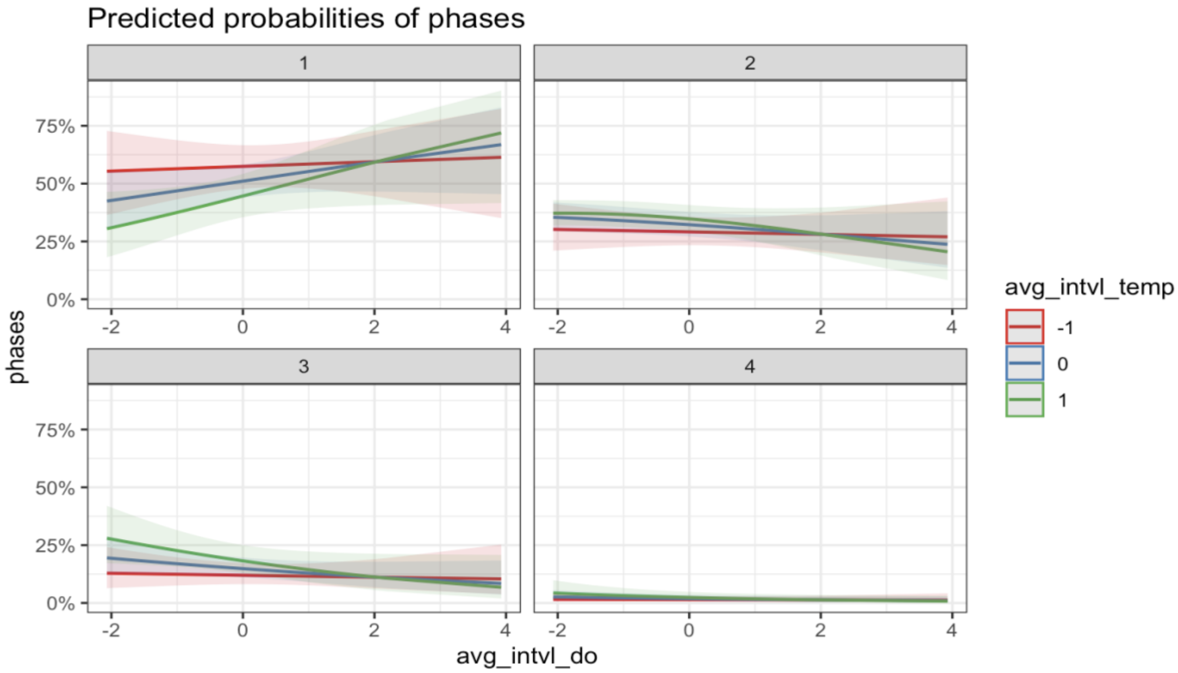


Figure 2.4: Predicted probabilities of developmental phases as functions of dissolved oxygen across temperature treatments.

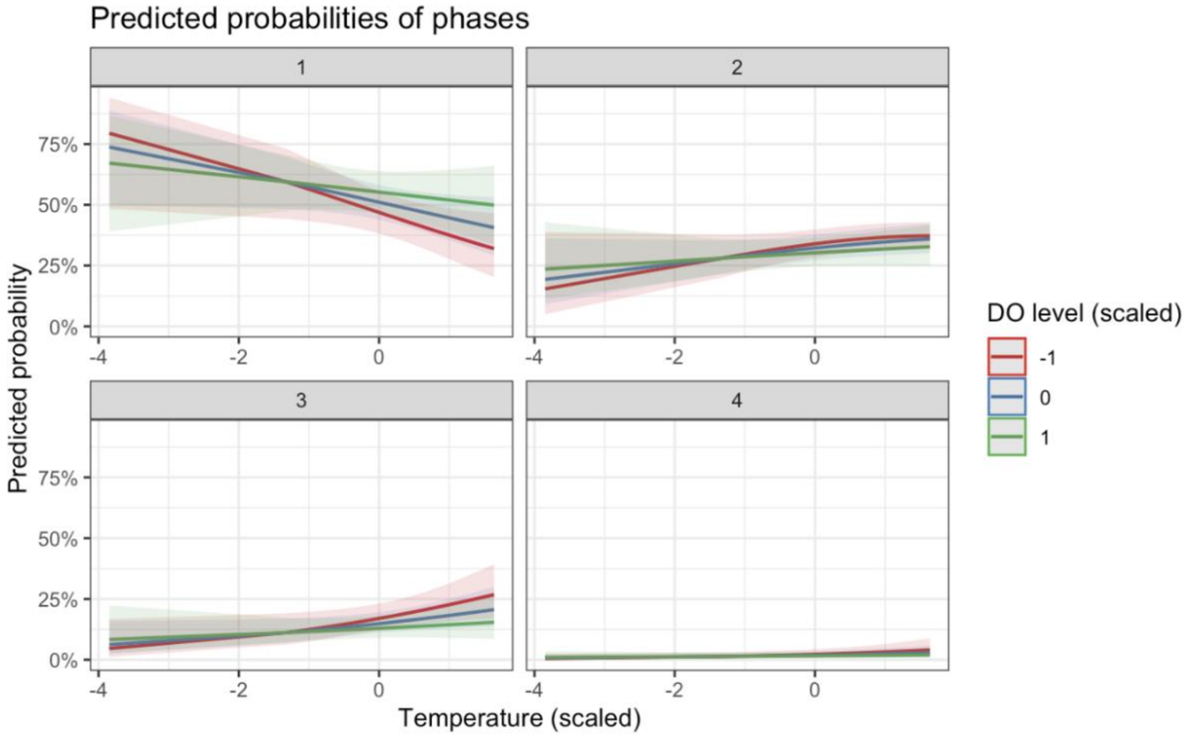


Figure 2.5: Predicted probabilities of developmental phases as functions of temperature across dissolved oxygen (DO) levels.

Interactions between temperature and oxygen (Figure 2.4) were strongest in the earliest stages. In Phase 1, higher oxygen increased the chance of embryos staying in that phase longer, suggesting that oxygen-rich, warm conditions supported longer cleavage activity. Phase 2 showed almost no interaction, while Phase 3 showed a small temperature-driven speed-up, and Phase 4 was not affected by either factor. Phase-specific probability curves (Figure 2.5) confirmed that temperature was the main driver of developmental rate, with oxygen slightly modifying its effect. In Phase 1, embryos left the phase faster as temperature increased, especially when oxygen was high, showing that warm, oxygen-rich water sped up development. In Phases 2 and 3, progress continued slowly with mild warming, and in Phase 4, no clear environmental effects were seen.

Across all analyses, three main results stood out. First, the starting developmental stage had the strongest effect; embryos beginning at earlier stages progressed faster. Second, temperature affected development in a curved, nonlinear way: moderate warming accelerated early stages, but too much heat reduced progress. Third, oxygen mainly affected the earliest phase, helping embryos develop faster when levels were high but having little effect later.

In summary, Bluehead Chub and its nest associate's embryo development was most sensitive to environmental conditions during the early cleavage and blastula stages, when both temperature and oxygen strongly shaped developmental rate. As embryos matured, development became more stable and guided mainly by internal physiological and genetic processes (Rombough 2011). I found that early embryos are the most vulnerable to environmental stress, especially warming and low oxygen, which may be important for understanding how future temperature and oxygen changes in streams could affect reproductive success.

Following the analysis of developmental progression, embryo survival was evaluated at hatch, 24 hours after hatch, and 48 hours after hatch to assess the effects of temperature, dissolved oxygen, and embryo size.

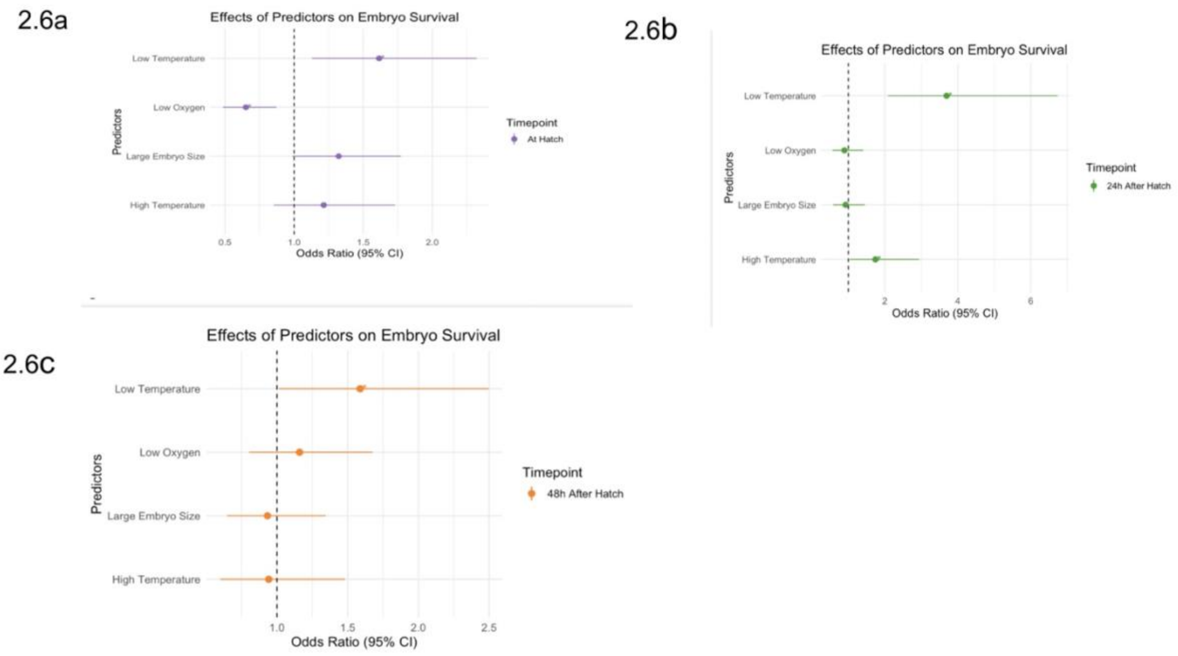


Figure 2.6. Effects of temperature, dissolved oxygen, and embryo size on embryo survival estimated from generalized linear models. Panels show odds ratios (±95% confidence intervals) for predictor effects on survival at (a) hatch, (b) 24 hours after hatch, and (c) 48 hours after hatch. The dashed vertical line indicates an odds ratio of 1.0, representing no effect.

Early model outputs (Figures 2.6a–2.6c) showed that survival was generally highest at low temperature and lowest under low oxygen, while embryo size effects were modest and often accompanied by wide uncertainty. Raw hatch counts and survival distributions are presented in Appendix Figures A2.1–A2.19 to provide descriptive context for model-based results. These figures illustrate variability in hatching and early survival across temperature, dissolved oxygen, and embryo size treatments and are included to support transparency and visualization of the underlying data.

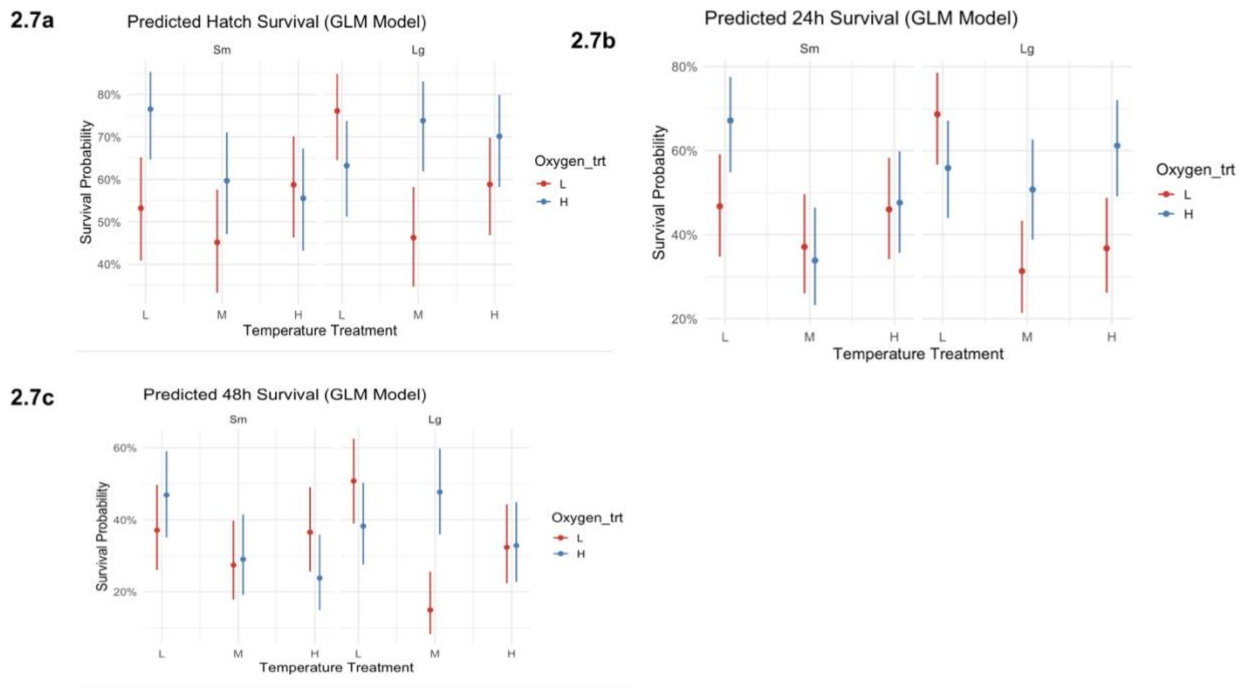


Figure 2.7. Predicted survival probabilities from generalized linear models across temperature and dissolved oxygen treatments for small (Sm) and large (Lg) embryos. Panels show predicted survival at (a) hatch, (b) 24 hours post-hatch, and (c) 48 hours post-hatch. Points represent model-predicted means and error bars indicate 95% confidence intervals. Oxygen treatments are shown as low (L) and high (H), and temperature treatments are shown as low (L), medium (M), and high (H).

Model-based predicted probabilities offered a complementary perspective. The GLM-based figures (2.7a–2.7c) showed that survival consistently declined as embryos progressed from hatch to 24 hours and then to 48 hours, with the strongest survival always occurring at low temperature. High oxygen and larger embryo size offered modest improvements, but confidence intervals overlapped broadly. Mixed-effects model predictions and predicted survival probabilities are provided in Appendix Figures A2.20-A2.23 to supplement the primary analyses.

These figures illustrate predicted hatch and post-hatch survival across temperature, dissolved oxygen, and embryo size treatments and are included to visualize model-based estimates and associated uncertainty.

Together, these findings reveal a consistent thermal pattern across survival stages: low temperature reliably supported the highest survival**,** particularly for small embryos, while high oxygen provided modest benefits**,** especially for large embryos at warmer temperatures. Although size and oxygen effects were secondary and often variable, the combined figure set demonstrates that temperature was the dominant factor shaping early survival**,** with medium and high temperatures reducing survival and increasing variability. These results indicate that Bluehead Chub and nest-associate embryos are most vulnerable to elevated temperatures, particularly when oxygen availability is limited, and that early developmental stages show clear thermal sensitivity that may influence recruitment in warming stream systems.

Discussion

This study demonstrates that temperature and dissolved oxygen (DO) jointly shape the developmental rate and survival of Bluehead Chub *Nocomis leptocephalus* embryos and those of its nest associates. Across all analyses, temperature emerged as the primary driver of developmental progression, with embryos advancing most rapidly at intermediate temperatures and more slowly at both cooler and warmer conditions. At the same time, survival consistently declined as temperature increased, particularly when oxygen levels were low. These findings reinforce long-standing physiological principles: metabolic demand in ectothermic embryos increases with temperature, while the capacity to meet that demand is constrained by available oxygen (Bradford 1990; Wetzel 2001; Schulte 2015). Together, temperature and oxygen define the aerobic window within which embryos can sustain normal development.

The interaction between warming and hypoxia was especially evident in the sharp reduction in survival under warm, low-oxygen conditions. As temperature rises, oxygen solubility in water declines, and the gradient for oxygen diffusion into the embryo becomes weaker. In this experiment, high-oxygen treatments mitigated some of the negative effects of warming, suggesting that improved oxygen availability can buffer embryos against moderate thermal stress. However, this buffering capacity was limited: at the warmest temperature, survival decreased regardless of oxygen level. These patterns highlight the synergistic nature of thermal and oxygen stress during early development, a relationship documented across many freshwater fishes and clearly expressed here in both raw survival outcomes and model-based predictions.

An important component of the study was the use of degree days to quantify cumulative thermal exposure. Because embryonic development depends not only on temperature but also on the duration of exposure, degree-day metrics offered a biologically meaningful representation of the total thermal energy experienced by embryos. Models incorporating degree days performed as well as, or better than, those using mean temperature, supporting the suitability of this measure for predicting developmental timing. The results align with earlier work demonstrating that cumulative thermal input is a robust predictor of developmental progress in many fish species (Chezik et al. 2014). Although degree days did not significantly improve survival predictions, they helped clarify developmental trends by reflecting the true physiological time experienced by embryos across treatments.

The responses observed here have clear ecological implications for Bluehead Chub nesting systems. Gravel nests naturally create vertical gradients in temperature and oxygen, with surface layers receiving more flow and deeper layers experiencing reduced oxygen availability. The experiment showed that embryos tolerated moderate hypoxia at cooler temperatures but experienced substantial mortality when low oxygen coincided with warmer conditions. These patterns suggest that nest microhabitat quality including substrate permeability, flow-through, and oxygen exchange plays a critical role in shaping embryonic outcomes. Small variations in flow or sedimentation could therefore have disproportionate effects on survival during warm periods of the spawning season.

Broader environmental trends further underscore the importance of these findings. Climate-driven warming of streams, combined with episodic or chronic reductions in dissolved oxygen resulting from flow alteration, nutrient enrichment, or organic loading, may compress the range of suitable developmental environments available to nest-spawning fishes. For species such as Bluehead Chub, which construct communal nests used by multiple species, declines in oxygen or increasing temperatures could influence not only their own reproductive success but also the reproductive dynamics of the entire nest-associate community. Reduced survival in warm, low-oxygen conditions may force shifts in spawning timing, settlement depth within nests, or reliance on cooler microhabitats responses that may not always be possible in degraded or thermally altered systems.

Overall, this study shows that temperature and oxygen jointly determine embryonic outcomes by influencing metabolic demand, diffusion capacity, and physiological tolerance. Development proceeded most consistently under cooler, well-oxygenated conditions, while warm, hypoxic conditions produced marked developmental stress and mortality. Degree-day metrics provided valuable insight into the cumulative effects of temperature on developmental progression, reinforcing their usefulness in studies of ectotherm embryology. Combined, these results emphasize the importance of maintaining well-oxygenated, thermally stable stream habitats to support successful reproduction and recruitment of Bluehead Chub and its nest associates.

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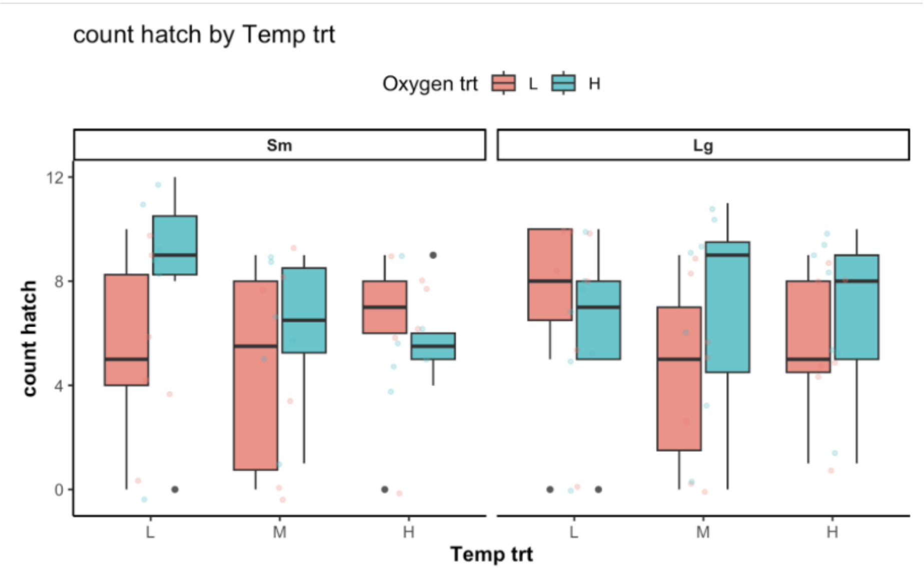
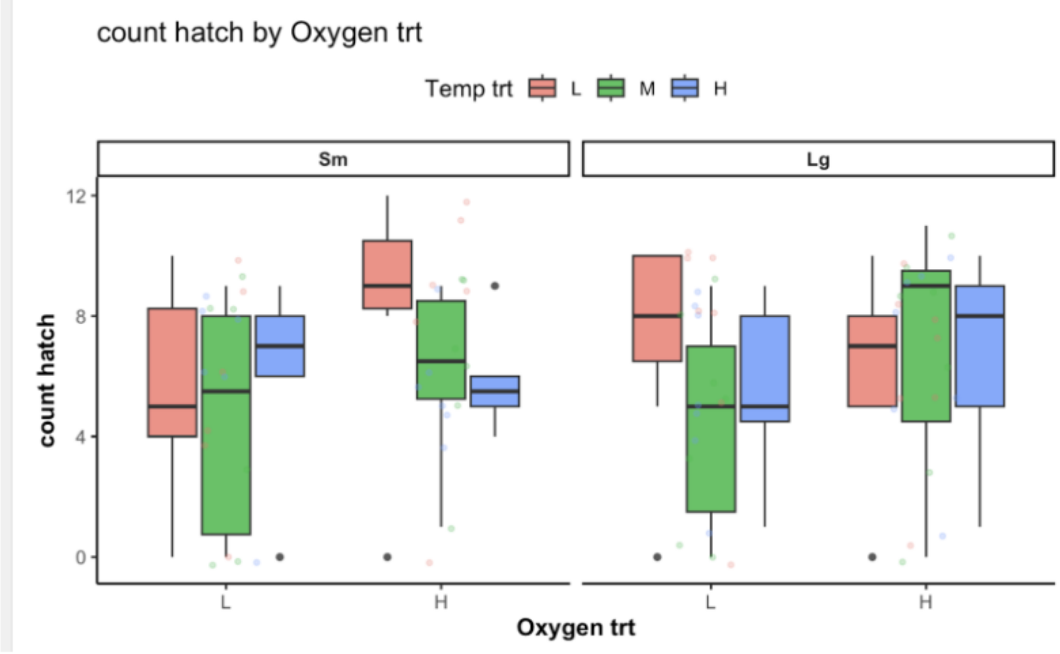
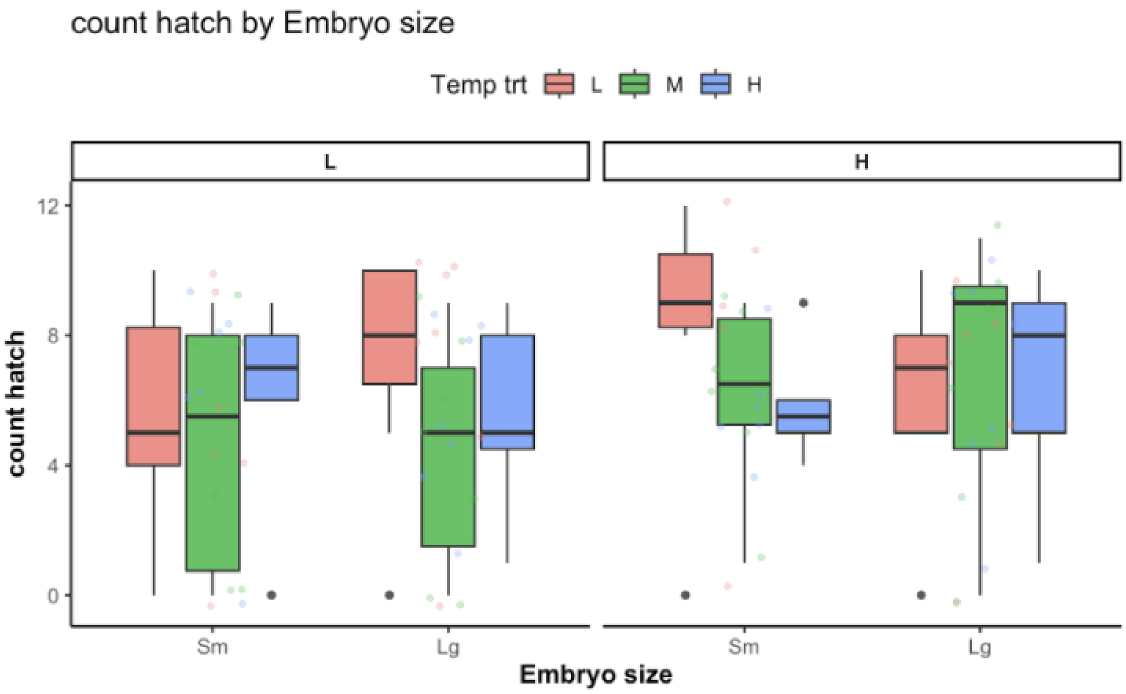
Appendix Figures



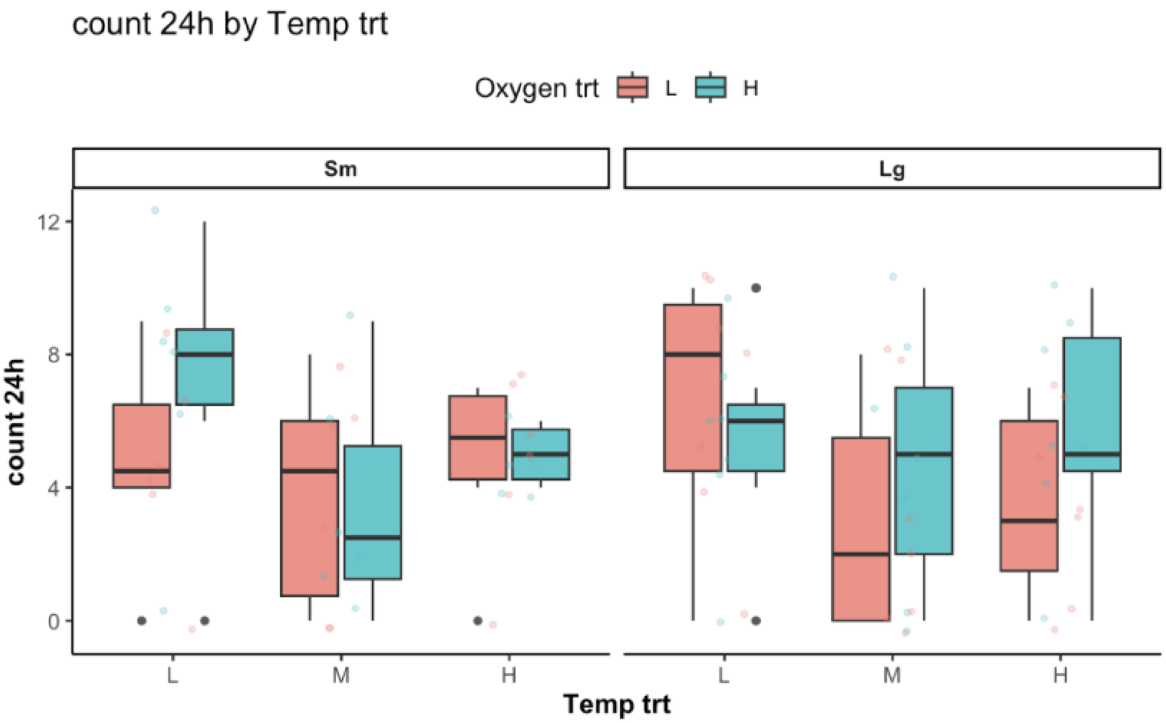
Appendix Figure A2.1. Count of Hatchlings by Temperature and Oxygen Treatments Across Embryo Size Classes. Hatch counts were generally higher under high oxygen, with small embryos performing best at low temperature and large embryos at medium temperature.



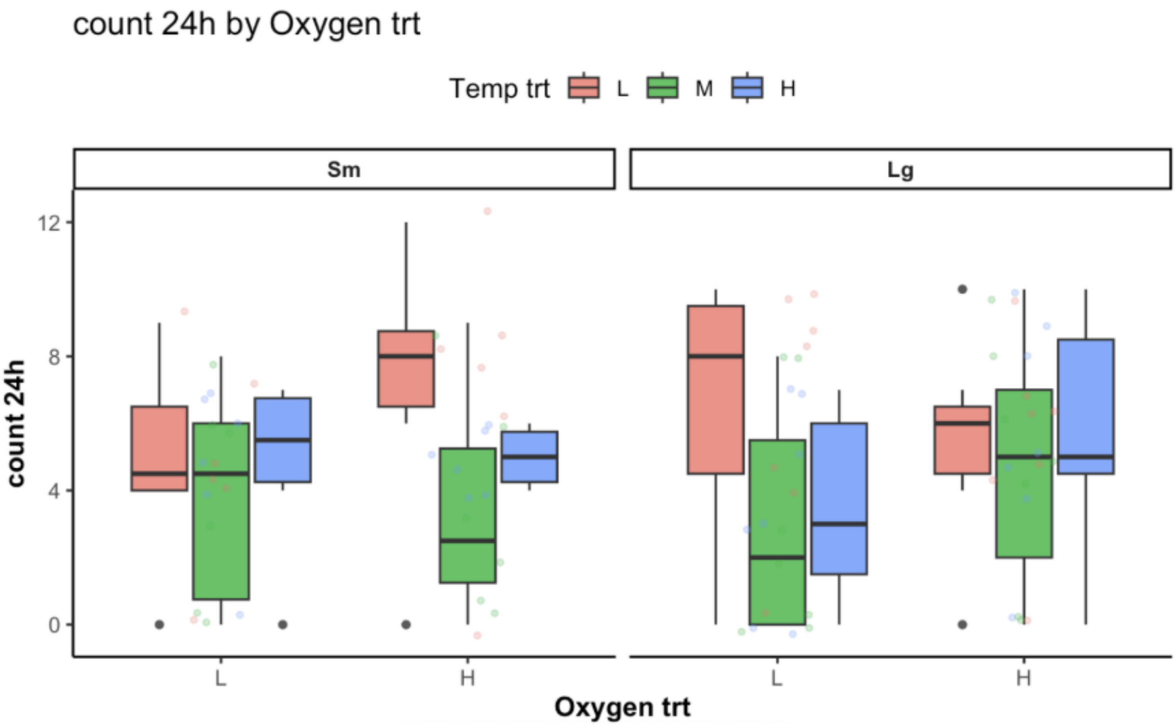
Appendix Figure A2.2. Count of Hatchlings by Oxygen and Temperature Treatments Across Embryo Size Classes. Hatch success declined under low oxygen and higher temperatures, indicating size-dependent responses to combined thermal and oxygen stress.



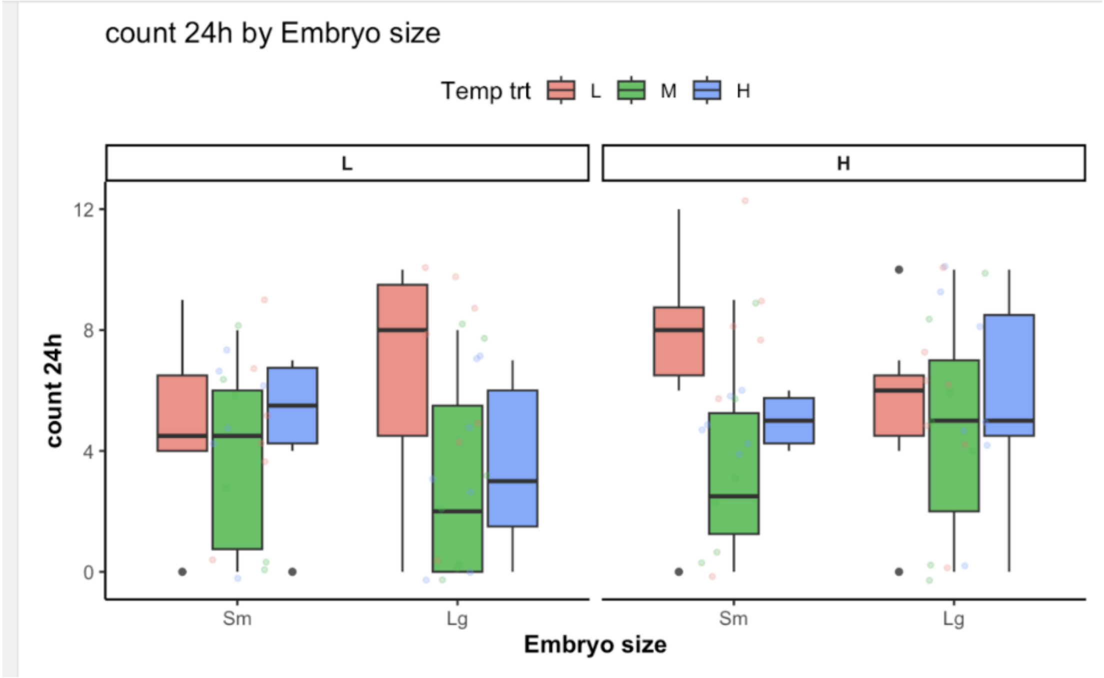
Appendix Figure A2.3. Count of Hatchlings by Embryo Size Across Temperature and Oxygen Treatments. Under low oxygen, hatch counts declined sharply at medium temperature for both size classes, whereas under high oxygen, hatching success increased across all temperatures. Small embryos performed best at low temperature, while large embryos achieved the highest hatch counts at medium temperature when oxygen was abundant.



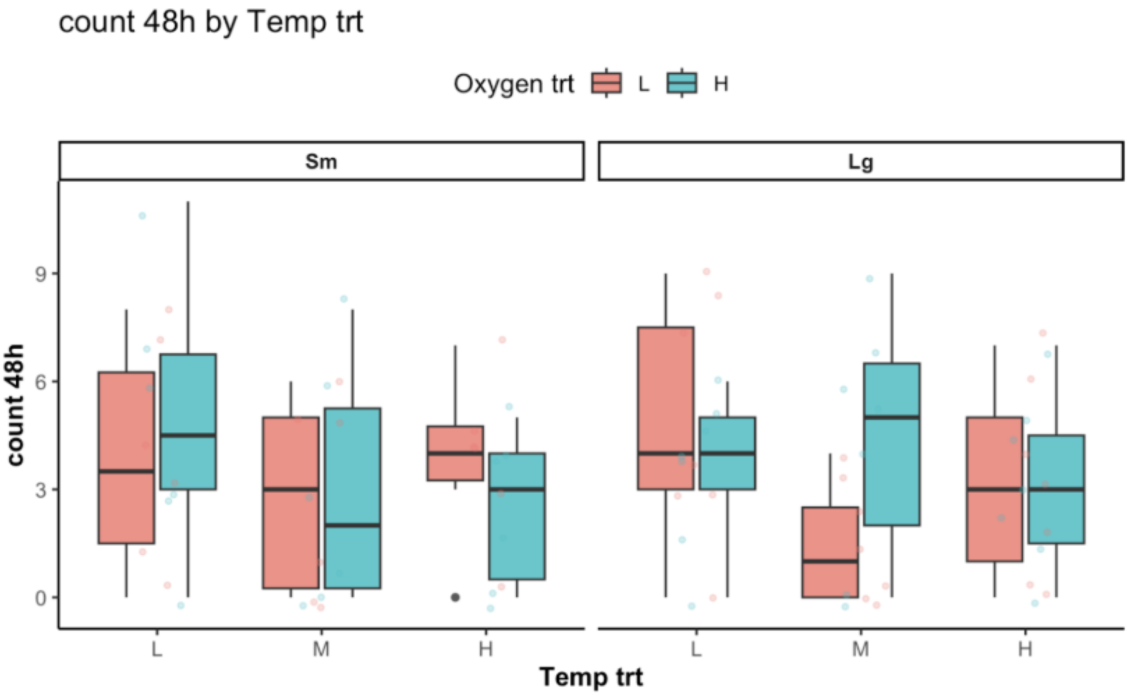
Appendix Figure A2.4. Count of Surviving Larvae 24 Hours Post-Hatch by Temperature and Oxygen Treatments Across Embryo Size Classes. Survival was highest at low temperature, particularly under high oxygen, and declined at medium and high temperatures. Oxygen availability partially mitigated temperature stress, with stronger effects for small embryos.



Appendix Figure A2.5. Count of Hatchlings Across Temperature and Oxygen Treatments by Embryo Size Class. Small embryos achieved the highest hatch counts at low temperature across oxygen levels, whereas large embryos showed higher hatch success at warmer temperatures when oxygen was abundant. These patterns indicate strong size-dependent interactions between temperature and oxygen in regulating hatching success.



Appendix Figure A2.6. Median Hatch Counts Across Temperature and Oxygen Treatments by Embryo Size Class. Hatch success declined with increasing temperature, particularly under low oxygen, while high oxygen increased hatch counts across temperatures. Small embryos performed best at low temperature, whereas large embryos showed highest hatch success at medium temperature when oxygen was abundant.



Appendix Figure A2.7. Count of Surviving Larvae 48 Hours Post-Hatch by Temperature and Oxygen Treatments Across Embryo Size Classes.

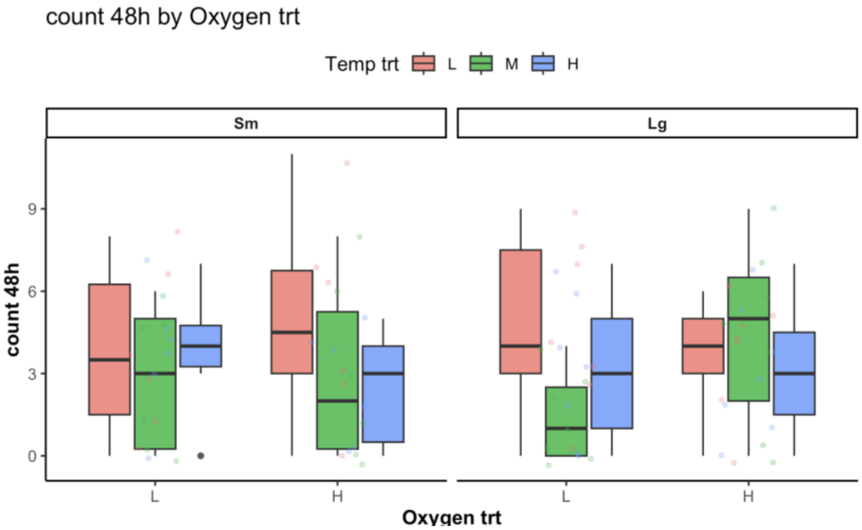
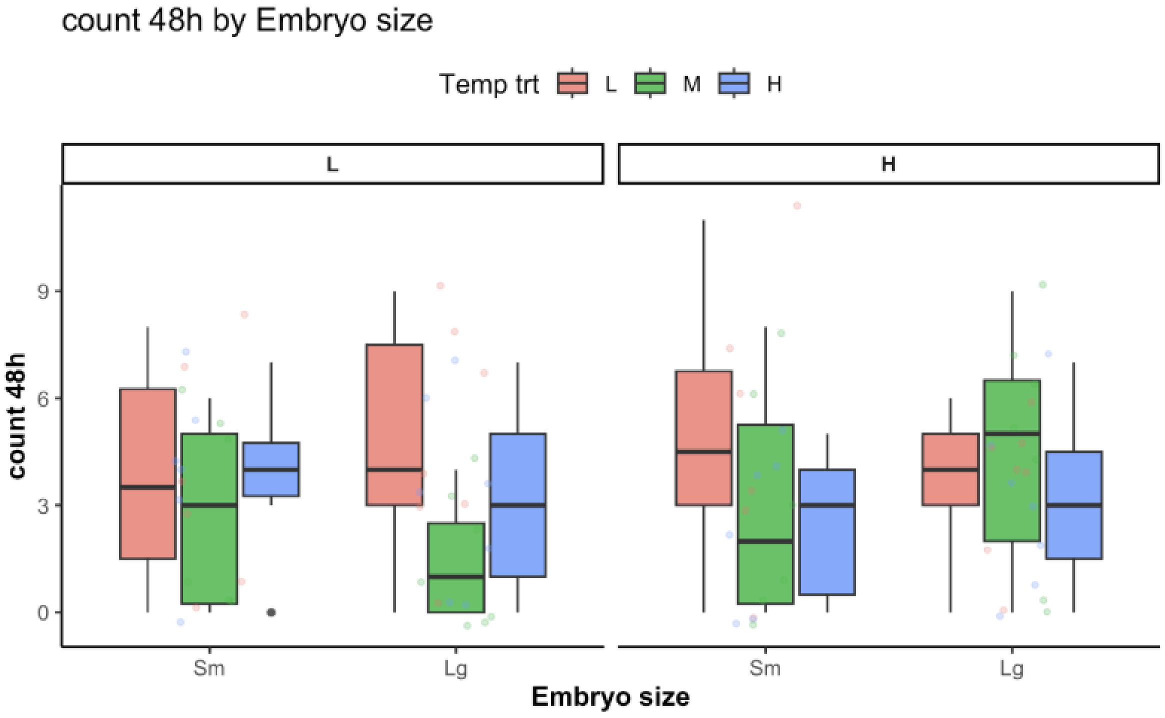
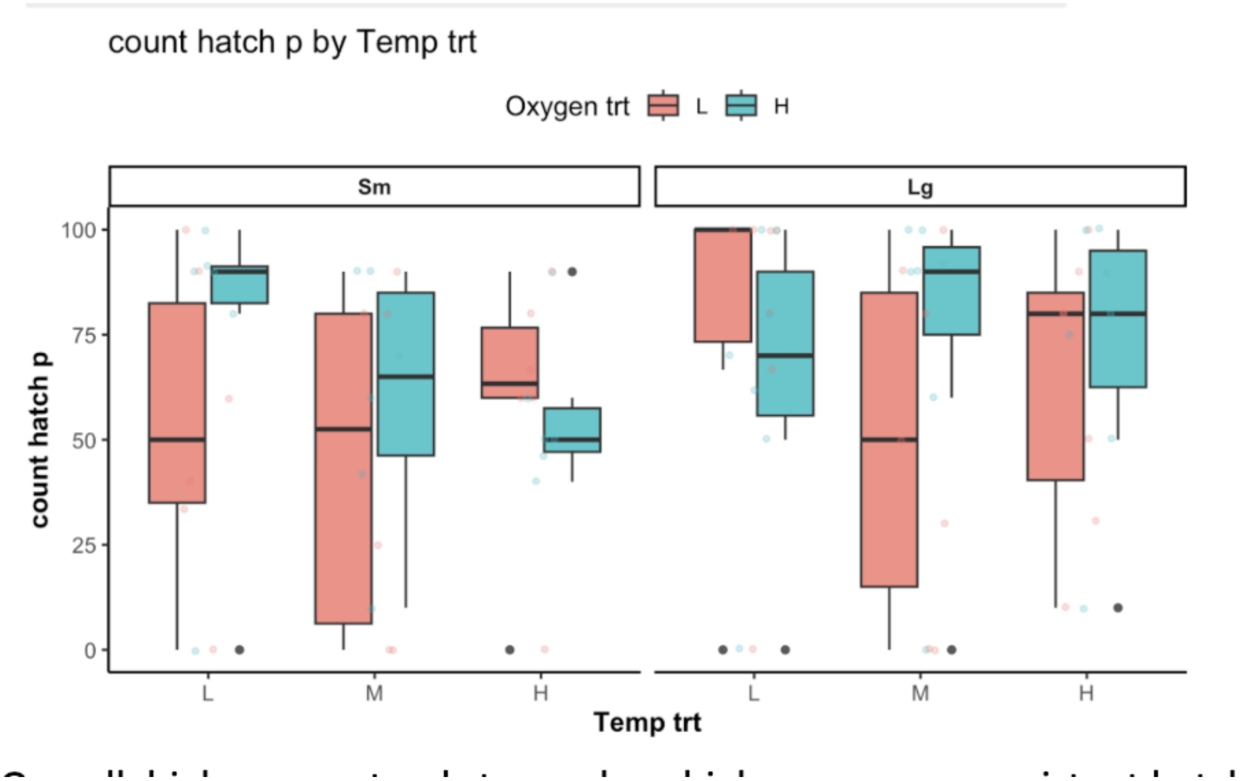


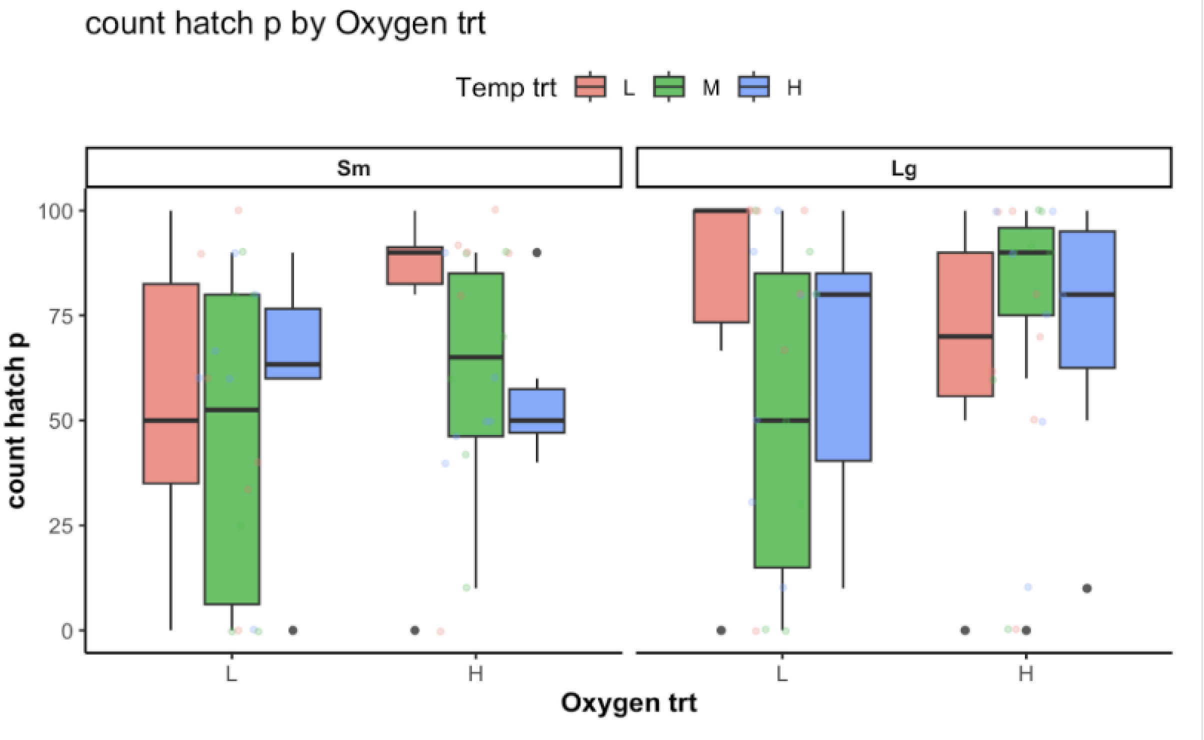
Figure 2.8. Count of Surviving Larvae 48 Hours Post-Hatch by Oxygen Treatment, Temperature, and Embryo Size.



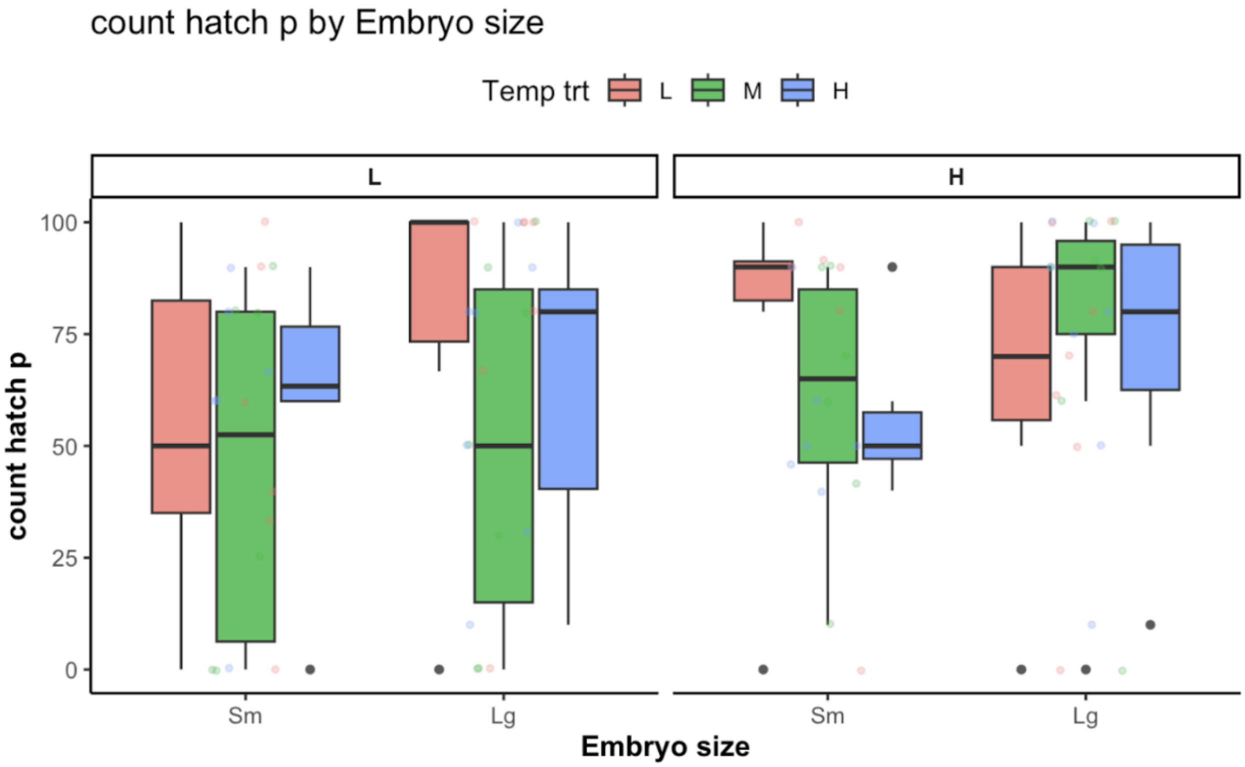
Appendix Figure A2.9. Count of Surviving Larvae 48 Hours Post-Hatch by Embryo Size, Temperature, and Oxygen Treatments.



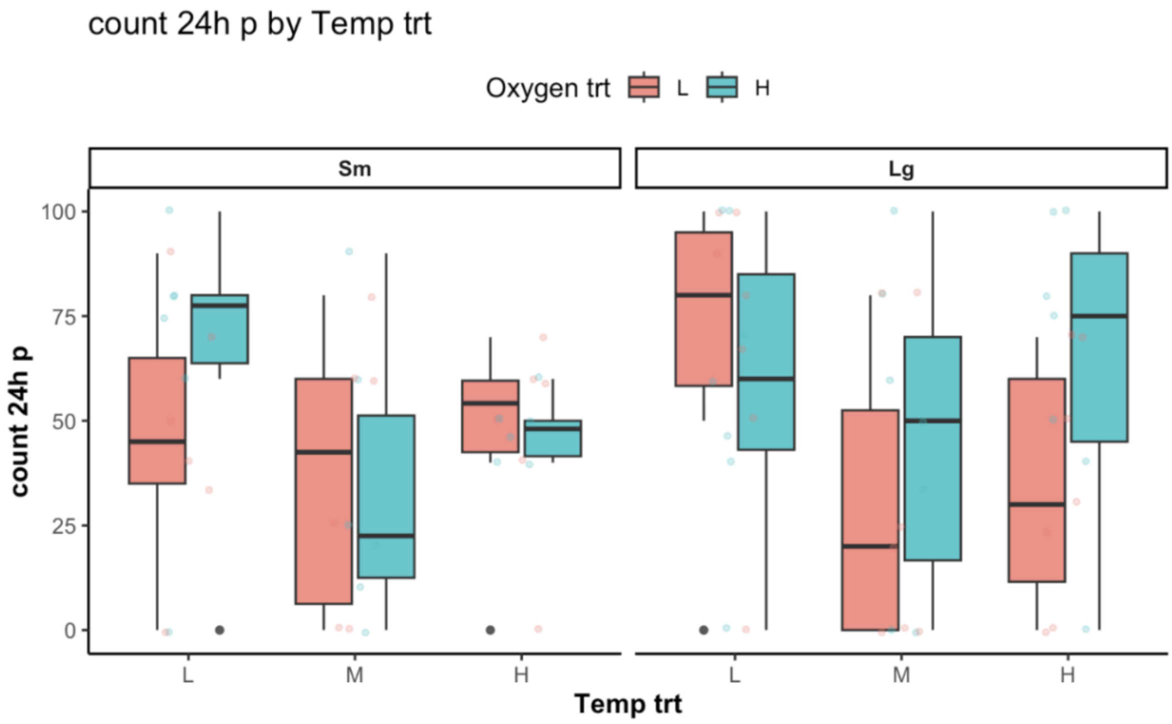
Appendix Figure A2.10. Hatch Proportion Across Temperature and Oxygen Treatments by Embryo Size Class.



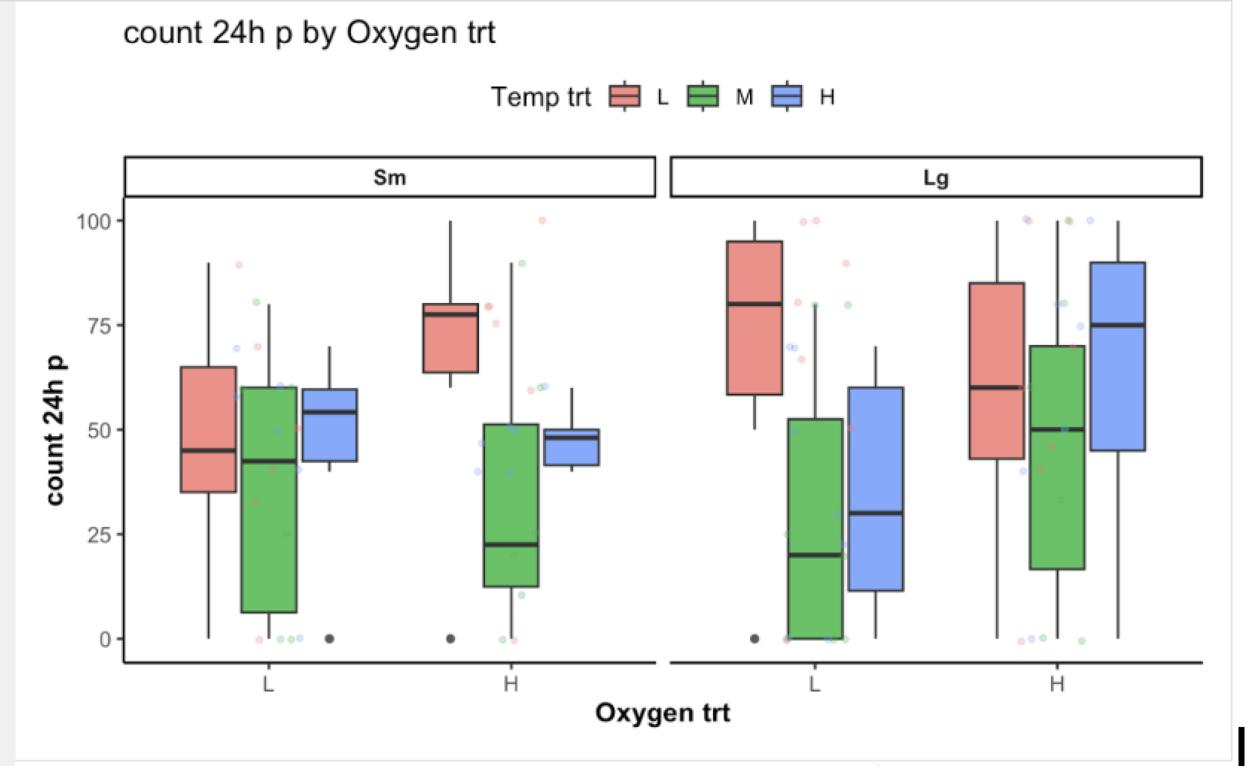
Appendix Figure A2.11. Hatching Percentage Across Oxygen Treatments by Temperature and Embryo Size Class.



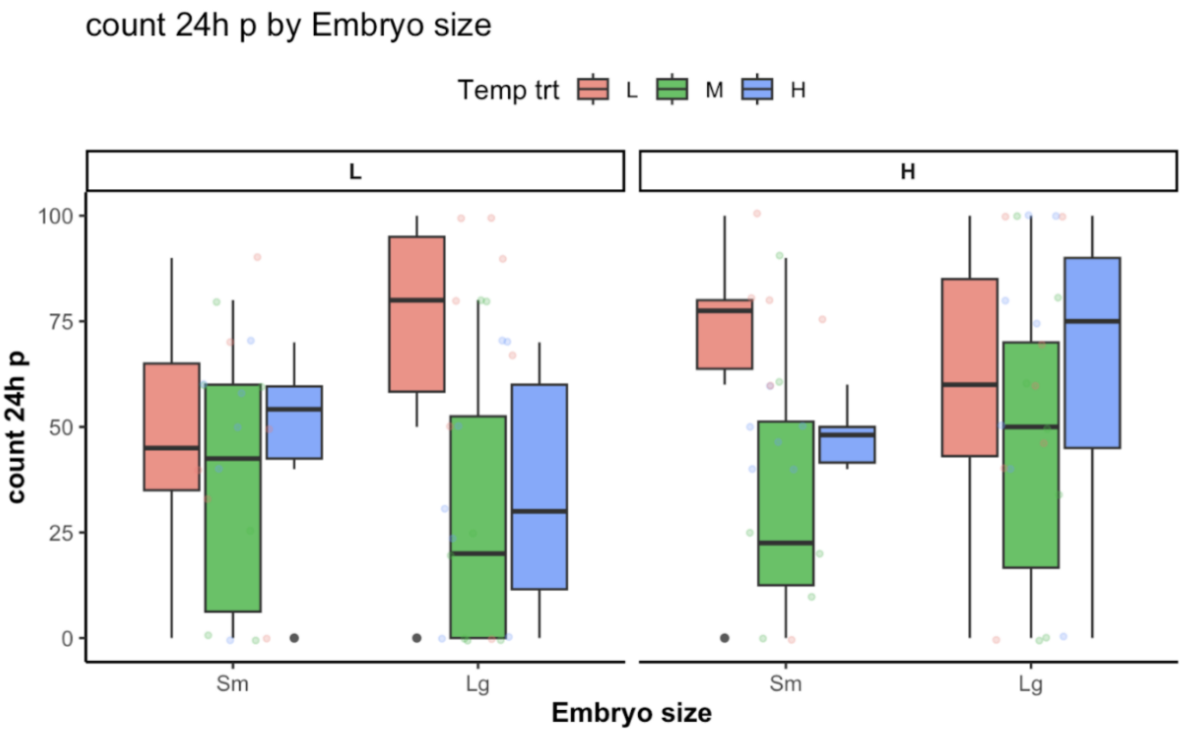
Appendix Figure A2.12. Hatching Percentage Across Embryo Size, Temperature, and Oxygen Treatments.



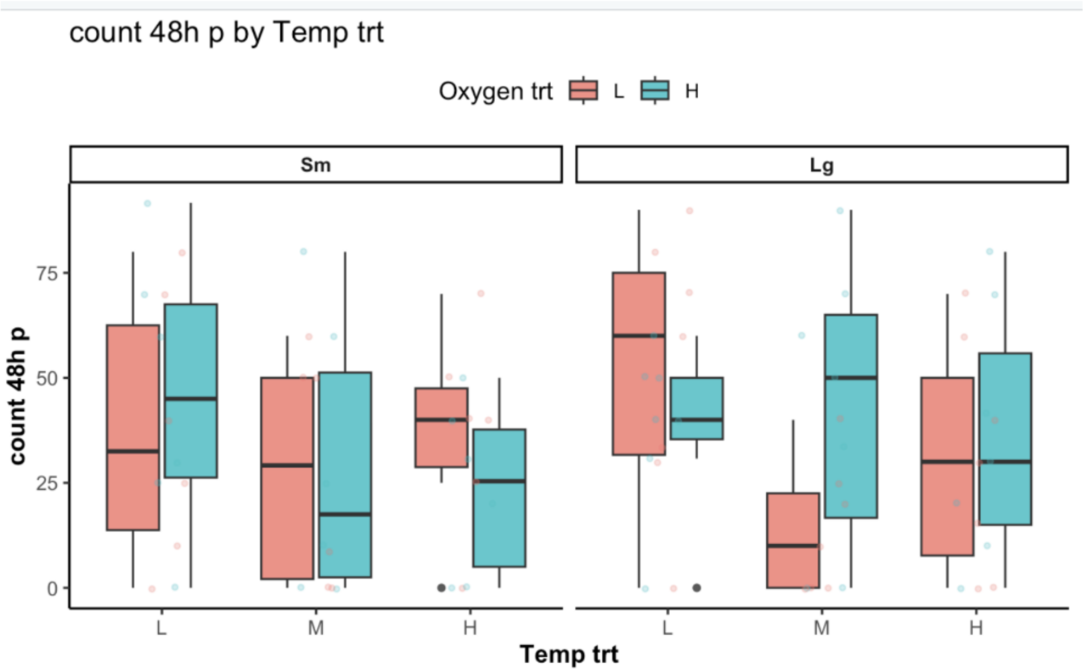
Appendix Figure A2.13. Percentage of Larvae Surviving 24 Hours Post-Hatch Across Temperature and Oxygen Treatments by Embryo Size.



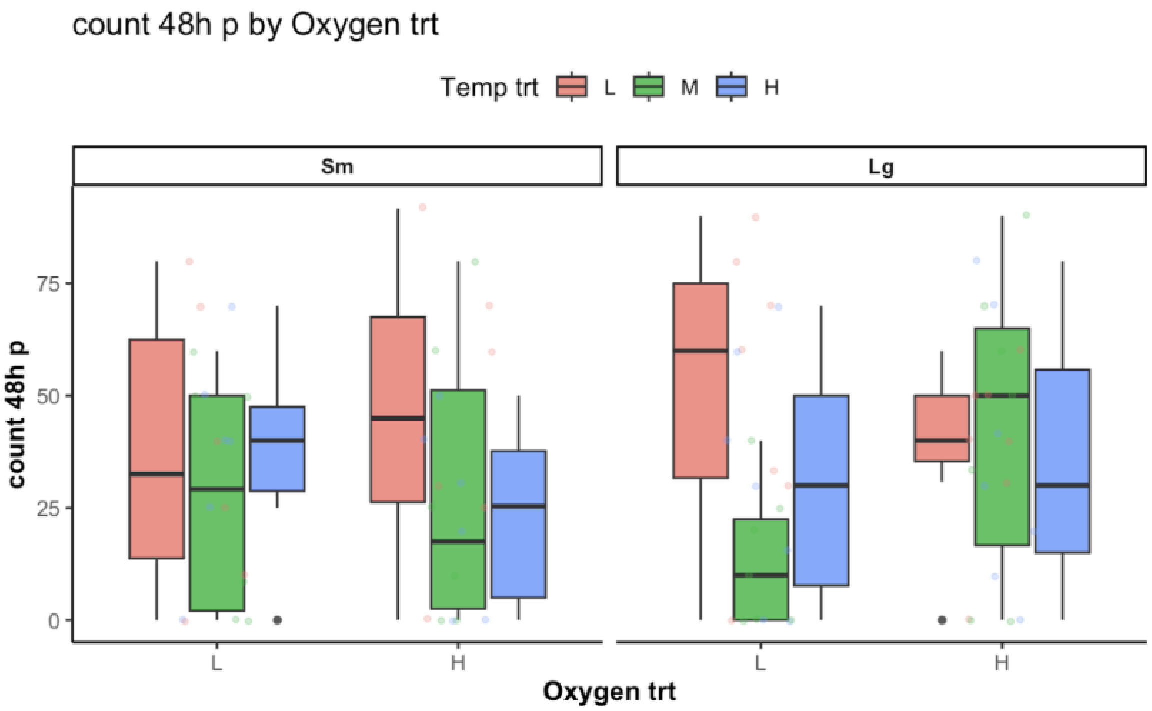
Appendix Figure A2.14. Percentage of Larvae Surviving 24 Hours Post-Hatch Across Oxygen and Temperature Treatments by Embryo Size.



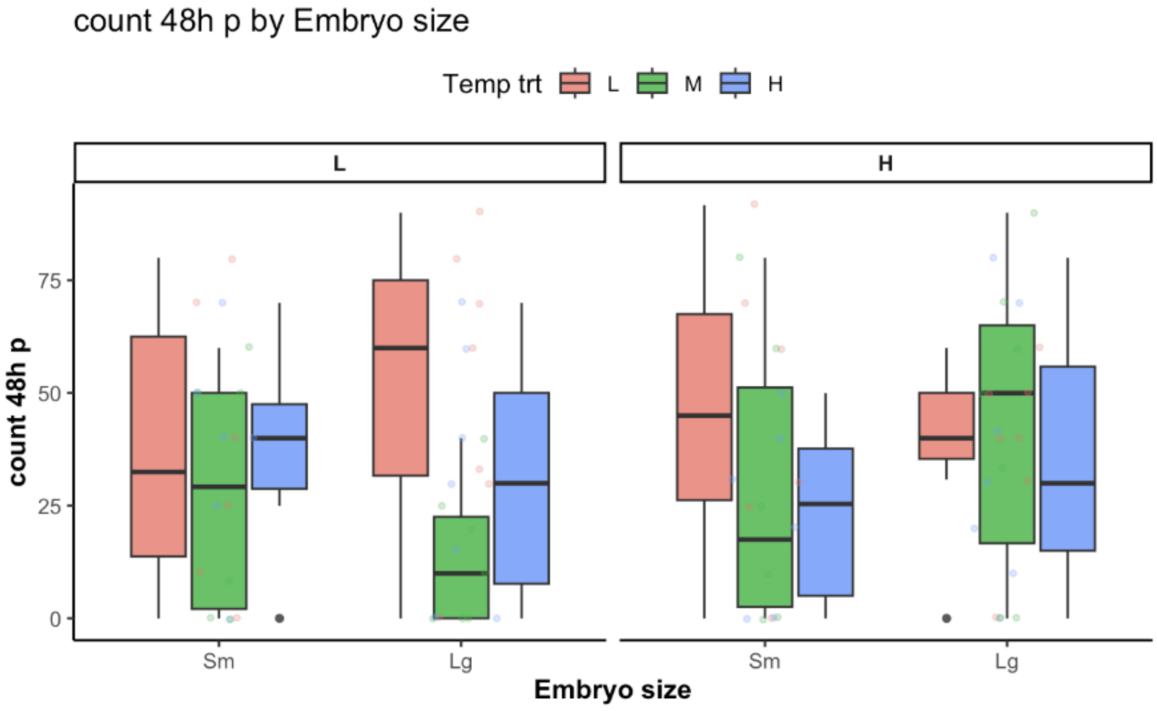
Appendix Figure A2.15. Percentage of Larvae Surviving 24 Hours Post-Hatch Across Embryo Size, Temperature, and Oxygen Treatments.



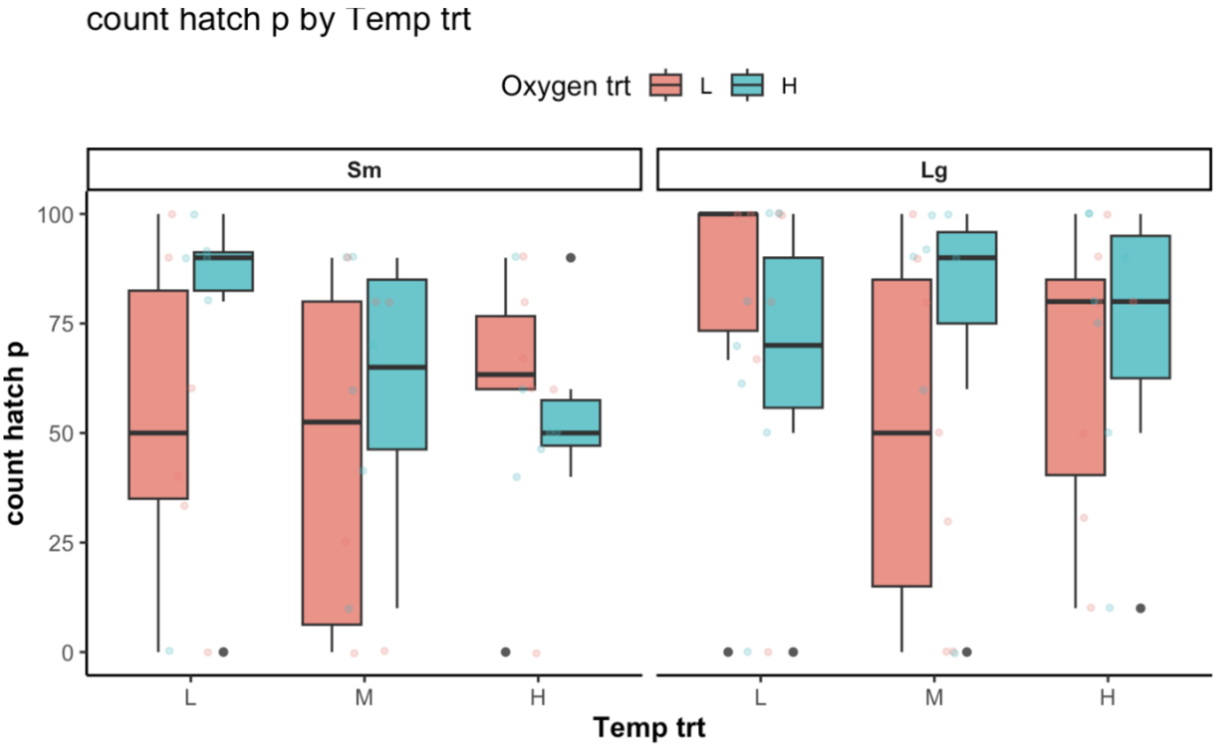
Appendix Figure A2.16. Percentage of Larvae Surviving 48 Hours Post-Hatch Across Temperature and Oxygen Treatments by Embryo Size.



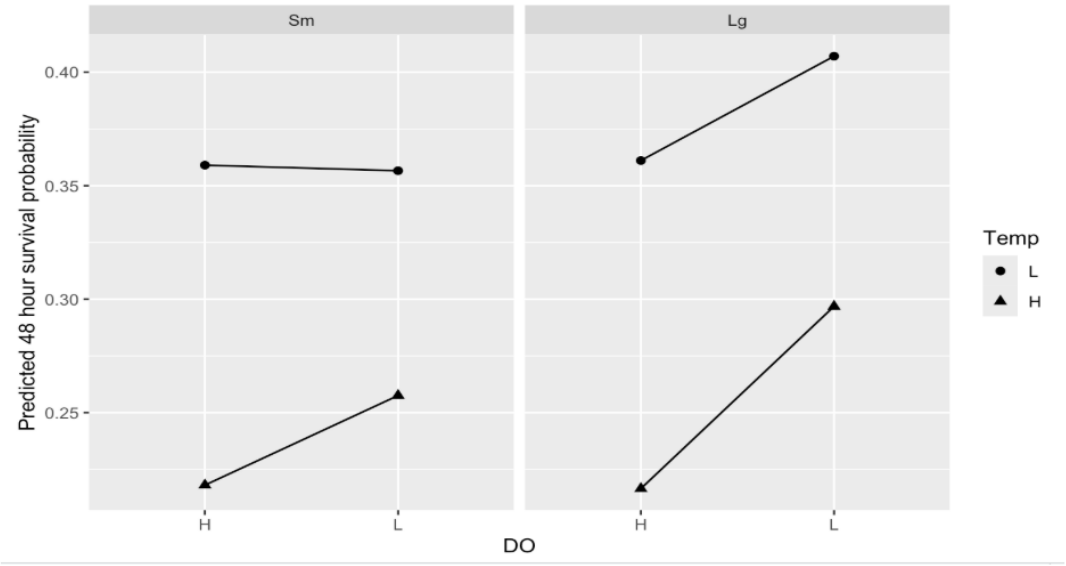
Appendix Figure A2.17. Percentage of Larvae Surviving 48 Hours Post-Hatch Across Oxygen and Temperature Treatments by Embryo Size.



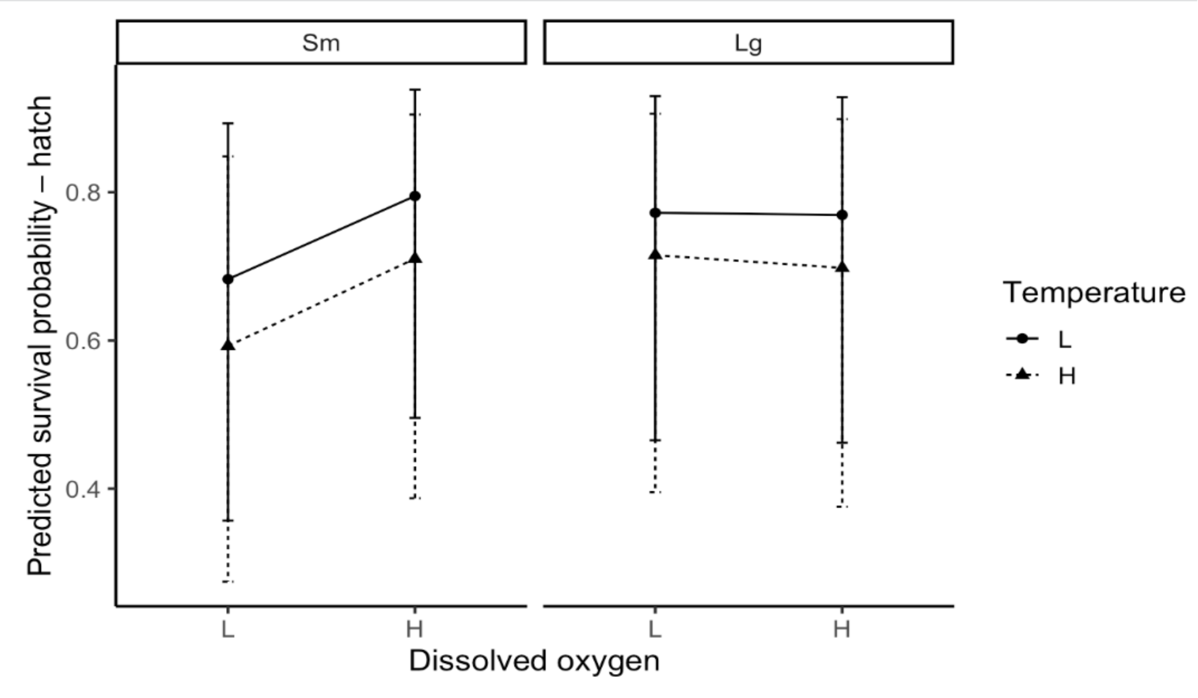
Appendix Figure A2.18. Percentage of Larvae Surviving 48 Hours Post-Hatch Across Embryo Size, Temperature, and Oxygen Treatments.



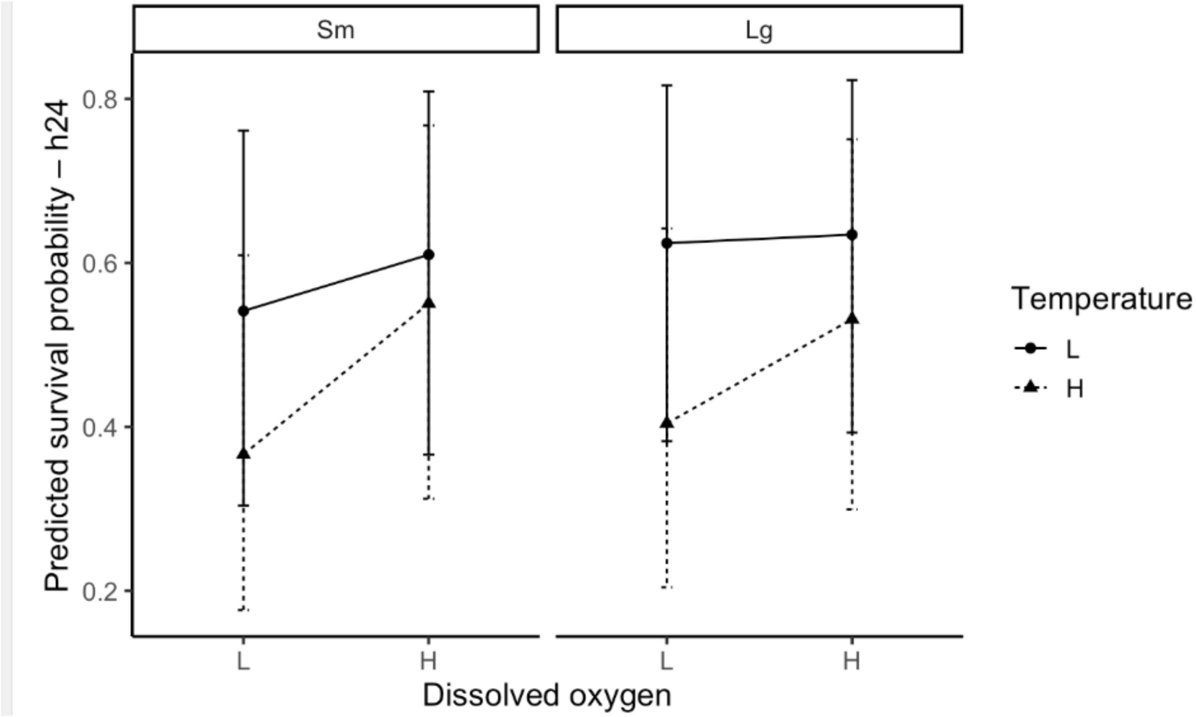
Appendix Figure A2.19. Percentage of embryos hatched across temperature and oxygen treatments by embryo size.



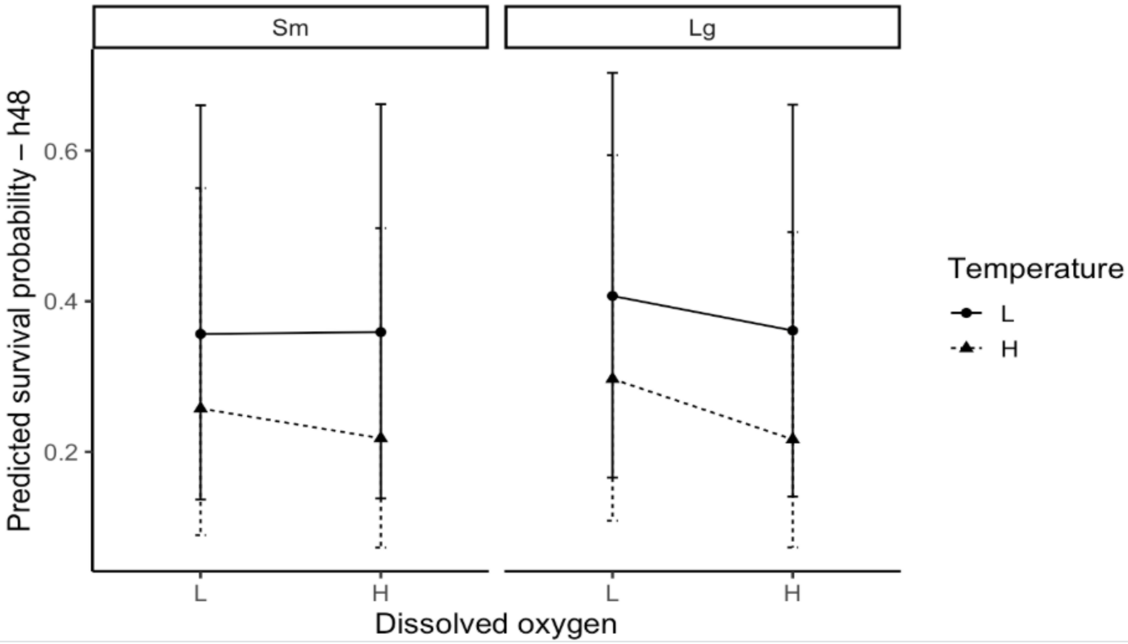
Appendix Figure A2.20. Predicted 48-hour survival probability across dissolved oxygen (DO), temperature, and embryo size classes.



Appendix Figure AFigure 2.21. Predicted hatch survival probability under temperature, dissolved oxygen, and embryo size treatments.



Appendix Figure A2.22. Predicted 24-hour post-hatch survival probability under temperature, dissolved oxygen, and embryo size treatments.



Appendix Figure A2.23. Predicted 48-hour post-hatch survival probability under temperature, dissolved oxygen, and embryo size treatments.

Appendix Tables

Appendix Table A2.1 Pearson correlations between temperature and degree-day metrics

Pair	r	p	CI_L	CI_U
Avg Intvl temp vs Degree Days 1	0.034	0.504	-0.133	0.066
Avg Intvl temp vs Degree Days 2	0.42	0	0.335	0.499

Interpretation:

Pearson correlation analysis revealed distinct relationships between average interval temperature and the two degree-day formulations. The correlation between average interval temperature and Degree Days 1 was weak and non-significant (r = –0.034, p = 0.504), indicating that this degree-day calculation was largely independent of average temperature variation across treatments. In contrast, Degree Days 2 showed a moderate, positive, and significant correlation with average interval temperature ($r = 0.420$, $p < 0.001$), suggesting that this formulation better captures the cumulative influence of temperature over time.

Appendix Table A2.2 Model comparison between linear and quadratic temperature–degree-day models using AIC, ΔAIC, and Akaike weights.

Model	AIC	ΔAIC	Weight_AIC	AICc	ΔAICc	Weight_AICc
Quadratic: Temp + DD1 + Temp^2	901.79	0	0.749	902.62	0	0.745
Quadratic: Temp + DD2 + Temp^2	904.66	2.87	0.178	905.49	2.87	0.177
Linear: Temp + DD2	907.37	5.58	0.046	908.07	5.46	0.049
Linear: Temp + DD1	908.4	6.61	0.027	909.1	6.48	0.029

Model selection showed that the quadratic temperature model using Degree Days 1 (Temp + DD1 + Temp^2) fit the data best (AIC = 901.79, weight = 0.75). The quadratic model using Degree Days 2 also received some support, but was weaker (ΔAIC = 2.87). Linear models performed poorly (ΔAIC > 5), indicating that a quadratic temperature term better captures the relationship between temperature and embryo development.

Appendix Table A2.3. Final model selection for temperature–degree-day (DD) models based on the significance of the quadratic temperature term (Temp^2).

Track	Decision	p(Temp^2)
Temp with DD1	Quadratic (Temp + Temp^2 + DD)	0.0037
Temp with DD2	Quadratic (Temp + Temp^2 + DD)	0.0404

Interpretation:

This table shows that both temperature–degree-day models (Temp + DD1 and Temp + DD2) retained a quadratic temperature term ($Temp^2$) because it was statistically significant ($p < 0.05$). The Temp with DD1 model had stronger support ($p = 0.0037$) than the Temp with DD2 model ($p = 0.0404$), indicating that the relationship between temperature and developmental progression was nonlinear in both cases. This means embryo development increased with temperature up to an optimal range, after which it slowed down a pattern consistent with a thermal optimum effect.

Conclusion:

This thesis examined how temperature and dissolved oxygen influence embryo oxygen consumption, development, and survival within communal nests formed by Bluehead Chub and their nest associates. The overarching goal was to clarify how key environmental factors shape embryo performance in multi-species nesting systems, a component of freshwater fish ecology that remains poorly understood.

Chapter 1 demonstrated that incubation temperature was the dominant predictor of embryo oxygen consumption across both large and small embryos. Oxygen consumption increased significantly with temperature, whereas ambient dissolved oxygen concentration had no detectable effect within the range tested. Embryo size and developmental stage exerted only marginal positive influence, with larger embryos and later stages of development exhibiting slightly higher oxygen consumption. These results indicate that metabolic demand within communal nests is primarily driven by thermal conditions rather than short-term variation in dissolved oxygen availability.

Chapter 2 showed that temperature was also the strongest factor influencing embryo survival. Survival was highest at 18 °C, intermediate at 21 °C, and declined sharply at 24 °C, indicating a clear temperature threshold beyond which survival costs increased substantially. Although higher oxygen conditions reduced some negative effects of warming, they did not fully prevent survival loss at elevated temperatures. Survival declined over time across all treatments, suggesting cumulative physiological stress associated with continued exposure to warmer conditions.

Taken together, Chapters 1 and 2 consistently identify temperature as the primary environmental constraint on embryo performance, influencing both metabolic demand and survival. Oxygen availability provided only limited buffering and did not offset the strong effects of temperature within the range of conditions typically experienced inside nests.

When synthesized across chapters, patterns of oxygen consumption, development, and survival make ecological and evolutionary sense in relation to where the nest host and major nest manipulators concentrate their embryos. Large embryos, primarily those of the host and Central Stoneroller, exhibited slightly higher metabolic demand and greater sensitivity to elevated temperature. These embryos are known to be concentrated near the center and bottom of the nest (Betts et al. *In Press*), a location that reduces predation risk (Betts et al., 2025) but does not appear to confer strong disadvantages with respect to oxygen availability as oxygen consumption does not depend on ambient DO levels. In contrast, smaller nest-associate embryos are more frequently distributed toward peripheral nest regions, where predation risk is higher but physiological conditions are not substantially different.

These findings support an extension of selfish-herd theory from adult spatial positioning to early life stages (Betts et al., 2025). While the host and major nest manipulators benefit from occupying central nest locations that minimize predation risk, embryo performance within the nest appears to be constrained primarily by temperature rather than ambient oxygen availability.

This interpretation is reinforced by field measurements from our Civil Engineering collaborators, which showed little to no variation in dissolved oxygen within natural nests, except near the nest bottom–hyporheic interface. Thus, within the range of conditions typically experienced inside nests, oxygen availability is relatively uniform, and temperature-driven metabolic demand emerges as the dominant physiological constraint. The significance of this work lies in showing that nest-spawning fishes, despite the potential protective benefits of communal nesting, may still be highly vulnerable to environmental change. Small increases in temperature can substantially elevate metabolic demand and reduce survival across embryo types, regardless of their spatial position within the nest. Overall, this thesis adds new understanding of how communal nesting works at the embryo stage. The results show that embryos placed near the center of the nest mainly benefit from lower predation risk, not from better oxygen or lower stress. This extends selfish-herd ideas to early life stages in freshwater fishes. At the same time, the study shows that warming poses a serious risk for gravel-nesting species, because higher temperatures increase oxygen demand and reduce survival across all nest locations. Together, these findings help explain how behavior, nest structure, and environmental conditions interact to shape reproductive success. Future work in this system could combine long-term field data on nest timing and stream temperature with detailed measurements of sediment and groundwater flow to better understand nest microhabitats. Experiments that change nest structure or water flow under warmer conditions would also help determine whether nest-building behaviors can reduce thermal stress or whether these systems remain highly sensitive to climate warming.